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Production and characterization of biodiesel from microalgae cultivated in a photobioreactor at laboratory scale.

Proyecto de investigación

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Production and characterization of biodiesel from microalgae cultivated in a photobioreactor at laboratory scale.

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RESUMEN

Una promisoria fuente de biomasa para la producción de combustibles alternativos son las microalgas, ya que cuentan con un gran metabolismo fotosintético y son capaces de crecer rápidamente en términos de producción de biomasa con el fin de producir celulosa, almidones y aceites en grandes cantidades. El objetivo de este estudio fue completado obteniendo y caracterizando biodiesel producido a partir de microalgas nativas cultivadas en condiciones de laboratorio controladas. En primer lugar, se produjo biodiesel a partir de lípidos extraídos por dos metodologías (lípidos neutros y totales) y directamente de la biomasa de algas se seca. El aceite de soja comercial fue utilizado para producir biodiesel de soja como control positivo. La reacción de transesterificación se llevó a cabo en presencia de un catalizador homogéneo hidróxido de potasio (KOH) y metanol (CH_3OH). Cromatografía de capa fina (TLC) y valores Rf se aplicaron para evaluar la eficiencia de la reacción de transesterificación en primera instancia. Por otra parte, cromatografía de gases con detector de espectrometría de masas (GC-MS) y cromatografía de gases con detector de ionización de llama (GC-FID) fue aplicado para la identificación y cuantificación de los ésteres metílicos de ácidos grasos (FAME). Las muestras de biodiesel de microalgas, están compuestas por: tetradecanoato de metilo (14:0) (2.94 %), palmitato de metilo (16:0) (7.18 %), 9,12,15 éster metílico octadecanoico (18:3) (1.16 %), 9,12 éster metílico octadecanoico (18:2) (39.47 %), 9 éster metílico octadecanoico (18:1) (33.29 %), estearato de metilo (18:0) (5.37 %), fitol (2.48 %) y tetratriacontano (8.11 %). La cuantificación se llevó a cabo empleando dodecanoato de metilo (12:0) como el estándar interno y dos estándares linoleato de metilo (18:2) y palmitato de metilo (16:0). Curvas de calibración se construyeron relacionando la masa (m) y el área (A) entre m(16:0)/m(12:0) vs. A(16:0)/A(12:0) y m(18:2)/m(12:0) vs A(18:2)/A(12:0). Un factor de respuesta 1:1 entre los ésteres que contienen de 14 a 20 átomos de carbonos fue demostrado. En conclusión, el proceso de producción de biodiesel a partir de lípidos neutros de microalgas es el proceso más eficiente. El porcentaje de rendimiento de la reacción de transesterificación para la muestra de biodiesel producido a partir de lípidos neutros de microalgas fue 5,04%.

Palabras clave: biodiesel, microalgas, biomasa, lípidos, transesterificación, caracterización, cromatografía.

ABSTRACT

One promising source of biomass for alternative fuel production is microalgae since they have a large photosynthetic metabolism and grow rapidly in terms of production of biomass and great ability to produce cellulose, starches and oils in large quantities. The objective of this study was completed by producing and characterizing biodiesel produced from native microalgae cultivated in laboratory-controlled conditions. First, biodiesel was produced from lipids extracted by two methodologies (neutral and total lipids) and directly from dried algal biomass. Commercial soja oil was employed to produce soja biodiesel as a positive control. The transesterification reaction was accomplished in presence of a homogeneous catalyst potassium hydroxide (KOH) and methanol (CH_3OH). Thin Layer Chromatography (TLC) and Rf values were applied to firstly evaluate the transesterification reaction efficiency. On the other hand, gas chromatography with mass spectrometry (GC-MS) and gas chromatography with flame ionization detector (GC-FID) were employed to identify and quantify fatty acid methyl esters (FAME). The microalgae biodiesel samples, are composed of: methyl tetradecanoate (14:0) (2.94 %), methyl palmitate (16:0) (7.18 %), 9,12,15 octadecanoic acid methyl ester (18:3) (1.16 %), 9,12 octadecanoic acid methyl ester (18:2) (39.47 %), 9 octadecanoic acid methyl ester (18:1) (33.29 %), methyl stereate (18:0) (5.37 %), phytol (2.48 %), and tetratriacontane (8.11 %). Quantification was conducted employing methyl dodecanoate (12:0) as the internal standard and two standards methyl linoleate (18:2) and methyl palmitate (16:0). Calibration curves were constructed by relating the mass (m) and area (A) of: m(16:0)/m(12:0) vs. A(16:0)/A(12:0) and m(18:2)/m(12:0) vs A(18:2)/A(12:0). A 1:1 response factor among esters ranging from 14 to 20 carbons was demonstrated. In conclusion, biodiesel production process from microalgae neutral lipids is the most efficient process. The percent yield of the transesterification reaction for the microalgae neutral lipids biodiesel sample was 5.04 %.

Key words**:** biodiesel, microalgae, biomass, lipids, transesterification, characterization, chromatography, standards, Fatty Acid Methyl Esters (FAME).

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1. Introduction

Fossil fuel such as petroleum, coal and natural gas are used for endless activities around the world such as heating homes, factories, industries, transport, electricity generation, emong others [20]. However, the issues of utilizing fossil fuels are related to the fact that obtaining them involves the emission and accumulation of greenhouse gases (GHGs) into the atmosphere, contamination of environmental matrices, acidification of water bodies, etc. According to Cusick, it has been estimated that about 30% of CO_2 in the atmosphere is due to the use of fossil fuels [7]. Furthermore, this type of fuel is usually characterized by having a significant content of sulfur and nitrogen. Evidentially, the global consumption of fossil fuels tends to increase, and eventually the diminishing of these is warrantied.

The challenge nowadays is to find a supply of clean, sustainable and renewable energy that contributes to the prevention of the environment pollution. In recent years, many researchers have focused on the production of biodiesel because its production is made from renewable and biodegradable raw material. As stablished by Alam *et al.,* biofuel production from renewable sources is considered one of the most sustainable alternatives to fossil fuels, it is viable for environmental and economic sustainability because it maintains natural resources through energy efficient methods [2].

One of the most dynamic activities of the early 21st century is related to the rapid expansion of biofuel production. Governments around the world have developed new biofuel policies that directly or indirectly provide incentives to companies and banks to invest in production facilities and processing biofuels [16]. Reports that predict the future development of global biofuel markets conclude that biofuel is a booming business.

One promising source of biomass for alternative fuel production is microalgae [15]. The production of biodiesel from microalgae is an important alternative when choosing

among the best sources of extraction. Algae, unlike other plants, have a large photosynthetic metabolism, which is capable of fixing CO_2 and use solar energy. They grow rapidly in terms of production of biomass and great ability to produce cellulose, starches and oils in large quantities [10]. In addition, they grow in a liquid medium, which is handled very easily, and also, can grow in wastewater. Microalgae commonly double their biomass within 24 hours, and during the exponential growth phase, the period could be 3.5 hours [5]. In addition, soja oil is the dominant oil produced in the U.S., so the development of biodiesel has focused around soy oil [12].

The biological cycle in the production and use of biodiesel reduces approximately 80% of CO_2 emissions, and almost 100% less of SO_2. Burning biodiesel reduces in 90% the amount of unburned total hydrocarbons, and in 75-90% aromatic hydrocarbons [5]. It also provides significant reductions in the emission of particulates and carbon monoxide. In the final balance there is no increase in emissions of carbon dioxide since the reduced emissions, compared to petrodiesel, are offset by the absorption of CO_2 by the oil crops.

Biodiesel is defined by ASTM as "a fuel comprised of monoalkyl esters of long-chain fatty acids derived from vegetable oils or animal fats, designated B100" [12]. In the same manner, the US Congress has adopted a similar definition for "biomass-based diesel," with the additional requirement that the fuel have life-cycle greenhouse gas (GHG) emissions that are at least 50% less than baseline lifecycle GHG [12]. Biodiesel is commonly produced by a chemical process known as transesterification, by which the triglycerides are reacted with alcohols, in the presence of a catalyst, to produce fatty acid alkyl esters. Since the most common alcohol used to produce biodiesel is methanol, another name for biodiesel is fatty acid methyl esters (FAME) [9].

The objective of this study was to characterize biodiesel produced from native microalgae cultivated in laboratory-controlled conditions. Different biodiesel production

methods were applied looking to improve the efficiency of the transesterification process. Gas chromatography analysis were carried out to characterize biodiesel samples in order to quantify the content of fatty acid methyl esters (FAME) in the samples and find the yield of the transesterification process.

2. Materials and Methods

2.1 Chemicals

Sodium nitrate [$NaNO_3$], calcium chloride [$CaCl_2.2H_2O$], magnesium sulphate [$MgSO_4.7H_2O$], dipotassium phosphate [K_2HPO_4], sodium chloride [NaCl], chloroform [$CHCl_3$], n-hexane [C_6H_6], potassium hydroxide [KOH], petroleum ether, and dietylether [$(C_2H_5)_2O$] were obtained from Reactivos H.V.O (Quito, Ecuador). Sodium bicarbonate [$NaHCO_3$] was obtained from Vamarth Representaciones (Quito, Ecuador). Methanol [CH_3OH] was obtained from HR Representaciones (Quito, Ecuador). Monopotassium phosphate [KH_2PO_4] was obtained from Judex Laboratory (Sudbury Middlesex, England). Acetic acid [CH_3COOH] (100% purity) was obtained from Merck Chemicals (Darmstadt, Germany). Proteose peptone was obtained from Becton, Dickison and Company (Le pont de Claix, France). Methyl dodecanoate (12:0) (99% purity) was obtained from Alfa Aesar (USA). Merhyl palmitate (16:0) (99% purity) was obtained from MP Biomedicals, LLC (Illkirch, France). Methyl linoleate (18:2) (99% purity) was obtained from Acros Organics (New Jersey, USA). All the reagents were of analytical grade and were used in the state in which they were received.

2.2 Microalgae cultivation

The native microalgae *Chlorella sp.* strain was donated from "ESPE", Escuela Politécnica del Ejército. Microalgae were cultivated in a nutrient medium in a 10 L bench-scale tubular photobioreactor (TPBR) with an inlet upwards airflow of 3 mL min^{-1} of atmospheric air provided by an aquarium pump [11]. The nutrient medium composition of the autotrophic algae cultivation was 250 mg L^{-1} $NaNO_3$, 25 mg L^{-1} $CaCl_2.2H_2O$, 75 mg L^{-1} $MgSO_4.7H_2O$, 75 mg L^{-1} K_2HPO_4, 175 mg L^{-1} KH_2PO_4, 25 mg L^{-1} NaCl, 100 mg L^{-1}

$NaHCO_3$, and 1000 mg L^{-1} proteose peptone [11]. The nutrient medium was sterilized in the autoclave during 15 minutes at 121 ºC prior use. Every day, 300 mL of medium were taken out from the TPBR and 300 fresh nutrient medium were added. pH was measured *in-situ* everyday with a Thermo Scientific Orion 5-Star portable multiparameter meter and its corresponding probe (Thermo Specific Electrode, Orion). Artificial light was offered by 5 fluorescent 20W OSRAM with 12 hours photoperiods to assure light optimal cultivation conditions at room temperature (23±0.5ºC). Additionally, a CO_2 air Aluminum cylinder tank was used to provide CO_2 to six different flasks in order to evaluate three different duplicated concentrations in batch reactors (3%, 5%, and 7%). A gas proportioner instrument (Model G, AALBORG) was used to regulate the inlet mixture flow (CO2 anda air).

2.3 Biomass production

Every day, 6 pre-weighted falcon tubes containing 45 mL samples were centrifuged at 5000 rpm for 10 minutes. The supernatant of each tube was discarded and the tubes were dried in an oven at 105ºC for 12 hours.

2.4 Lipids extraction

Two different methods, total and neutral lipids extraction, were carried out to compare the efficiency of each one in the biodiesel production from microalgae.

2.4.1 Total lipids extraction

A solvent extraction method using chloroform and methanol was applied to obtain the total lipids from microalgae. Briefly, 1 g of dried biomass was grounded to powder and transferred to 15 mL falcon tubes; 2 mL of chloroform and 1 mL of methanol were added and centrifuged at 5000 rpm for 10 minutes. The supernatant containing the total lipids was

transferred to another 15 mL falcon tube, and 5 mL of distilled water was added in order to obtain a biphasic layer. This sample was centrifuged at 5000 rpm for 10 minutes, and the organic layer containing chloroform and the extracted lipids was transferred to a pre-weighted boiling flask. The process was repeated three times until a clear supernatant was obtained. The boiling flask was then settled into a rotary evaporator to recover the chloroform and obtain pure total lipids. Finally, the boiling flask containing the total lipids was weighted. The content of dried biomass was calculated with Eq. 1 and the content of total lipids was calculated with Eq. 2.

$$Dried\ lipids\ (g) = glass\ flask\ with\ lipids\ - glass\ flas \qquad \text{Eq.1}$$

$$Lipidis\ content\ \ \% \left(\frac{w}{w}\right) = \frac{Dry\ lipids\ (g)}{Dry\ biomass\ (g)} \times 100 \qquad \text{Eq. 2}$$

2.4.2 Neutral lipids extraction

Neutral lipids extraction was achieved by using a 150 mL Soxhlet apparatus and n-hexane as solvent. The Soxhlet extractor connection was placed on the top of the round-bottomed boiling flask. The thimble containing 7 g. of dried biomass sample was placed in the extractor fitting and 2 g. of boiling chips were added to a 250 mL flask. Approximately 110 mL of hexane were poured into the pre-weighted boiling flask. The Allihn condenser was connected on top of the Soxhlet extractor and the water flow was turned on. The lipids were extracted for 3 hours. Finally, the boiling flask was settled into a rotary evaporator to recover the n-hexane and obtain pure neural lipids. The content of neutral lipids was calculated with Eq. 2.

2.5 Biodiesel production

Biodiesel was produced by carrying out a transesterification process. As described by Geris *et al.* (2007), transesterification process was accomplished in presence of a

homogeneous catalyst potassium hydroxide (KOH) and methanol (CH_3OH) [9]. In order to compare the effectiveness of the transesterification process from microalgae lipids, commercial soja oil was employed to produce soja biodiesel as a positive control. All biodiesel samples were prepared at room temperature.

2.5.1 Biodiesel produced from commercial soja oil

50 g. of commercial soja oil were added to a solution of 0.5 g. of potassium hydroxide (KOH) and 14 mL of methanol (CH_3OH). This solution was stirred for 3 hours. The mixture was transferred to a separatory funnel and the washing process started with the removal of the aqueous phase. 10 mL of NaCl saturated solution (6M) were added three times to the separatory funnel and then removed. Subsequently, 10 mL of distilled water were added three times to the funnel, and then they were removed in order to completely clean the aqueous phase from the biodiesel. pH measurements were conducted in order to obtain neutral values in the aqueous phase. The solution was left in contact with approximately 3 g. of magnesium sulfate anhydrous in order to dry the biodiesel until it turned apparently white. Finally, the biodiesel sample was transferred to a falcon tube.

2.5.2 Biodiesel produced from total and neutral lipids

Previously extracted lipids were added to a solution of potassium hydroxide (KOH) and methanol (CH_3OH). The solution was stirred for 3 hours to complete the transesterification reaction. The mixture was transferred to a separatory funnel to achieve the washing process. This process started with the removal of the aqueous phase and then 2 mL of NaCl saturated solution (6M) were added three times to the separatory funnel and then removed. Subsequently, 2 mL of distilled water were added three times to the funnel, and then they were removed in order to completely clean the aqueous phase from the biodiesel.

pH measurements were conducted in order to obtain neutral values in the aqueous phase The solution was left in contact with approximately 3 g. of magnesium sulfate anhydrous in order to dry the biodiesel until it turned apparently white. Finally, the biodiesel sample was transferred to a falcon tube and mixed with 10 mL of n-hexane (C_6H_6) to store.

For total lipids, 0.3224 g were added to a solution of 3.224 mg of potassium hydroxide (KOH) and 3 mL of methanol (CH_3OH). On the other hand, for neutral lipids 0.3426 g were added to a solution of 3.426 mg of potassium hydroxide (KOH) and 3 mL of methanol (CH_3OH).

2.5.3 Biodiesel produced from dried biomass (Direct transesterification process)

1 g. of dried microalgae biomass was added to a solution of 0.05 g of potassium hydroxide (KOH) and 3 mL of methanol (CH_3OH). This solution was stirred for 3 hours. The mixture was transferred to a separatory funnel and the washing process started with the removal of the aqueous phase. 2 mL of NaCl saturated solution (6M) were added three times to the separatory funnel and then removed. Subsequently, 2 mL of distilled water were added three times to the funnel, and then they were removed in order to completely clean the aqueous phase from the biodiesel. pH measurements were conducted since it is necessary to obtain neutral values in the aqueous phase. The biodiesel obtained was dried with approximately 3 g. of magnesium sulfate anhydrous and stored in 10 mL of n-hexane (C_6H_6).

2.6 Biodiesel characterization

2.6.1 Thin Layer Chromatography (TLC) and Rf values

TLC silica plates EMD Millipore Company were prepared by marking a solvent front at the upper part of the plate and a reference line at the bottom. A solvent mixture was prepared using 8 mL of petroleum ether, 1.9 mL of ethyl ether, and 0.1 mL of acetic acid [9].

Silica worked as the stationary phase and the solvent mixture worked as a mobile phase. The biodiesel sample was placed at the reference line of the plate with a capillary tube. Finally, the TLC plate was revealed with an iodine chamber. TLC plates for soja biodiesel and microalgae biodiesel were run. R_f values are the measurements of the travelled distance by the solvent, and the distance travelled by individual spots. R_f values were calculated using Eq. 3.

$$R_f = \frac{distance\ travelled\ by\ component}{distance\ travelled\ by\ solvent} \qquad \text{Eq. 3}$$

2.6.2 Chromatography Analysis

The identification and quantification of fatty acid methyl esters (FAME) from biodiesel samples were quantified using a Shimadzu GCMS-QP 2010 Ultra gas cromatograph with autoinjector AOC-20i for liquid samples, mass spectrometry detector (GC-MS) and flame ionization detector (GC-FID), respectively.

2.6.2.1 Gas chromatography–mass spectrometry (GC-MS)

Qualitative (GC-MS) analysis for the biodiesel samples were carried out using a low-polarity phase (Crossbond™ silarylene phase 1,4-bis (dimethylsiloxy) phenylene dimethyl polysiloxane) SH-Rxi™-5Sil MS column (30 m length, 0.25 mm internal diameter, 0.25 µm stationary phase film thickness). The software used was GCMSsolution Version 4.11 SU2 equipped with commercial mass spectral libraries. The sample injection volume was 0.2 µL and was configured with injector in split mode 1:10. The injection temperature was 250 °C. The column oven program was configured as follows: the initial temperature was 150 °C; ramp to 250 °C at a rate of 25 °C min^{-1}; ramp to 253 °C at a rate of 1°C min^{-1}; ramp to 275 °C at 25°C min^{-1}, held isothermally at 275 °C for 2 min. The pressure of helium carrier gas was 99.5 kPa and the velocity was 13 mL min^{-1} [23].

2.6.2.2 Gas chromatography-flame ionization detector (GC-FID)

Based on what was found in the qualitative (GC-MS) analyses, an internal standard (IS), methyl dodecanoate (12:0) and two standards methyl linoleate (18:2) and methyl palmitate (16:0) were used. Quantitative (GC-FID) analysis for the biodiesel samples were carried out based on the method EN 14103 [8] using a TR-WAX-20M column (30 m length, 0.25 mm internal diameter, 0.25 µm stationary phase film thickness). The chromatograph was configured with injector in split mode 1:10 coupled to auto-sampler autoinjector AOC-20i for liquid samples. The sample injection volume was 0.2 µL. The injector and detector temperatures were kept at 260 and 280 °C, respectively. The column oven program was configured as follows: the initial temperature was 190 °C; ramp to 200 °C at a rate of 4 °C/min; ramp to 225 °C at a rate of 2°C min^{-1}; ramp to 260 °C at 15°C min^{-1}, held isothermally at 260 °C for 2 min. The pressure of helium carrier gas was 142.6 kPa and the velocity was 3 mL min^{-1} [23].

For the methyl dodecanoate (12:0) (S1), methyl palmitate (16:0) and methyl linoleate (18:2) standards preparation, n-hexane (C_6H_6) was used as solvent (10 mg mL^{-1}) [3].

Seven different mixture solutions of the prepared standards were injected in the chromatograph to obtain two calibration curves.

$$\frac{m_{16:0}}{m_{12:0}} = m\,\frac{A_{16:0}}{A_{12:0}} + b \quad \text{Eq. 4}$$

$$\frac{m_{18:2}}{m_{12:0}} = m\,\frac{A_{18:2}}{A_{12:0}} + b \quad \text{Eq. 5}$$

where:

$m_{16:0}$, is the mass of the methyl palmitate (16:0) standard.

$m_{12:0}$, is the mass of the methyl dodecanoate (12:0) internal standard.

$m_{18:2}$, is the mass of the methyl linoleate (18:2) standard.

$A_{16:0}$, is the area of the methyl palmitate (16:0) standard.

$A_{12:0}$, is the area of the methyl dodecanoate (12:0) internal standard.

$A_{18:2}$, is the area of the methyl palmitate (16:0) standard.

m, is the slope and,

b, is the intercept.

Based on the internal standard method [25], two equations were constructed to quantify methyl oleate (18:1) and methyl linolenate (18:3).

The solutions were prepared by varying the volume of the different prepared standards, as follows:

Table 1. Mixture solutions of standards to construct a calibration curve.

	Standards		
	Methyl linoleate [µL]	Methyl palmitate [µL]	Methyl dodecanoate [µL]
Point 1	400	400	500
Point 2	600	600	450
Point 3	800	800	350
Point 4	1000	1000	250
Point 5	1200	1200	150
Point 6	1400	1400	100
Point 7	2000	2000	100

Additionally, 100 mg of soja biodiesel were weighted in a 10 mL volumetric flask and completed with n-hexane (C_6H_6) to obtain a 10 mg mL^{-1} solution (S2).

Before injecting the samples in the GC-FID, they were prepared following the procedure described by Aranda [3]. For soja biodiesel, 1000 µL of S2 were mixed with 250 µL of S1 and completed with n-hexane (C_6H_6) to obtain a 10 mL sample. On the other hand, for each microalgae biodiesel samples 4.5 mL of them were mixed with 125 µL of S1 and completed with n-hexane (C_6H_6) to obtain a 5 mL sample.

3. Results and Discussion

3.1 Microalgae cultivation and biomass production

As reported by Hinojosa *et al.*, nutrient medium was used to cultivate *Chlorella sp.* in a 10 L bench-scale tubular photobioreactor (TPBR); it provided many essential elements for the microalgae growth such as C, O, H, N, K, Ca, Mg, P, and trace elements [11]. Daily monitoring of pH was carried out to assure the microalgae strain was always in neutral values. According to Yusuf Chisti, pH is one of the most influencing factors for the microalgae optimal growth and the values typically range from 6 to 7 [4]. The TPBR pH remained between values of 6-8 throughout all the experiments during the 150 days of algae cultivation. Biomass concentration was measured before and after the experiments and it was 42.85±1.86 mg L^{-1} d^{-1}. This value is below the values reported by Zhao *et al.*, who found 58.4±0.57 mg L^{-1} d^{-1} for *Chlorella sp.* biomass concentration [22]. The effect of CO_2 injection in microalgae growth was evaluated in batch reactors. In this study, the nutrient medium used already had a C-source, which was $NaHCO_3$ (0.1 mg L^{-1}). Zhu's *et al* study demonstrated that $NaHCO_3$ can be used as a buffering agent to control the pH. However, in this study 5 % of CO_2 was found to be the most efficient concentration as shown in Annex C.

3.2 Lipid content

An important part of the research was to determine the lipid content because these are used to produce biodiesel. Microalgae oil content for *Chlorella sp.* varies from 28-32 % (v/v) [24]. Indeed, Zhao *et al.* report in their study a lipid content of 32.03±2.82 % (v/v) for *Chlorella sp.* microalgae [22]. In our study, the mean percentage of total lipids was 34.01±1.13 %, while the mean percentage of neutral lipids was 7.02±0.44 %, which is in

accordance with the previous mentioned studies [22]. This means that about 21% of the total lipids found in the microalgae strain were neutral lipids.

3.3 Biodiesel production

Biodiesel production was conducted by a catalytic transesterification reaction in which the triglycerides of fatty acids (microalgae lipids) react with alcohol in the presence of a homogeneous catalyst (KOH) to produce methyl esters [9,13].

Three different experiments were carried, two ways of lipid extraction (neutral and total) and direct transesterification process from dried biomass were applied. The samples were analyzed in the GC-MS apparatus and the biodiesel produced from the neutral lipids extraction sample was found to be the most efficient since at the end the percent yield of the transesterification reaction was 5.04 %.

3.4 Characterization of biodiesel from microalgae

3.4.1 Thin Layer Chromatography (TLC) and Rf values

The transesterification reaction efficiency was firstly evaluated using TLC silica plates. In this case, silica worked as the stationary phase and the solvent mixture worked as a mobile phase. After obtaining the chromatograms for all the biodiesel samples, the analysis confirmed the transesterification process effectively produced methyl esters.

Retardation factor (R_f) values were calculated for all the samples and are presented in Table 2. The relative R_f value for soja biodiesel is 0.83 [9], which is similar to the R_f value for the soja biodiesel sample obtained in this study, 0.821. On the other hand, microalgae biodiesel has a relative R_f value between 0.7-0.8; however, in this study we obtained a value of 0.847 for the biodiesel produced from the microalgae total lipids, 0.843 for the biodiesel

produced from the neutral lipids, and 0.807 for the biodiesel produced directly from the microalgae dried biomass (Table 2).

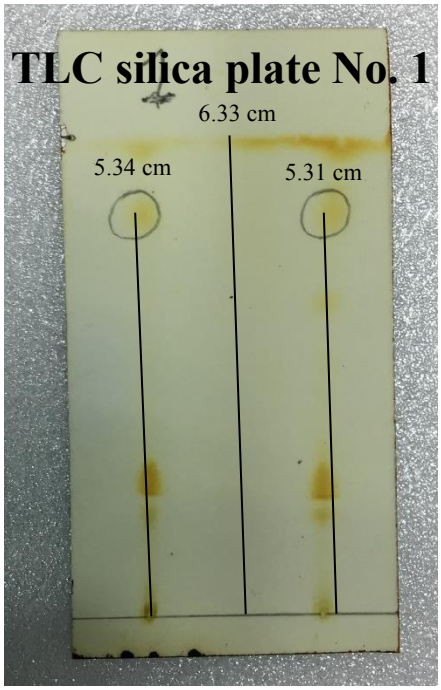


Figure 1. TLC silica plate No. 1. Biodiesel sample produced from commercial soja on the left and biodiesel sample produced from microalgae neutral lipids on the right.

Table 2. Retardation factor (Rf) determined for soja biodiesel and microalgae biodiesel by using Thin Layer Chromatography (TLC).

Substance	Rf
Soja biodiesel	0.838
Microalage biodiesel (total lipids)	0.847
Microalage biodiesel (neutral lipids)	0.843
Microalgae Biodiesel (dried biomass)	0.807

3.4.2 Chromatography Analysis

3.4.2.1 Gas chromatography–mass spectrometry (GC-MS)

Qualitative (GC-MS) analysis was applied to identify the methyl esters present in the biodiesel samples. A soja biodiesel sample and three different samples of microalgae biodiesel were analyzed with GC-MS.

The biodiesel produced from commercial soja oil was injected as a positive control. The chromatogram showed five peaks (Fig. 2) and were identified by the mass spectral library NIST11 by using the software GCMSsolution Version 4.11 SU2 as: methyl dodecanoate (12:0), methyl palmitate (16:0), 9,12,15 octadecanoic acid methyl ester (18:3), 9,12 octadecanoic acid methyl ester (18:2), and methyl stereate (18:0). Those components were in accordance with the reported composition of soybean oil in the study of Hoekman *et al.* [12]. The ester 18:1 is not observed since the column used is not able to separate compounds with 18 carbons; unlike, they are all observed together. In the following spectrum mass (Fig. 3), the presence of the m/z ratios for each of the identified esters are observed. It is remarkable the presence of the following fragments of ions: $\frac{m}{z} = 74$ (-CH_2COOCH_3, penthyl), $\frac{m}{z} = 87$ (-$(CH_2)_5CH_3$, hexyl), $\frac{m}{z} = 43$ (-$(CH_2)_2CH_3$, propyl), $\frac{m}{z} = 143$ (-$(CH_2)_9CH_3$) for those esters without instaurations. While for those presenting instaurations, the fragmentation patterns are more complex, in which the principal fragments have a: $\frac{m}{z} = 67$ (M-$(CH_2)_{15}CH_3$) in methyl linoleate (18:2), and $\frac{m}{z} = 55$ (M-$(CH_2)_{15}CH_3$) in methyl oleate (18:1).

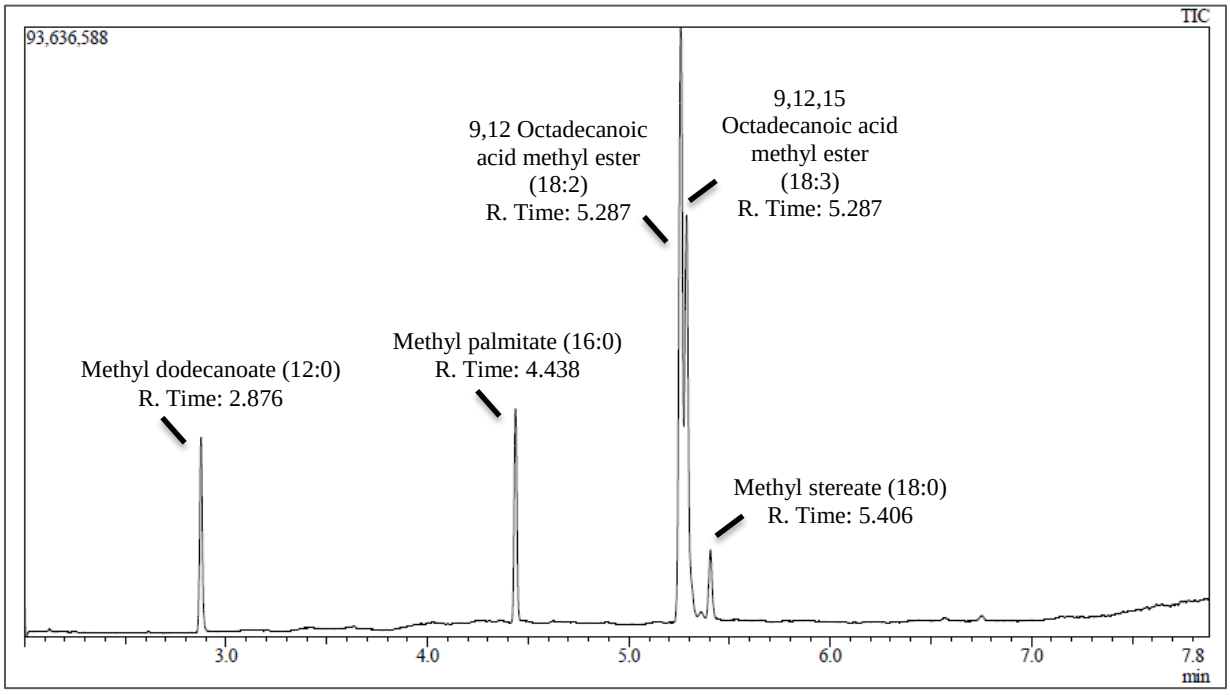


Figure 2. GC-MS chromatogram of soja biodiesel sample.

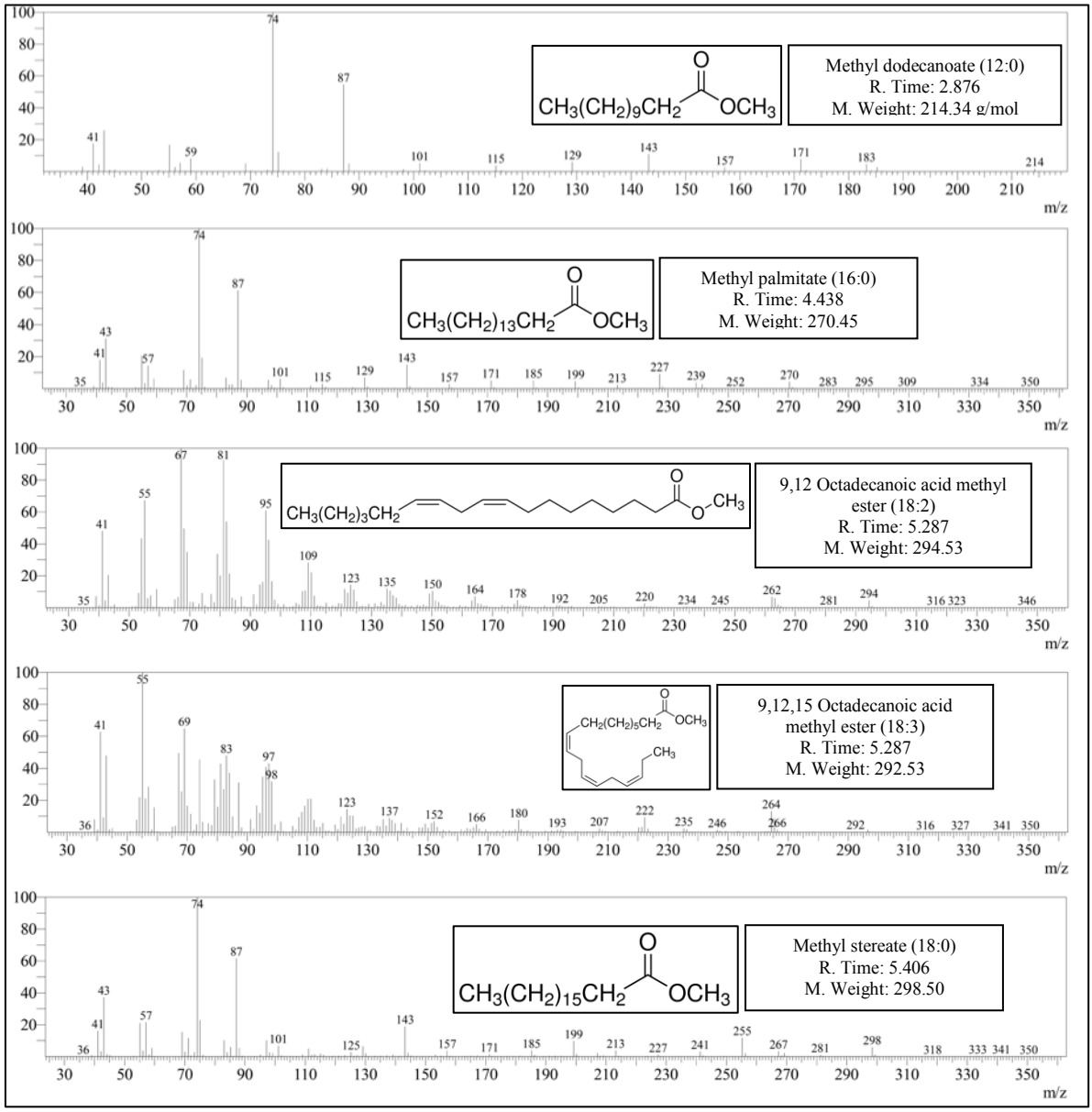


Figure 3. Spectrum mass of esters present in soja biodiesel.

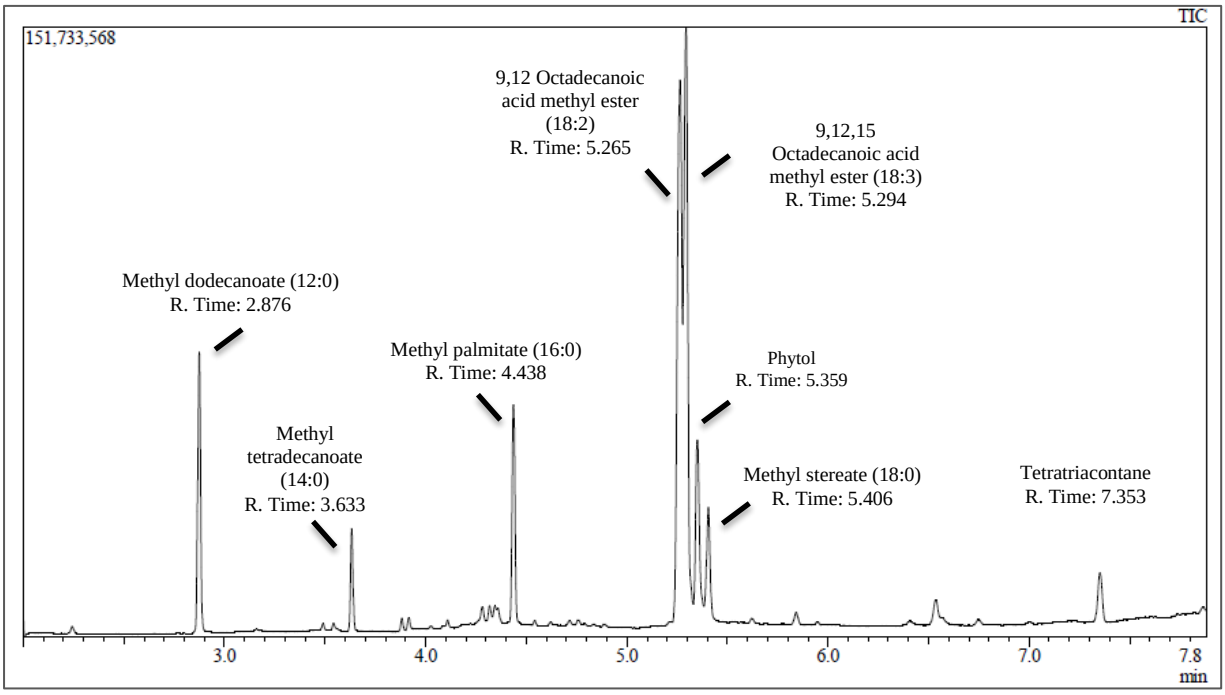


Figure 4. GC-MS chromatogram from microalgae neutral lipids sample.

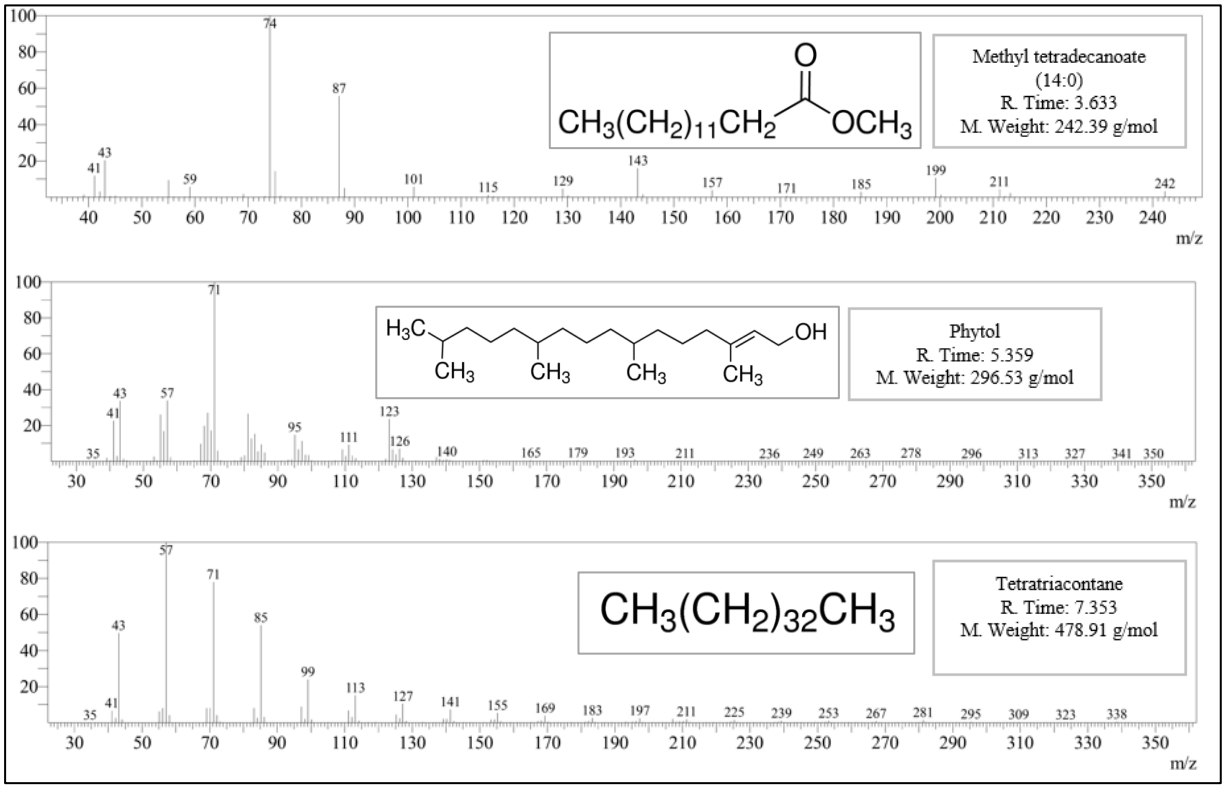


Figure 5. Spectrum mass of additional esters and compounds present in microalgae biodiesel.

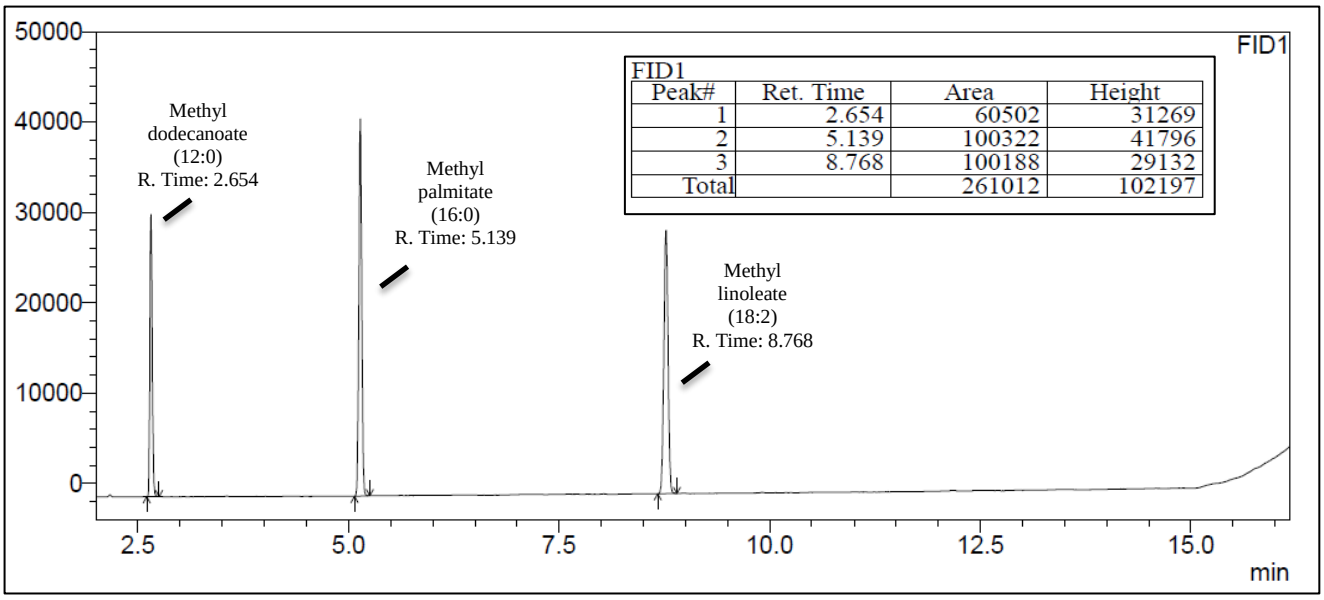
For the three microalgae biodiesel samples, eight peaks were observed (Fig. 4) and identified by the mass spectral library NIST11 by using the software GCMSsolution Version 4.11 SU2 as: methyl dodecanoate (12:0), methyl tetradecanoate (14:0), methyl palmitate (16:0), 9,12 octadecanoic acid methyl ester (18:2), 9,12,15 octadecanoic acid methyl ester (18:3), methyl stereate (18:0), phytol, and tetratriacontane. As in the soja biodiesel sample, in the microalgae biodiesel samples the ester 18:1 was not observed as described above. In figure 5, m/z ratios for the additional esters and compounds present in microalgae biodiesel are illustrated. Additionally, the ion fragmentation follow a similar pattern of the one presented in soja biodiesel.

Comparing to the soja biodiesel sample, in all microalgae biodiesel samples the following compounds were observed: methyl dodecanoate (12:0), methyl palmitate (16:0), 9,12 octadecanoic acid methyl ester (18:2), 9,12,15 octadecanoic acid methyl ester (18:3), and methyl stereate (18:0). However, in the biodiesel from microalgae, methyl tetradecanoate (14:0), phytol, and tetratriacontane were also foud.

Additionally, its remarkable that with the SH-Rxi™-5Sil MS column, methyl stereate (18:0) is observed behind the 9,12 octadecanoic acid methyl ester (18:2) and 9,12,15 octadecanoic acid methyl ester (18:3). Finally, it is observable that this column is not able to separate all the compounds with 18 carbons.

3.4.2.2 Gas chromatography– flame ionization detector (GC-FID)

Considering the methyl esters found in the qualitative (GC-MS) analyses, methyl linoleate (18:2) and methyl palmitate (16:0) were used as standards to confirm the presence of these components in the biodiesel samples. Additionally, methyl dodecanoate (12:0) was used as an internal standard since it is no present in the samples.



FID1

Peak#	Ret. Time	Area	Height
1	2.654	60502	31269
2	5.139	100322	41796
3	8.768	100188	29132
Total		261012	102197

Figure 6. GC-FID chromatogram from a sample injected to construct the calibration curves (Point 2 of Table 1).

Quantitative (GC-FID) analysis was applied to quantify the methyl esters present in the biodiesel samples based on the method EN 14103 [8]. Two calibration curves were constructed with the seven different mixture solutions of the standards with regression line coefficients $R^2 > 0.99$.

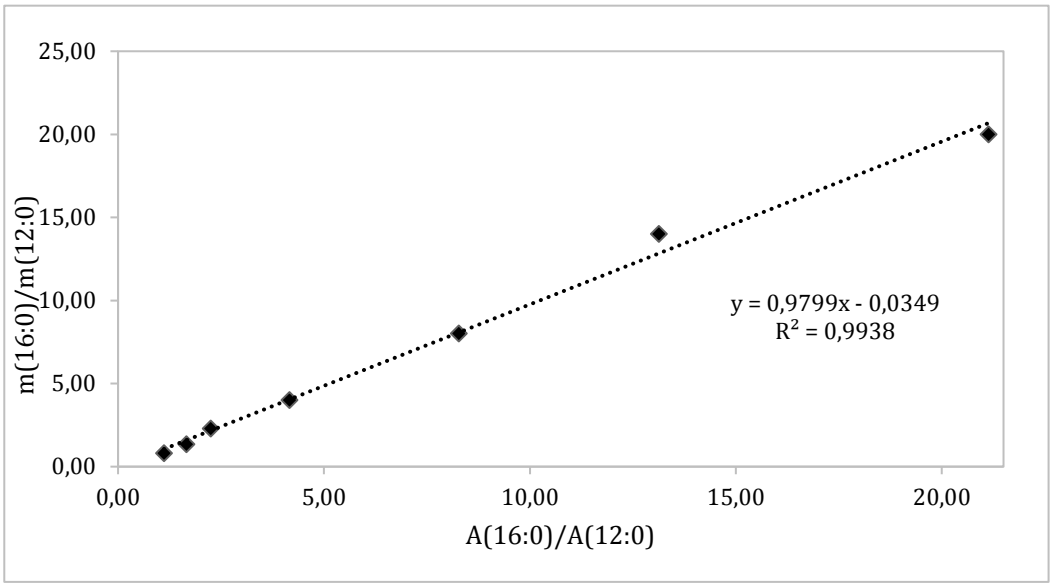


Figure 7. Calibration curve for methyl palmitate standard.

$$\frac{m_{16:0}}{m_{12:0}} = 0.9799\frac{A_{16:0}}{A_{12:0}} - 0.0349 \qquad \text{Eq. 6}$$

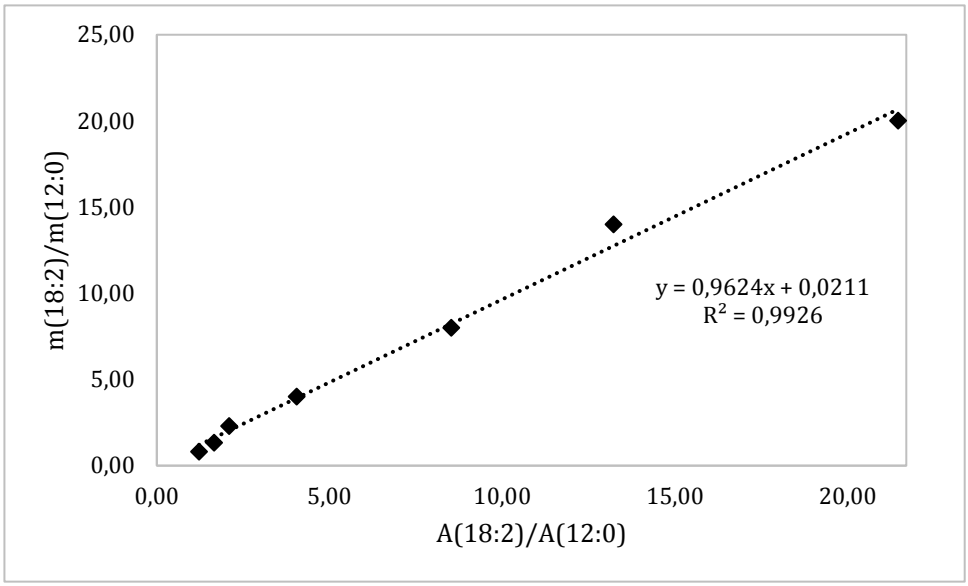


Figure 8. Calibration curve for methyl linoleate standard.

$$\frac{m_{18:2}}{m_{12:0}} = 0.9624\frac{A_{18:2}}{A_{12:0}} + 0.0211 \qquad \text{Eq. 7}$$

The response factor is a measure of the relative mass spectral response of an analyte compared to its internal standard [26]. When injecting methyl palmitate (16:0) and methyl linoleate (18:2) standards in the GC-FID, similar areas were obtained (Table 1, Fig. 5). Therefore, it was concluded that a 1:1 response factor was obtained since the slope of the figure $\frac{m_{18:2}}{m_{16:0}} vs. \frac{A_{18:2}}{A_{16:0}}$ is 1.

Additionally, in order to prove that the other esters found in the biodiesel samples also follow a 1:1 response factor, soja biodiesel was used as reference. In this way, considering the reported values by the literature for the percentages of esters present in soja biodiesel [12], a linear regression between these values and the areas obtained in our analysis was applied. This means that the percentage of each ester was calculated by the Eq 8.

$$E_i(\%) = \frac{A_i}{A_T} * 100 \qquad \text{Eq. 8}$$

where:

A_T, is the sum of the all ester areas present in soja biodiesel.

Table 3. Percentages of each ester present in soja biodiesel. Values reported in literature and experimental values.

Ester	Literature Values [12]		Experimental values	
	Mean Percentage (%)	Standard Deviation	Mean Percentage (%)	Standard Deviation
Methyl palmitate (16:0)	11.6	2	12.71	0.18
Methyl oleate (18:1)	23.7	2.4	25.49	1.65
Methyl linoleate (18:2)	53.8	3.5	57.36	1.58
Methyl linolenate (18:3)	5.9	2.6	6.00	0.90
Methyl stearate (18:0)	3.9	0.8	4.44	0.11

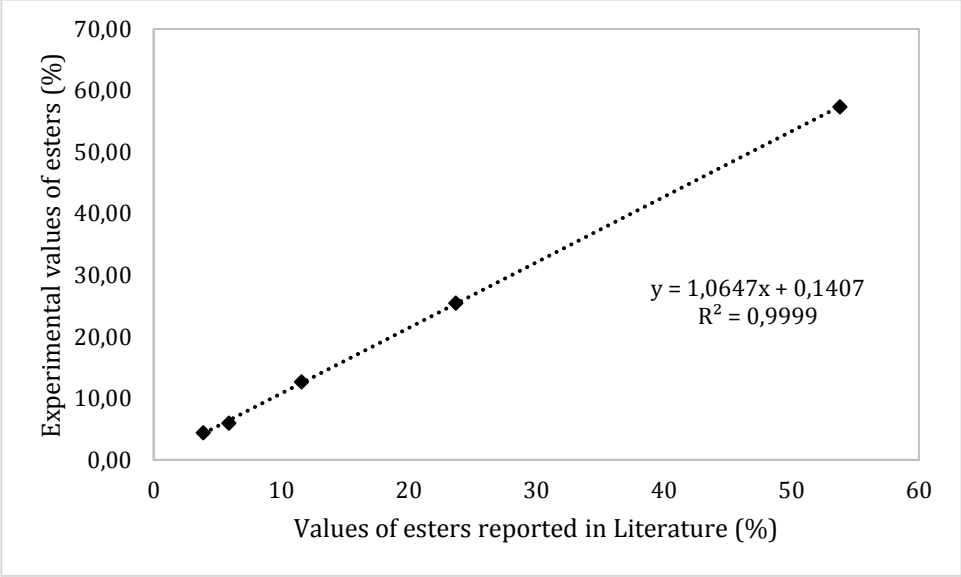


Figure 9. Regression line between experimental values of esters (%) vs. values of esters reported in literature (%) [12].

Finally, a line with slope of 1 was obtained and a regression line coefficients R^2 > 0.99. Hence, for esters ranging from 14 to 20 carbons we can conclude that a 1:1 response factor among them was obtained.

Therefore, the equations to quantify the other esters present in biodiesel sample such as: methyl tetradecanoate (14:0), methyl oleate (18:1) and methyl linolenate (18:3), methyl stearate (18:0) were constructed.

$$\frac{m_{Ci}}{m_{Cy}} = \frac{A_{Ci}}{A_{Cy}} \; where, if \begin{cases} i = 14 - 16 \rightarrow y = 16 \\ i = 17 - 20 \rightarrow y = 18 \end{cases} \qquad \text{Eq. 9}$$

where:

m_{Ci}, is the mass of the ester containing from 14 to 20 carbons.

m_{Cy}, is the mass of the standard (methyl palmitate (16:0) or methyl linoleate (18:2)).

A_{Ci}, is the area of the ester containing from 14 to 20 carbons.

A_{Cy}, is the area of the standard (Methyl palmitate (16:0) or Methyl linoleate (18:2)).

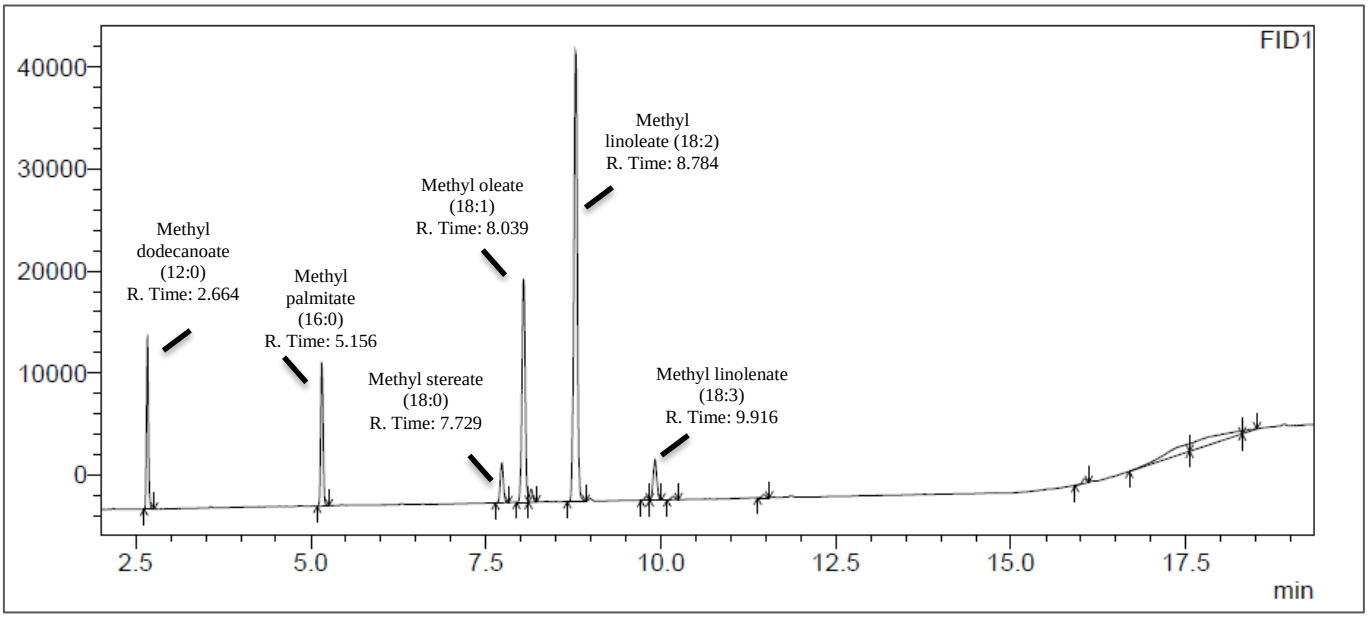


Figure 10. GC-FID chromatogram for soja biodiesel sample.

The soja biodiesel was analyzed with GC-FID apparatus. The chromatogram showed six peaks (Fig. 10). The area and the retention time are shown in Table 4. The percent yield of the transesterification reaction was 100 %.

Table 4. Soja biodiesel composition.

Ester	Retention Time	Area	Mass	Percentage (%)
Methyl dodecanoate (12:0)	2.664	32535		
Methyl palmitate (16:0)	5.156	33404	2.43	11.81
Methyl oleate (18:1)	8.039	70773	5.21	25.34
Methyl stearate (18:0)	7.729	12008	0.88	4.30
Methyl linoleate (18:2)	8.784	149320	10.99	53.46
Methyl linolenate (18:3)	9.916	14239	1.05	5.10
		Total Mass (mg)	20.00	

The microalgae neutral lipids biodiesel was analyzed and the chromatogram showed nine peaks (Fig. 11). The area and the retention time are shown in Table 5. The percent yield of the transesterification reaction was 5.04 %.

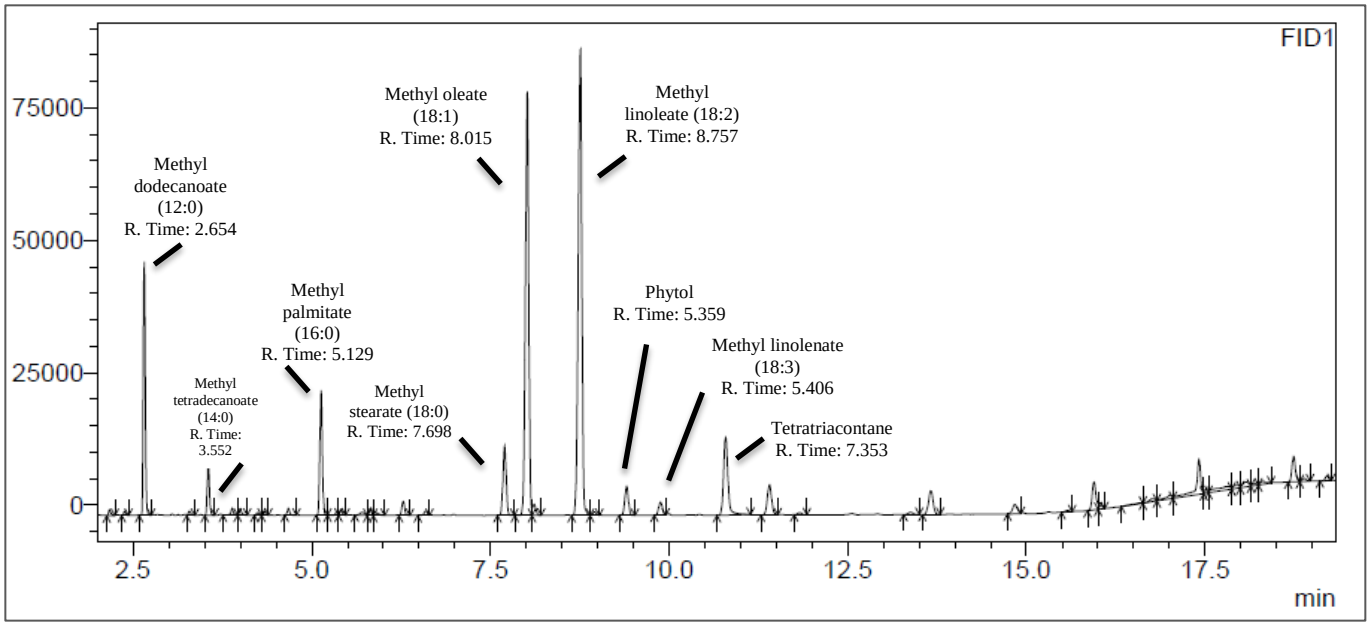


Figure 11. GC-FID chromatogram for microalgae neutral lipids biodiesel sample.

Table 5. Microalgae neutral lipids biodiesel composition.

Ester	Retention Time	Area	Mass	Percentage (%)
Methyl dodecanoate (12:0)	2.654	93002		
Methyl tetradecanoate (14:0)	3.552	21991	0.30	2.94
Methyl palmitate (16:0)	5.129	58132	0.72	7.18
Methyl oleate (18:1)	8.015	260418	3.35	33.29
Methyl stearate (18:0)	7.698	41991	0.54	5.37
Methyl linoleate (18:2)	8.757	308739	3.97	39.47
Phytol	9.405	19398	0.25	2.48
Methyl linolenate (18:3)	9.880	9070	0.12	1.16
Tetratriacontane	10.791	63452	0.82	8.11
		Total Mass (mg)	10.05	

In the case of the microalgae biodiesel samples, ester 1, ester 2, and compound 1 were found with a significant abundance value. GC-MS analysis confirmed compound 1 as phytol (Figure 4), and the two esters as methyl tetradecanoate (14:0) tetratriacontane (Fig. 4).

Unlike the SH-Rxi™-5Sil MS column used for GC-MS analysis, with the TR-WAX-20M column, methyl stereate (18:0) is observed before methyl oleate (18:1)ester, methyl linoleate (18:2), and methyl methyl linolenate (18:3). Finally, it is observable that the TR-WAX-20M column was able to separate compounds with 18 carbons.

4. Conclusions

The feasibility of producing biodiesel from native Ecuadorian microalgae was successfully studied based on microalgae cultivation, different lipid extraction methods and the transesterification process. Chlorella sp. is a good lipid producer with optimal lipid content; however, its biomass production is not the optimal to produce high amount of biodiesel. The biomass production measured before and after the experiments in the TPBR was 42.85±1.86 mg L^{-1} d^{-1}, below the values reported by Zhao *et al.* for *Chlorella sp.* biomass concentration [22]. On the other hand, the mean percentage of total lipids found in this study was 34±0.12 %, while the mean percentage of neutral lipids was 7±0.8%, which is in accordance with the Zhao *et al.* study [22].

Considering the qualitative (GC-MS) analysis, in all the biodiesel samples we can find the compounds: methyl dodecanoate (12:0), methyl palmitate (16:0), 9,12 octadecanoic acid methyl ester (18:2), 9 octadecanoic acid methyl ester (18:1), and methyl stereate (18:0). However, in the microalgae samples we can also find a methyl tetradecanoate (14:0), phytol, and tetratriacontane. Phytol is a compound that belongs to microalgae biodiesel samples.

Considering the quantitative (GC-FID) analysis, we conclude that biodiesel produced from microalgae neutral lipids is the most effective method to produce biodiesel compared to other experiments carried out in this study. The percent yield of the transesterification reaction was 5.04 %.

Additionally, it is observable that the SH-Rxi™-5Sil MS column, used for the GC-MS analysis, is not able to separate all the compounds with 18 Carbons in its chain, while the TR-WAX-20M column, used for the GC-FID analysis, was able to separate the compounds with 18 Carbons in its chain.

It is crucial to find alternatives to optimize the microalgae growth focusing on light and culture conditions. The present study provides important information regarding microalgae biodiesel characterization; nevertheless, more studies about optimization of biomass production and transesterification process optimization are needed.

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Annex A: GC-MS and Table composition Total Lipids

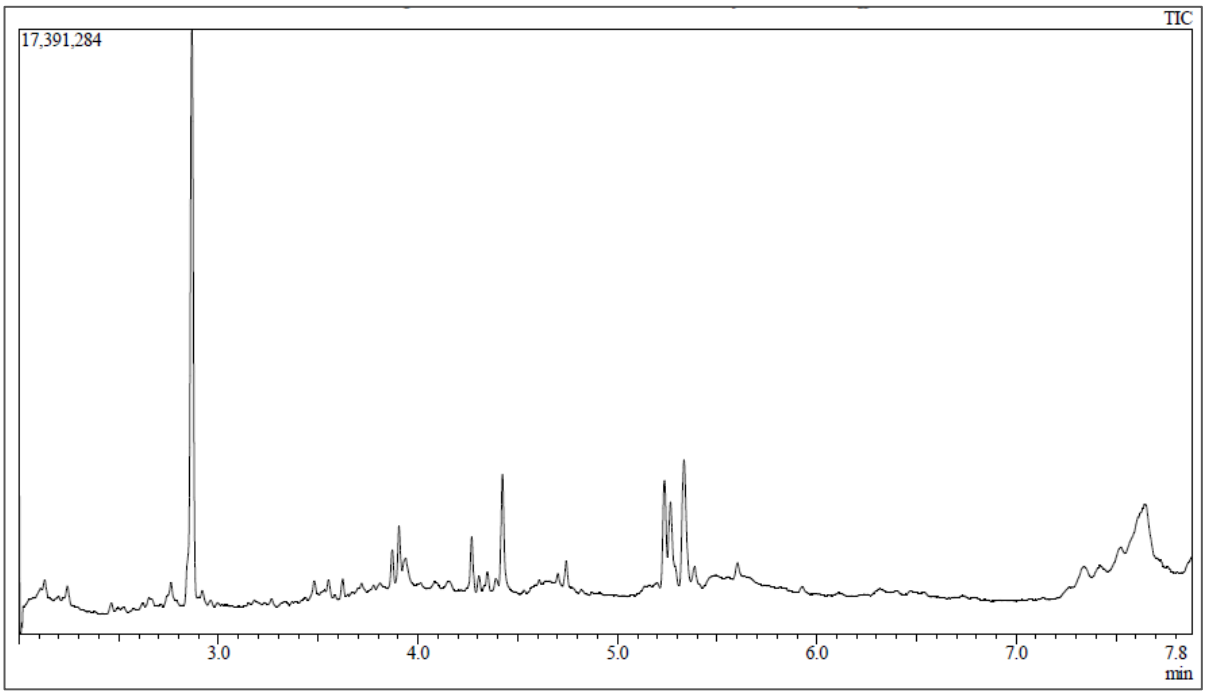

Figure 12. GC-MS chromatogram for microalgae total lipids biodiesel sample.

Table 6. Microalgae total lipids biodiesel composition.

Ester	Retention Time	Area	Mass	Percentage (%)
Methyl dodecanoate (12:0)	2.653	67286		
Methyl tetradecanoate (14:0)	3.551	7511	0.14	13.46
Methyl palmitate (16:0)	5.128	9463	0.13	12.41
Methyl oleate (18:1)	8.002	18747	0.28	27.05
Methyl linolenate (18:3)	7.694	2766	0.04	3.99
Methyl linoleate (18:2)	8.757	2766	0.14	13.03
Tetratriacontane	10.786	20834	0.31	30.06
		Total Mass	1.04	

Percent yield of the transesterification reaction was < 2 %.

Annex B: GC-MS and GC-FID Dried Biomass

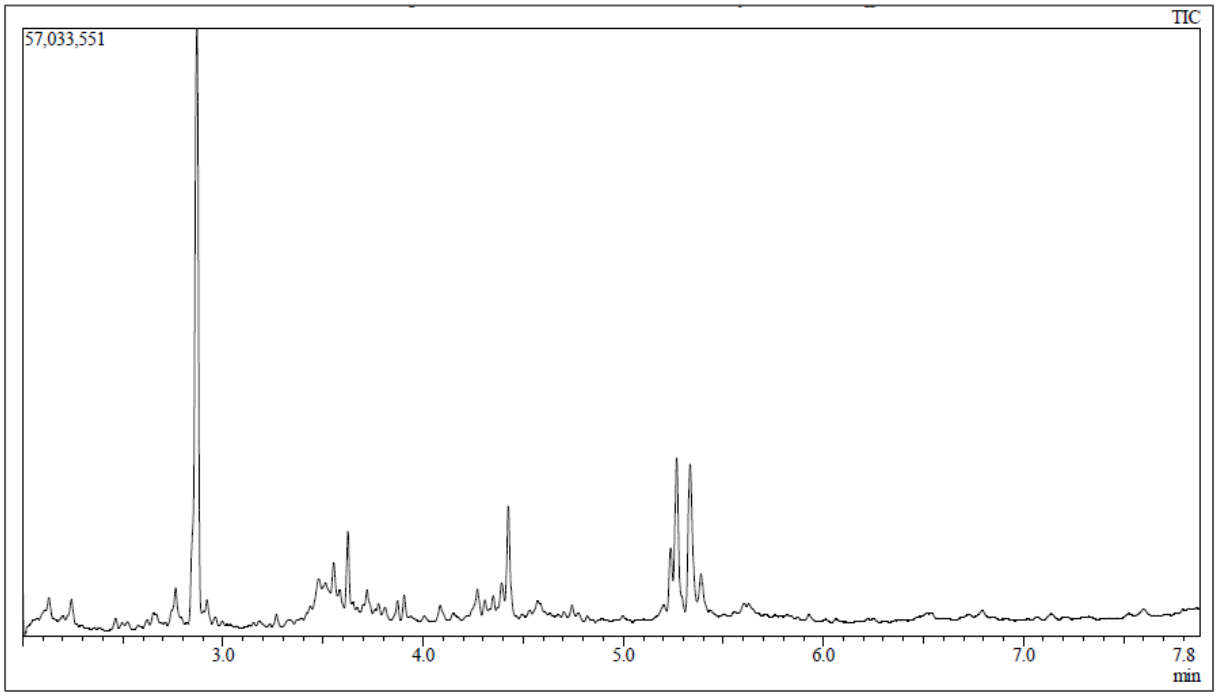

Figure 14. GC-MS chromatogram for microalgae dried biomass biodiesel.

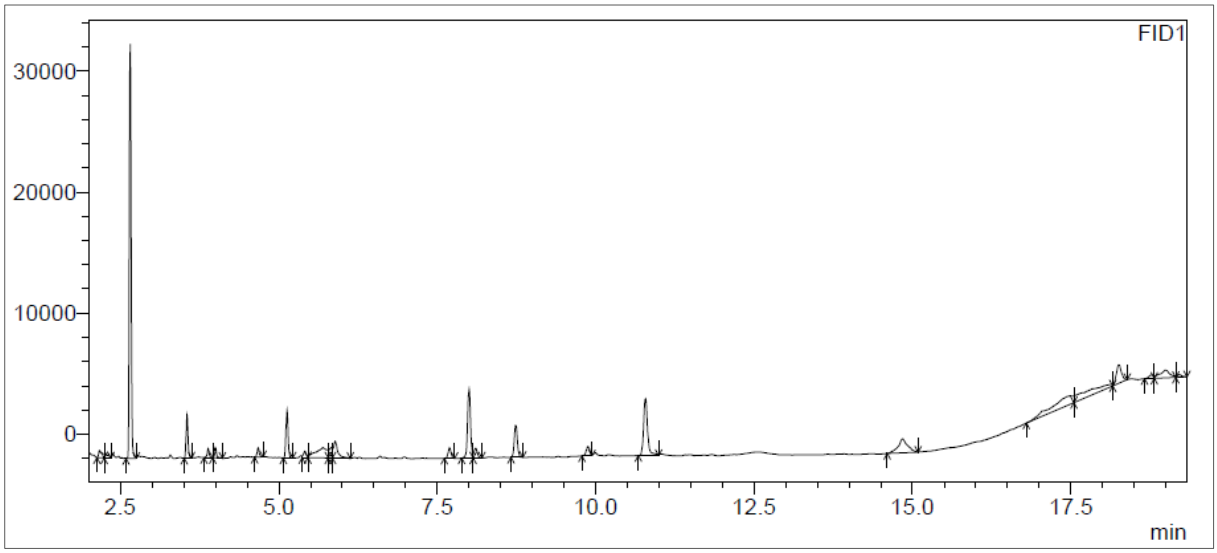

Figure 13. GC-FID chromatogram for microalgae dried biomass biodiesel sample.

Annex C: Effect of CO_2 injection in batch reactors

Table 7. Effect of CO2 injection in batch reactors

Biomass					
3 %		5 %		7 %	
Time [h]	Cellular Density [g mL-1]	Time	Cellular Density [g mL-1]	Time	Cellular Density [g mL-1]
72	280.36±55.14	72	249.81±43.62	72	294.64±93.93
120	428.79±47.21	120	449.11±33.68	120	423.81±39.34
168	508.93±38.41	168	789.29±46.84	168	884.53±56.78
216	804.67±59.53	216	1204.65±36.72	216	1576.79±44.56