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# FATTY ACID PROFILING THROUGH GAS CHROMATOGRAPHY MASS SPECTROPHOTOMETRY (GC-MS) OF *CHLORELLA VULGARIS* AS POTENTIAL FEEDSTOCK FOR BIOFUEL PRODUCTION

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## Abstract

Microalgae has emerged as promising feedstock for production of biodiesel compared to other vegetative resources primarily because of their high photosynthetic ability, high lipid content as well as rapid growth. As an alternative fuel, biodiesel is very imperative for the sustainable development of mankind. The production of Biodiesel process is carried out through upstream and downstream processing to generate cellular biomass to produce fatty acids and lipids via transesterification reaction. In our present study, *Chlorella vulgaris* was grown autotrophically in BG-11 medium as a batch culture. After proper incubation of algae, harvesting, drying and extraction of lipid were carried out with the help of standard procedure *i.e.* Modified Bligh & Dyer's method. Extracted lipid was 28.5% and it was further converted into biodiesel with the help of base catalysed trans-esterification reaction. The identification and quantification of fatty acids was performed using gas chromatography mass spectrometer (GC-MS). The identified fatty acids were dominated by those with carbon chain of C16 and C18; *i.e.* 13-Docosenoic acid, Oleic acid, 13-Eicosenoic acid, Benzenepropanoic acid, Glycidyl oleate, 9-Octadecanoic acid and Erucic acid.

***Key words:*** *Chlorella vulgaris*, Transesterification, Fatty acids, Biofuel, GC-MS.

## Introduction

Energy demand worldwide is increasing continuously at a rapid rate with the increase of urbanization in developing countries. Majority of the world's energy needs are supplied through petrochemical sources, coal and natural gases, with the exception of hydroelectricity and nuclear energy and these sources are finite and at current usage rates will be consumed shortly (Srivastava and Prasad, 2000). Fuels represent around 70% of the total global energy requirements, particularly in transportation, manufacturing and domestic heating (Mata *et al.,* 2010). In near future there will be a 60% increase in global energy requirement by 2030 over its present consumption level leading to more environmental damage. Out of this increase, 45% is accounted by developing countries like India and China as they are new growing economies. Search for clean and renewable energy sources ranks as one of the most daunting challenges for mankind which is intimately linked with economic development, global stability and quality of life (Gouveia and Oliveira, 2009).

First generation biofuels which have now attained economic levels of production, have been mainly extracted from food and oil crops including rapeseed oil, sugarcane, sugar beet and maize as well as vegetable oils and animal fats using conventional technology (FAO, 2008). But these have negative impact on global food markets and on food security (Brennan and Owende, 2010). But in the case of second generation biofuels evolve where biodiesel are produced from the whole plant matter of dedicated energy crops or agricultural residues, forest harvesting residues or wood processing waste (Lim and Teong, 2010). However, the technology for conversion in the most part has not reached the scales of significant commercial exploitation (Demirbas, 2002). In contrast, transition to third generation biodiesel, which are derived from Microalgae have been reported as one of the best sources of biodiesel (Shay, 1993). They can produce up to 250 times the amount of oil per acre compared to soybeans

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(Shay, 1993). In fact, producing biodiesel from microalgae may be the only way to produce sufficient automotive fuel to replace current petro-diesel usage (Chisti, 2007). Furthermore, other biodiesel fuel resources presently used for biodiesel production, algae can be grown on non-arable land with different streams of wastewater and do not compete with the agricultural production of food crops (Brennan and Owende, 2010).

This study mainly focuses on the identification of fatty acids present in *Chlorella vulgaris* through GC-MS as potential feedstock for the development of biofuel production.

## Materials and Methods

### Collection and Isolation of Micro algal Strain

Freshwater sample containing local strains of *Chlorella vulgaris* Fig. 2 was collected from the bloomed freshwater pond of Sheorinarayan, Janjgir- Champa district Chhattisgarh (21.7218° N, 82.5949° E), India during summer season. The pH of the water sample was measured and the alga collected was identified by cross checking with authentic illustration of standard monograph (N. Anand 1998). Serial dilution of water sample followed by micro capillary isolation method and streak plate method were used for the isolation of the alga in pure form. The isolated strain was cultured in BG- 11medium at pH of 7.30.

### Microalgae and culture media

The microalgae used in this study were *Chlorella vulgaris* (SN159) from the cultures collection of the Bioresources Tech Laboratory Guru Ghasidas Vishwavidyalaya Bilaspur (CG). Microalgae were maintained and grown in BG11 medium (Rippka *et al.,* 1979) containing (g/$L^{-1}$): $NaNO_3$ (1.50); $K_2HPO_4$, $3H_2O$ (0.04); $MgSO_4$,$7H_2O$ (0.075); $CaCl_2$, $2H_2O$ (0.036); $C_6H_{11}FeNO_7$ (0.006); disodium EDTA (0.001); $Na_2CO_3$ (0.02); $C_6H_8O7$ (0.006); $H_3BO_3$(2.86); $MnCl_2$, $4H_2O$ (1.81); $ZnSO_4$, $7H_2O$ (0.222); $Na_2MoO_4$, $2H_2O$ (0.39); $CuSO_4$, $5H_2O$ (0.079); $Co(NO_3)_2$, $6H_2O$ (0.0494).

### Preliminary qualitative screening of lipids from Microalgae

#### Chemicals and Reagents

Nile red fluorescent dye (9-(diethyl amino) benzo (a) phenoxazin- 5(5H) - one) was purchased from HiMedia Pty. Ltd. (India). Organic solvent *i.e.* acetone, isopropanol and n-hexane were purchased from commercial suppliers.

### Nile red staining for lipid oil determination in Micro algal

Nile red is a specific stain to quantify the intracellular lipids present in microalgae cells. Nile red stock solution was prepared according to Mohamady *et al.,* (2012). A quantity of Nile red was dissolved in brown bottle containing (0.01g) in 100 ml of acetone (0.25mg/) and this was stored at dark. Each 1 ml of microalgae culture broth was centrifuged at 10000 rpm for 10 minutes and the pellets were ringed with sterile distilled water (equal volume) for several times. After washing with distilled water the cell pellets were mixed with 0.5 ml of Nile red solution incubated for 10 min at room temperature. After stained with Nile red the stained cells observed under fluorescence microscopy.

### Extraction of fatty acid from microalgae

**Fig. 1:** Algal bloom of *Chlorella vulgaris* on water surface.

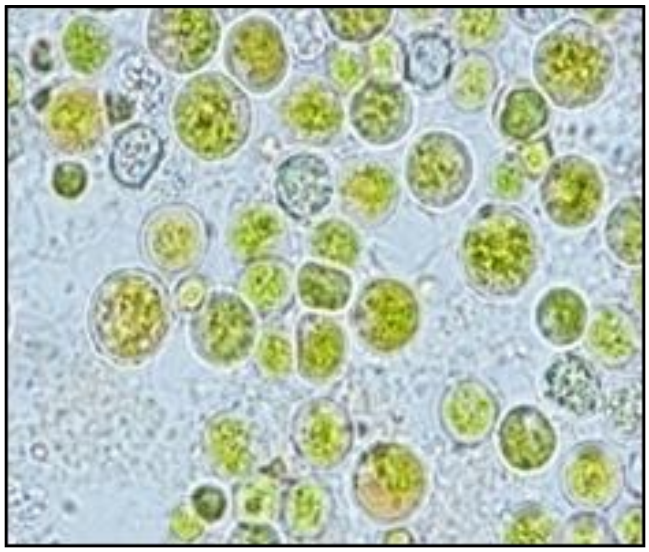

**Fig. 2:** Microphotograph of *Chlorella vulgaris*.

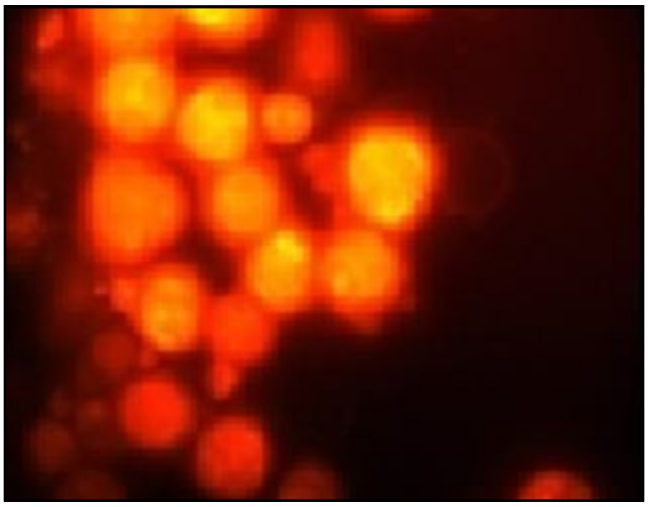

**Fig. 3:** Nile red staining of cellular micro algal cells under fluorescent microscope.

Changes in fatty acids of the well suspended micro alga cells were detected through FAME (Fatty acid methyl ester) analysis by using the protocol of NN with minor modifications. For that, micro alga cells were harvested by centrifugation at 12,000 rpm for 15 min. Approximately 1.0 g dried cell biomass were taken and crushed using pestle and mortar in chloroform: methanol (2:1) reagents. The crushed biomass was filtered through Whatman's no.1 filter paper and was collected in different 15 ml screw cap tube. Then after, the mouth of screw cap tube was remained open till the filtrate became dried and concentrated at room temperature (25ºC). Then after, an equal volume of methanol was added to all the tubes.

**Fig. 4:** Tranesterified lipid oil.

**Transesterification of extracted algal oil**

In the transesterification process fixed volume of the extract was taken and 5 ml sodium methoxide was added for saponification. Later on few drops of boron triflouride-methanol reagent were added and placed in a boiling water bath (100°C) for 10 min and it was immediately placed onto the ice bath. 5 ml of n-Hexane was added microalgae sample respectively and was further incubated at room temperature for 30 min. after some times, two distinct layers were visible. Two thirds of the upper layer containing methyl esters of fatty acid extracted was transferred to the small gas chromatography (GC) vials. 3 µl of the extracted fatty acid methyl ester mixture were subjected to GC-MS analysis.

**Calculation of Fatty Acid Composition of Algal Oil**

The present study calculation of the lipid concentration was defined as dry weight ratio of extracted lipids to biomass. According to Suganya *et*

**Table 1:** List of fatty acids identified through Gas Chromatography-Mass Spectrometry.

| S. No. | Compounds name | Retention time (min) | Area % | Area |
|---|---|---|---|---|
| 1. | Dodecanoic acid | 7.95 | 0.36 | 273016 |
| 2. | Hexadecanoic acid | 13.21 | 0.18 | 132827 |
| 3. | Benzenepropanoic acid | 18.41 | 3.88 | 2902550 |
| 4. | Palmitic acid | 21.06 | 0.14 | 103188 |
| 5. | 9,12-Octadecadienoic acid | 22.34 | 0.40 | 295925 |
| 6. | 9-Octadecenoic acid | 22.50 | 0.34 | 254320 |
| 7. | Triacontanoic acid | 23.14 | 0.21 | 156029 |
| 8. | Oleic acid | 25.17 | 16.59 | 12415609 |
| 9. | 13-Eicosenoic acid | 29.40 | 11.47 | 8585757 |
| 10. | Arachidic acid | 29.804 | 0.93 | 697976 |
| 11. | Undec-10-ynoic acid | 30.574 | 0.97 | 722793 |
| 12. | 9-Octadecenoic acid | 31.016 | 2.25 | 1686681 |
| 13. | 13-Docosenoic acid | 33.121 | 55.57 | 41599986 |
| 14. | Behenic acid | 33.597 | 1.43 | 1067367 |
| 15. | Glycidyl palmitate | 34.789 | 0.86 | 647034 |
| 16. | Erucic acid | 35.847 | 1.63 | 1218916 |
| 17. | Glycidyl oleate | 38.256 | 2.36 | 1770304 |
| 18. | Octadecanoic acid | 40.794 | 0.43 | 324218 |

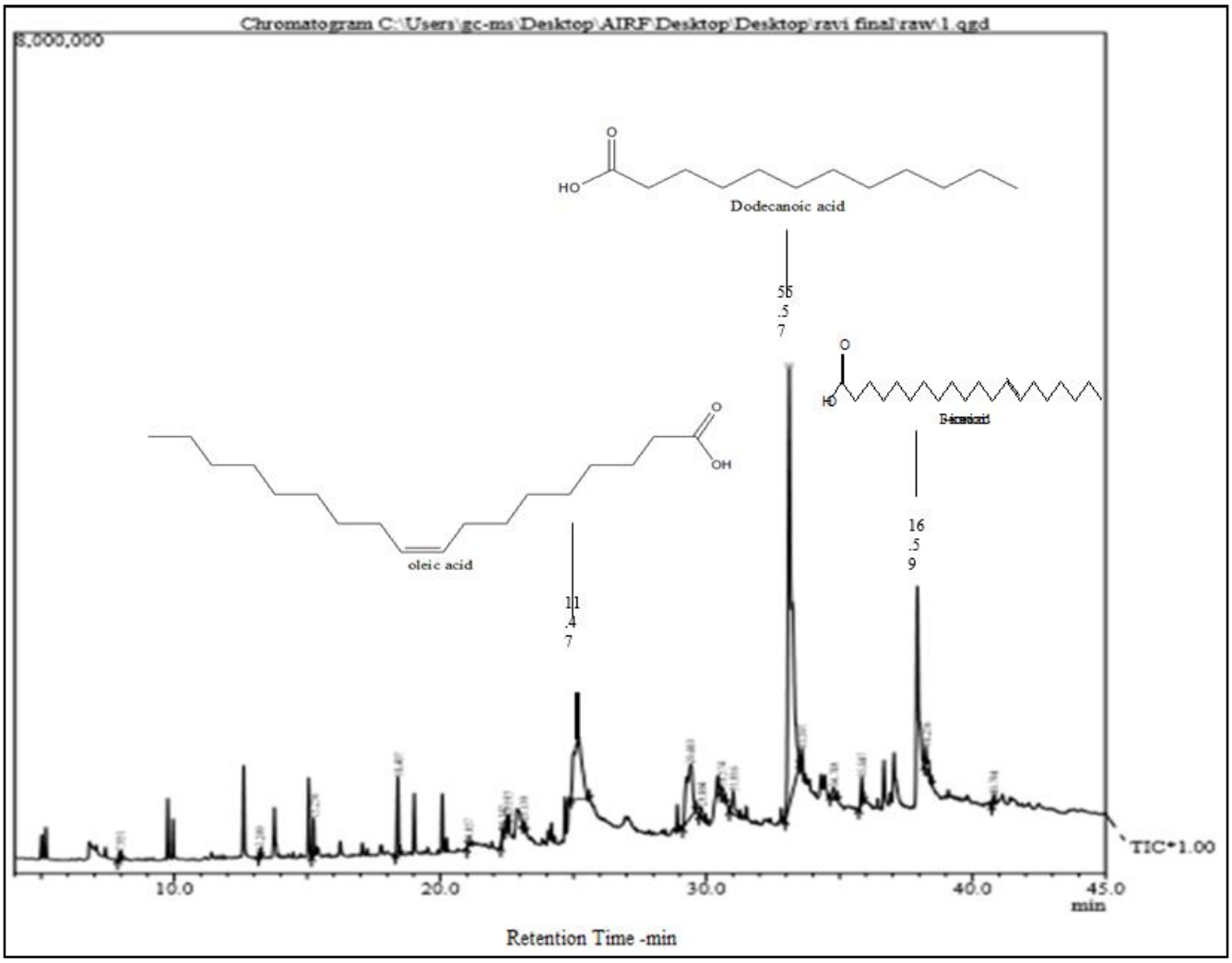


**Fig. 5:** Representative gas chromatogram of fatty acid methyl esters of *Chlorella vulgaris*.

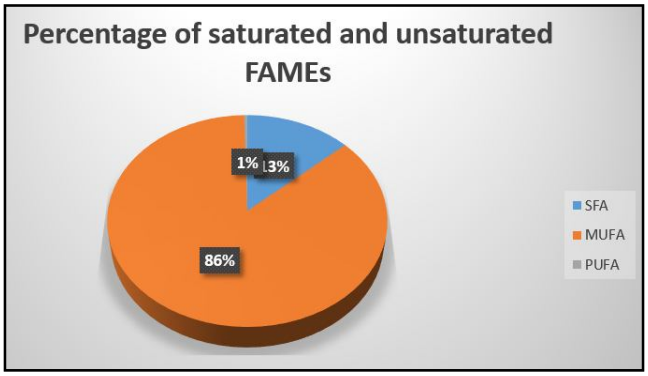


**Fig. 6:** Percentage of SFAs, MUFAs and PUFAs from microalgal strain *Chlorella vulgaris*.

*al.*, (2012), the oil extraction yield (%w/w) was determined by following formula;

Percentage of oil in algae (%) =

$$\frac{Weight\ of\ oil\ (g)}{Weight\ of\ sample\ (g)} \times 100$$

## Fatty acid profiling of microalgae through Gas Chromatography- Mass Spectrometry (GC- MS)

For the quantitative study of transesterified micro algal lipid profiling were done by Gas chromatography coupled with mass spectrometry (GC-MS). Chromatography was performed using a Shimadzu Mass Spectrometer 2010 series system (AIRF, JNU, New Delhi) equipped with a RTX-5 MS. qg. To observed GC MS detection, an electron ionization system with ionization energy of 70eV was used. Helium gas was used as a carrier gas at a flow rate of 1.2 ml per minute. Initial oven temperature is 100°C for 2 min, then 140°C for 1 min and is increased at a rate of 10°C $min^{-1}$ until it reaches 270°C, whereas, Injector temperature is 90°C and a constant helium flow of 1.0 mL $min^{-1}$ is used. A volume of 1 µL was injected. Total fatty acids are identified and quantified by comparing retention times and mass spectrum with known standards calibration curve with concentration ranging from 0.5 to 20 mg/mL Injector and mass transfer line temperature were set at 270°C and 280°C.

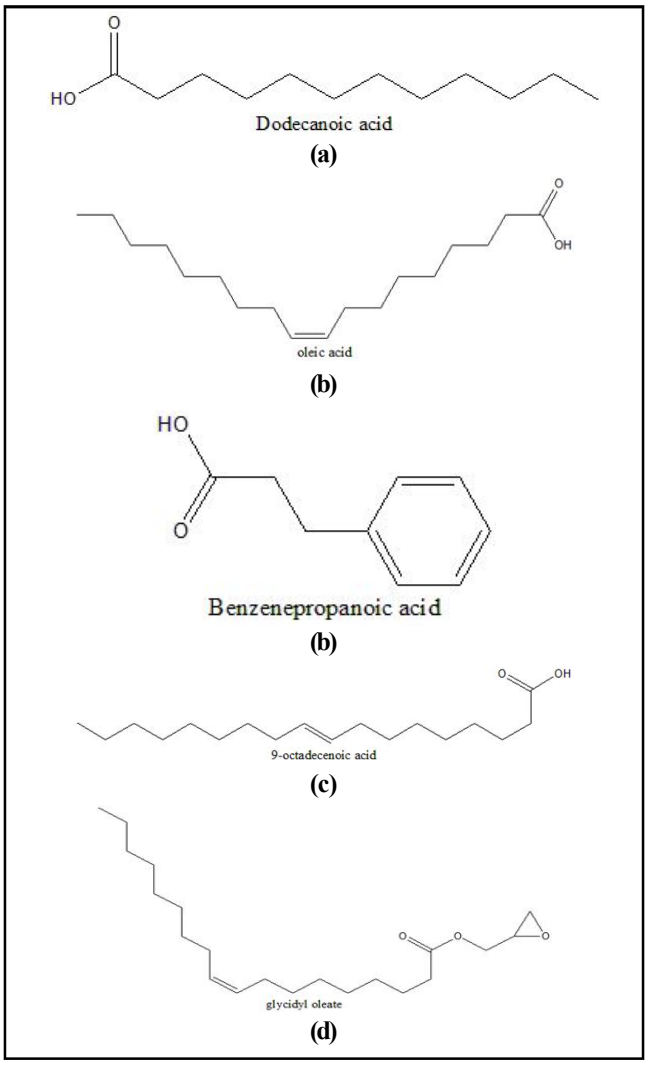


**Fig. 7:** Chemical structure of fatty acids (a) Dodecanoic acid, (b) Oleic acid,(c) Benzenepropanoic acid, (d) 9-Octadecenoic acid,(e) Glycidyl oleate.

## Results and Discussion

Microalgae are good candidate as potential source for sufficient production of renewable fuels to impact consumption of fossil fuels. In our present study, the isolate was studied under binocular microscope and identified with the help of standard monograph. The fatty acid profile of total polar lipid of *Chlorella species* was done by Gas chromatography coupled with mass spectrometry (GC-MS).

### Preliminary Nile red staining

Preliminary observation under the microscope indicated that the Nile red dye was able to interact with the intracellular lipid globules within the Chlorella vulgaris cells. As can be seen in the microscopic images Fig. 3, the lipid globules emitted orange/yellow fluorescence upon interaction with the Nile red dye, while chlorophyll emitted a red auto fluorescence. The choice of excitation and emission wavelengths (530nm and 660nm). Initially, Intracellular lipid droplets of were examined by Nile Red staining under fluorescent microscope with excitation at 450–490-nm and emission at 515-nm. Under the fluorescent microscope neutral lipid or triglycerides appeared as yellow dots, whereas polar lipid and chlorophyll were stained in red colour cells were observed by Nile Red staining shown in Fig. 3. The results indicated that not all algal species could be affected by Nile red staining since oil droplets were not clear and the whole cells were stained in red.

### GC-MS analysis of fatty acid methyl esters

The results pertaining to GC- MS analysis of the FAME composition and comparative fuel properties is play an important role for species selection for biofuel production. The identified fatty acid methyl esters (FAMEs) of the TKP159 isolate lipids were presented in table 1. There were total eighteen identified fatty acids *i.e*. Dodecanoic acid; Hexadecanoic acid; Benzenepropanoic acid; Palmitic acid; 9, 12 Octadecadienoic acid; 9-Octadecenoic acid; Triacontanoic acid; Oleic acid; 13-Eicosenoic acid; Arachidic acid; Undec-10-ynoic acid; 9-Octadecenoic acid; 13-Docosenoic acid; Behenic acid; Glycidyl palmitate; Erucic acid; Glycidyl oleate; Octadecanoic acid. The most abundant identified fatty acid was Glycidyl oleate with the content of 55.57 percent followed by Oleic acid 16.59% and 13-Eicosenoic acid 11.47 percent respectively. Fatty acid composition of microalgal lipid was analyzed by GC- MS Fig. 5. From the analysis, the saturated fatty acids (86%) were found to be more when compared to the unsaturated fatty acids MUFAs (13%) followed by PUFAs (1%) respectively shown in Fig. 6. Basu and Norris (1996) have studied that produce esters from feed stocks that have a high FFA content, diglycerides and monoglyerides, using calcium and barium acetate as a catalyst.

In the aspect production of biodiesel, the fatty acid profile is considered to be significant as that of the total fatty acid content. *Chlorella vulgaris* showed the major fatty acids are Glycidyl oleate, Oleic acid and 13-Eicosenoic acid. It was previously studied that Palmitic acid, stearic acid and linolenic acid were recognized as the most common fatty acids in biodiesel (Knothe, 2008). The present work investigated the efficiency of *Chlorella vulgaris* (SN159) as source of biodiesel with respect to total lipid content and lipid profile. The test strain showed moderate lipid content with promising lipid profile.

## Conclusion

*Chlorella vulgaris* have proven to be one of the

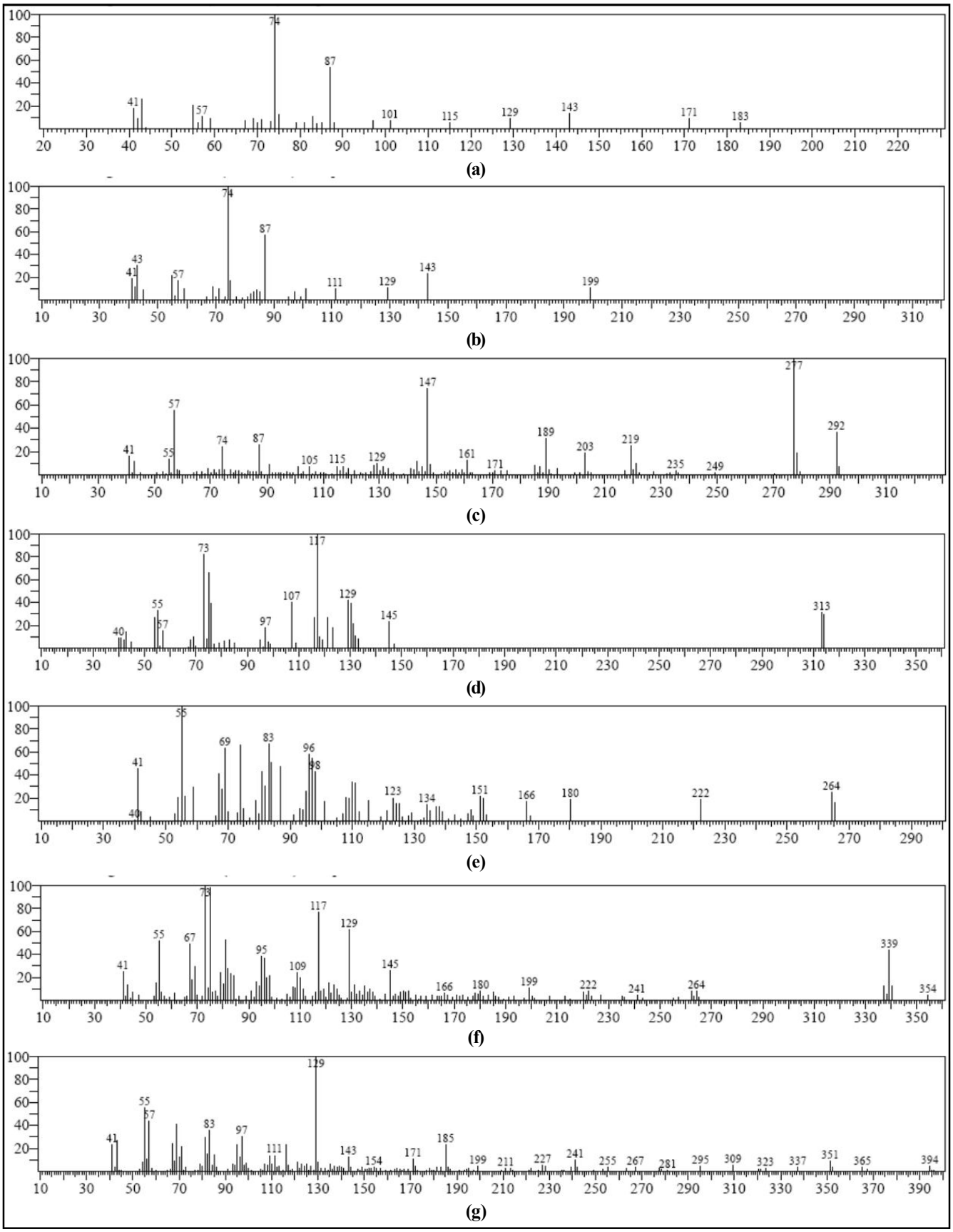


**Fig. 8:** Gas Chromatogram of fatty acids (a) Dodecanoic acid, (b) Hexadecanoic acid, (c) Benzenepropanoic acid, (d) Palmitic acid, (e) 9-Octadecenoic acid,(f) Oleic acid, (g) Glycidyl oleate.

best promising feed stocks for the production of third-generation biofuels that are both economically feasible and environmentally sustainable. Rapid, accurate, sustainable and cost-effective methods for the lipid extraction and quantification are essential for the rational application of microalgae-based biofuel production. The modified Bligh and Dyer method (1959) is suitable for total lipid extraction and may be applied for screening of *Chlorella vulgaris* for qualitative and quantitative analyses of total fatty acids. In conclusion, this paper highlights the role of qualitative composition of micro algal fatty acids and demonstrates the dependence of biodiesel fuel properties such as MUFA, PUFA and SFA on the FAME profile.

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### Conflict of interest

The authors hereby declare that there is no conflict of interest.

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