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Supercritical Carbon dioxide Treatment of the Microalgae Nannochloropsis oculata

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*Graphical Abstract

Nannochloropsis oculata

treated with supercritical CO_2 and co-solvents

produce a range of fatty acid methyl esters dependant on temperatures and pressures

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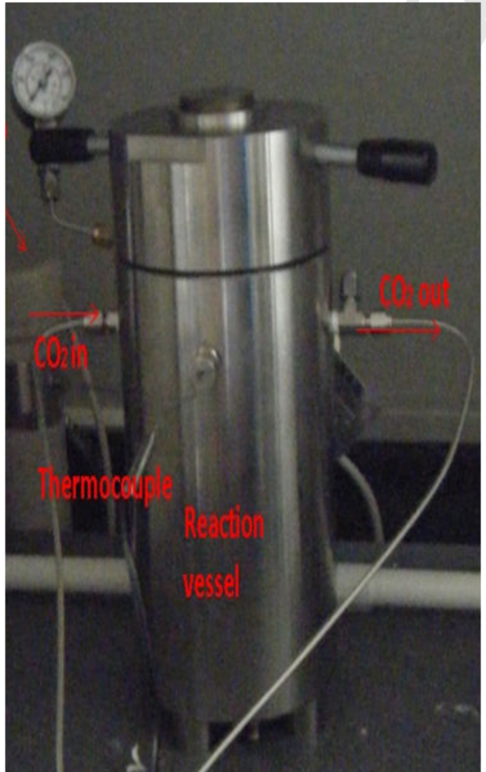

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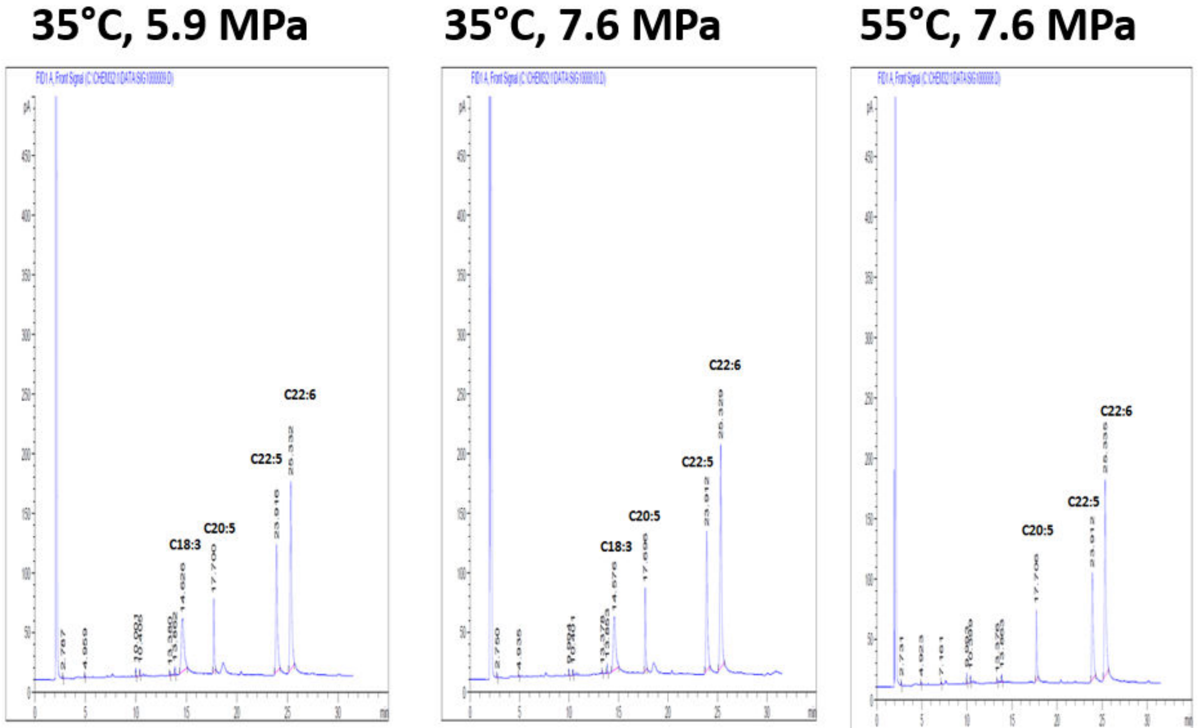

Highlights

- Extraction of fatty acid methyl esters from *Nannochloropsis oculata* using Supercritical fluids at low temperatures and pressures
- Using methanol as a co-solvent, extraction and transesterification was achieved in a single step
- Long chain FAMEs such as EPA and DHA were produced using methanol as a co-solvent
- Using hexane as a co-solvent, higher amounts of fatty acid methyl esters were obtained using 5.9 MPa than at 7.6 MPa

Supercritical Carbon dioxide Treatment of the Microalgae *Nannochloropsis oculata* for the production of Fatty Acid Methyl Esters

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Abstract

The aim of this work was to evaluate the potential of supercritical carbon dioxide (CO_2) to extract Fatty Acid Methyl Esters (FAME) from the microalgae *Nannochloropsis oculata* (*N. oculata*) at low temperatures (37 and 55 ℃) and pressures (5.9 and 7.6 megapascals (MPa)). A qualitative gas chromatography (GC) analysis showed that the individual FAMEs extracted varied depending on the co-solvent (methanol or hexane) used with supercritical CO_2. Using hexane, FAME compounds produced were similar to those extracted with soxhlet extraction alone while longer chain FAME were produced when methanol was the co-solvent. The effects of pressure and temperature variations were shown to be of statistical significance. The chromatograms produced in this work demonstrate that altering one of these parameters (co-solvent, temperature, pressure) can produce different compounds owing to the tunability of the technique.

Keywords: Supercritical fluids; microalgae; monounsaturated fatty acids; polyunsaturated fatty acids; biodiesel; gas chromatography

Abreviations

μm	micrometres.
N. oculata	*Nannochloropsis oculata*.
ANOVA	Analysis of Variance.
C	Carbon.
CO_2	Carbon dioxide.
DHA	Docosahexaenoic Acid.
DPA	Docosapentaenoic Acid.
EPA	Eicosapentaenoic Acid.
FAME	Fatty Acid Methyl Esters.
FID	Flame Ionization Detector.
g	grammes.
GC	Gas Chromatography.
He	Helium.
ml	millilitre.

MPa Megapascals.

MUFA Monounsaturated fatty acids.

PUFA Polyunsaturated fatty acids.

rpm revs per minute.

SCF Supercritical Fluids.

sp. species (singular).

TS total solids.

1 Introduction

In previously published work supercritical methanol [1] and CO_2 [2] have been used to extract and transesterify fatty acids to biodiesel in a single step using high temperatures and pressures. The aim of this work was to use supercritical CO_2 at low temperatures and pressures to convert microalgal oils to biodiesel in a single step, thus saving time and money. Milder conditions have been found to be less destructive to natural substrates such as algae [3]. Lower temperatures and pressures showed the production of long chain FAMEs which are of interest owing to their potential health benefits and other uses.

1.1 The Composition and Uses of Microalgae

Microalgae are rich sources of MUFA (monounsaturated fatty acids) and PUFA (polyunsaturated fatty acids) which are of interest in biodiesel production as well as human and animal health. Examples of MUFA and PUFA structures can be seen in figure 1. MUFAs are fatty acid chains containing one unsaturated C-C bond i.e double or triple bonds while PUFAs have more than one unsaturated C-C bond. Both compounds are of interest in the production of biodiesel [4]. There are a range of criteria which are required to identify a reliable substrate for biodiesel production including iodine value, heating value, and lubricity but a high MUFA and low PUFA composition is thought to be preferable [5]. However, sunflower oil has high PUFA content and can produce biodiesel which meets the requirements of the European legislation [6]. Of the total fats produced by *N. oculata* between 35 and 46% are MUFAs and between 8 and 22% are PUFAs [7].

Figure 1. Example of MUFA (oleic acid) and PUFA structure (linoleic acid) [8]

Nannochloropsis is a marine species in the Eustigmatophyceae class of microalgae [9] which has previously been studied as a source of biodiesel production [1], [10], [11], [12], [13], [14]. Under optimal growth conditions *N. oculata* has a lipid content above 50% [15] making it a substrate of interest in biodiesel production. Large amounts of microalgal biomass can be produced in a small space [16]. Biomass can double daily [17] and is high in the valuable fatty acid, EPA (eicosapentaenoic acid) when compared to other microalgae [18].

1.2 The Biodiesel Production Process

Biodiesel has been defined as the conversion of renewable animal and vegetable long chain fatty acids to FAMEs [19]. Transesterification reduces the viscosity of the fats so that it can be used in vehicle engines [20]. When compared to petroleum based diesel, the emissions from biodiesel are lower in pollutants such as unburned hydrocarbons, carbon monoxide and particulate matter [21], [22].

Before the biodiesel refinement process begins, the fatty acids must be separated from the other components of the substrate (in this case, the microalga). A number of techniques have been used to achieve this, among them microwave assisted extraction and ultrasound assisted extraction [23] but soxhlet extraction has traditionally been used and that is the technique referenced in this work. The soxhlet extraction procedure involves using a solvent such as hexane or petroleum ether to dissolve the oils of interest [24].

After the soxhlet extraction is completed the lipids are transesterified to biodiesel. Oils extracted from the substrate by soxhlet extraction consist of a glycerol backbone with 3 long chain fatty acids [25]. Glycerol is separated from the long chain fatty acids during transesterification by reacting the compound in excess alcohol and a catalyst. Water and free fatty acids residues have been shown to have a negative effect on FAME production. This effect is minimised by using an alkali catalysed reaction followed by an acid catalysed procedure [26], [27].

McDaniel and Taylor [28] demonstrated the feasibility of the incorporation of the transesterification step into the supercritical extraction process. In recent times the use of supercritical methanol (260 ℃ and 8.3 MPa), [1], [10] and supercritical ethanol (245 - 270 ℃ and 8.3 - 9.3 MPa) [29] to extract and transesterify fatty acids from *Nannochloropsis* species (sp.) in a single step has been achieved. Andrich et al. [30] found supercritical CO_2 was comparable to hexane soxhlet to extract bioactive lipids from *Nannochloropsis* sp. Supercritical CO_2 (40 ℃, 40 MPa) was followed by transesterification to extract the polyunsaturated EPA from *Nannochloropsis*. Some of the conditions investigated in the production of biodiesel by microalgae to date are outlined in table 1.

Table 1.Previously investigated Supercritical Fluids (SCF) conditions relating to biodiesel production from microalgae, * Pressure not reported but the pressure required for supercritical water is 22.1 MPa

In this work supercritical CO_2 at low temperatures and pressures and using a co-solvent was used to identify if soxhlet and transesterification steps could be skipped completely to produce biodiesel directly.

1.3 The use of supercritical fluids with natural substrates

The use of various SCFs have been compared favourably to other extraction techniques with plant and algal samples. High temperatures and pressures have been used in some cases [31], [32], [33], [34], [35], [36] and [37]. The use of co-solvents such as ethanol or methanol have been found to assist the extraction of non-polar lipids [35], [38].

Cheung et al. [37] found the treatment of the seaweed *Sargassum hemi-*

phyllum with supercritical CO_2 gave a lipid yield equivalent to a methanol/ chloroform soxhlet extraction and increased the proportion of EPA produced. When comparing soxhlet extraction and supercritical CO_2 Punín Crespo and Yusty [31] found extraction of aliphatic hydrocarbons from the brown seaweed, *Undaria pinnatifida* to be preferable using soxhlet extraction. Varying pressures were used to extract different components and different amounts of those components from the brown algae *Dilophus ligulatus* [32], [33]. Supercritical CO_2 and thermochemical liquefaction are compared in the extraction of biodiesel from the green seaweed *Chaetomorpha linum* by Aresta et al. [39]. A higher amount of long chain fatty acids and polyunsaturated fatty acids were obtained from the SCF procedure when compared to the thermochemical liquefaction process. In the work of Halim et al. [40] decreasing pressure and increasing temperature resulted in increased lipid production, while Mendes et al. [41] found that a decrease in pressure was more effective. When using supercritical ethanol Levine et al. [42] found that higher temperatures were more productive.

Reverchon [3] suggests that lower temperatures (40 - 50°C) and lower pressures (below 10.3 MPa) are more selective and less damaging for natural products. When extracting oil from ginger with supercritical CO_2, Roy et al. [43] observed that when higher pressures were used higher temperatures were preferable while at low pressures, lower temperatures were more effective. In this work relatively low temperatures and pressures were used to establish the effect of supercritical CO_2 on the extraction of FAME from *N. oculata* for use in biodiesel and human health products.

2 Materials and Methods

2.1 Supercritical Fluid Microalgal Study

Investigations were undertaken on the microalgae, *N. oculata* to evaluate the potential of using supercritical CO_2 to extract FAMEs for biodiesel and other applications.

The microalgae *N. oculata* used in this work was cultivated by researchers at Daithi O'Murchu Marine Research Station (DOMMRC) from stocks sourced from the Culture Collection of Algae and Protozoa (CCAP), based at the Scottish Association for Marine Science (SAMS) in Oban, Scotland. Cultures were grown in f/2 medium and after harvesting were freeze-dried for 24 hours. The average total solids (TS) of the freeze dried samples was 34.9%.

It was then subjected to a range of SCF treatments followed by soxhlet extraction in some instances. These extraction procedures were compared to traditional soxhlet extraction. Samples were analysed by liquid injection GC and compared to a previously run Carbon (C) standard containing all of the even FAMEs from C8 to C24 which are commonly found in oil samples used for biodiesel production.

Statistical significance was established by ANOVA (Analysis of Variance) in Microsoft Excel.

2.2 Supercritical Experimental conditions:

A supercritical fluid apparatus was used which was custom built by SCF Processing Ltd., Drogheda, Ireland. The supercritical fluid used was CO_2. Triplicate 10 grammes (g) samples of *N. oculata* were exposed to SCF treatment with co-solvents methanol (Fisher Scientific) or hexane (Fisher Scientific) using the temperatures and pressures outlined in table 2.

Table 2.Experimental conditions for SCF treatments used in *N. oculata* investigations

The following conditions were also used in all experiments:

Volume of co-solvent: 20 millilitres (ml)
Mode: static
Run time: 30 minutes
Depressurisation: 1-2 minutes

Traditional soxhlet extraction followed by transesterification was applied to a microalgal sample which was not treated with SCF. As outlined in table 2, where methanol was used as a co-solvent in the SCF treatment, samples were not subjected to the soxhlet or transesterification procedures. Transesterification was applied after the supercritical treatment where hexane was the co-solvent used.

Soxhlet extraction was carried out according to the method outlined in BS EN ISO 734:2015 [44] and oils were transesterified in accordance with BS EN

ISO 12966-2:2011 [45] using hexane to dissolve the sample in place of isooctane.

All samples were then centrifuged at 5000 revs per minute (rpm) for 5 minutes and the supernatant was analysed by GC.

2.3 Gas Chromatography Analysis

A method developed in compliance with European Standard EN14103 [46] which is suitable to analyse FAMEs between C_{14} and C_{24} was used to analyse the prepared microalgal samples. Qualitative GC analysis was carried out on an Agilent 7890A GC.

Samples were run using the following conditions:

Column: Carbowax 20M 30 m x 0.32 mm x 0.25 micrometres (μm)

Oven Program: Initial temperature 150 ℃ then 5℃ per minute to 220℃

Hold for 17.5 minutes

Injector split/splitless: 70:1 at 220℃

Pressure: 0.07 MPa Helium (He)

Detector: Flame Ionization Detector (FID)

Detector temperature: 250 ℃

3 Results and Discussion

This study was undertaken to evaluate the potential of supercritical CO_2 at low temperatures and pressures to extract and transesterify oils from *N. oculata* in a single step. This aim was achieved. Using supercritical CO_2 with methanol as a co-solvent FAMEs were obtained from *N. oculata* at 35 ℃ and 5.9 and 7.6 MPa. FAMEs were also produced at 55 ℃ and 7.6 MPa but at 55 ℃ and 5.9 MPa no supernatant was collected. The cost of biodiesel is debated currently [47] and any process which improves the economic feasibility and thus the use of renewable energy sources over fossil fuels is to be welcomed.

The effectiveness of traditional soxhlet extraction was compared to extraction using supercritical CO_2 with hexane as a co-solvent at low temperatures and pressures. Similar FAMEs were produced by both extraction techniques.

Chromatograms collected from the microalgal extracts were compared to a standard which contained even numbered FAMEs as these are commonly used in biodiesel production as outlined in EN14103.

3.1 Using methanol as a co-solvent with supercritical CO_2 for Long Chain FAMEs production

In the case of the methanolic/ SCF supernatant sample the chromatograms showed the production of different compounds from those collected using hexane. The relevant chromatograms are shown in figure 2. A number of peaks were detected later in these chromatograms than in the hexane chromatograms suggesting the presence of compounds with higher boiling points and therefore longer carbon chains.

In all 3 methanol derived chromatograms, peaks were detected which are not present in the standard or the other samples: at 17, 23 and 25 minutes. From the standard chromatogram and comparing to another GC application note [48], the unidentified peak at 17 minutes can be identified as a polyunsaturated C20 compound, potentially EPA (C20:5) which has previously been found in high concentrations in *N. oculata* [18]. The peaks at 23 and 25 minutes are found between the identified peaks - C22:1 and C24:0 which suggests that they are polyunsaturated C22 compounds - potentially DPA (docosapentaenoic acid) (C22:5) and DHA (docosahexaenoic acid) (C22:6).

The SCF with methanol treatment produced a higher proportion of longer chain fatty acids than either the soxhlet or SCF treated with hexane. In biodiesel production the fatty acid chain length together with degree of saturation are important quality criteria [49]. The SCF with methanol produced only PUFA and while present research suggests that high quantities of PUFAs are undesirable in biodiesel [4], many proven sources of biodiesel which meet the required standards have high levels of PUFA e.g. sunflower oil [6]. Further extensive testing in line with EN14214 [50] would identify the suitability of the SCF and methanol treated samples specifically for biodiesel production .

It is of note also that there is a peak at 14 minutes which corresponds to linolenic acid (C18:3) in figure 2 in both 35℃ samples which is absent in the 55℃ sample presented here. This implies that it is possible to change supercritical parameters to optimise the production of individual fatty acids. There is a maximum limit of 12.0% linolenic acid in biodiesel outlined in EN14103 [46].

EPA, DPA, DHA and linolenic acids are omega-3 fatty acids which have beneficial cardiovascular effects on human health [51]. They have been shown to have a positive impact on mental health [52] and are used in nutrient supplements and infant formulae [53]. MUFAs also have many beneficial health effects including diabetes treatment [54] and PUFAs are beneficial in heart disease [55], cancer prevention [56], and skin inflammation [57].

No chromatogram is shown for supercritical CO_2 and methanol at 55 ℃ and 5.9 MPa as no supernatant was produced by centrifugation. A larger sample size or increased intensity of centrifugation would increase the possibility of obtaining a supernatant for analysis.

Figure 2. Chromatograms from the methanol with supercritical CO_2 treatments of *N. oculata*

3.2 Using hexane as a co-solvent with supercritical CO_2 for biodiesel production

While the chromatograms collected from the supercritical CO_2 and hexane extractions (figures 4 and 5) showed peak retention times similar to the data collected with the soxhlet extraction (C14:0, C16:0, C16:1, C18:1), the intensity of the peaks were not as high. The total area response for the peaks of interest at the lower pressure (5.9 MPa) was found to be 5738 area counts. This was twice the response of of the higher pressure used (7.6 MPa) at 2585 area counts. An additional peak in both hexane SCF derived chromatograms which is not present in either the soxhlet extracted or the methanol chromatograms is found at 21.1 minutes. This was identified from the standard as C22:0, behenic acid.

Behenic acid is thought to have properties which have a negative effect on biodiesel at low temperatures [4] but which is valuable for its lubricating properties [58] and is also used in hair products [59].

Figure 3. Chromatogram from the hexane soxhlet extraction

Figure 4. Chromatogram from the hexane and supercritical CO_2 at 5.9 MPa and 35℃

Figure 5. Chromatogram from the hexane and supercritical CO_2 at 7.6 MPa and 35℃

3.3 Statistical Analysis of Results

Peak areas were compared and analysed using ANOVA. The results presented in table 3. Differences were considered significant at $p<0.05$. Accordingly, the changes in temperature and pressure were found to be statistically significant when the methanol samples were analysed and in the case of the hexane analysis a p-value of 0.05 was obtained suggesting that the null hypothesis at 95% cannot be rejected and it is also a statistically significant result.

Table 3: Statistical ANOVA results for FAME produced

4 Conclusion

This work provides evidence that it is possible to use supercritical CO_2 with various co-solvents at low temperatures and pressures to extract FAMEs for use in biodiesel and to produce the rarer longer chain fatty acids, of interest in human health and other commercial applications.

The presence of peaks in the methanolic extracts shows that extraction and transesterification is achievable in a single step process with supercritical CO_2 using methanol as a co-solvent at low temperatures and pressures. This is a more economical solution than the previously proven use of supercritical methanol at high temperatures and pressures as outlined by Patil et al. [1] and Jazzar et al. [10]. The temperatures required to obtain supercritical methanol (260 ℃) are almost 10 times higher than those used here, indicating that lower energy costs would be incurred using this process. Additionally, the cost of methanol ($549 per ton) [60] as the primary supercritical fluid is 5 times higher than that of CO_2 at $160 per ton [61]. Additionally, a wider range of compounds were produced in this work when compared to Patil et al. [1].

EPA, DPA, DHA and linolenic acid were found in the samples which were treated with methanol as a co-solvent. Linolenic acid was not present in the methanol sample collected at 55 ℃, 7.6 MPa.

SCF and soxhlet with hexane produced similar peaks demonstrating the possibilities of the SCF process in biodiesel production. The SCF treatment with a hexane co-solvent at 5.9 MPa produced double the quantities of FAME than

7.6 MPa demonstrating that higher pressures do not always produce higher extraction efficiencies.

The variation in the compounds produced using different co-solvents and pressures demonstrates the tunability of supercritical CO_2 to produce the product required.

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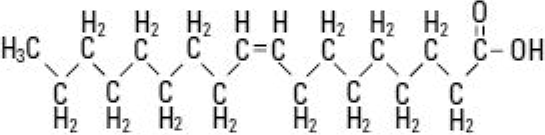
Figure

Oleic acid

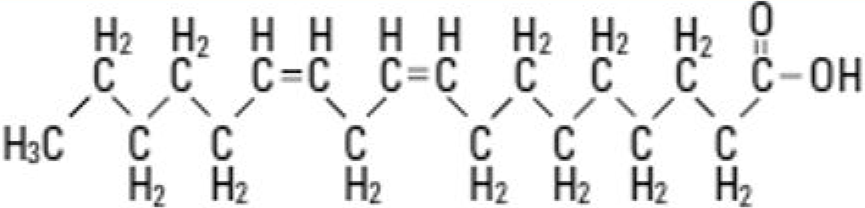
$C_{18}H_{34}O_2$



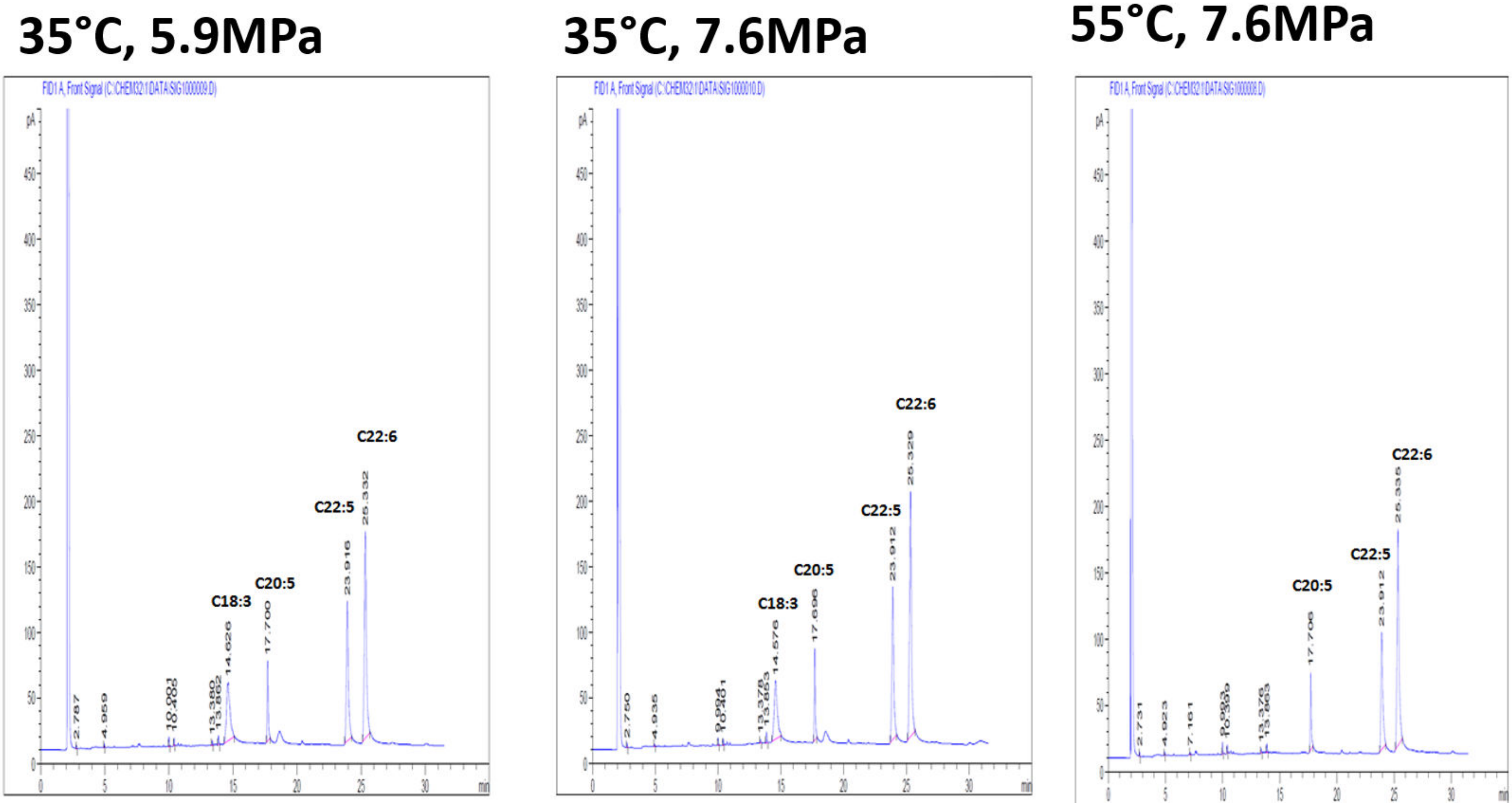
Figure

Linoleic acid

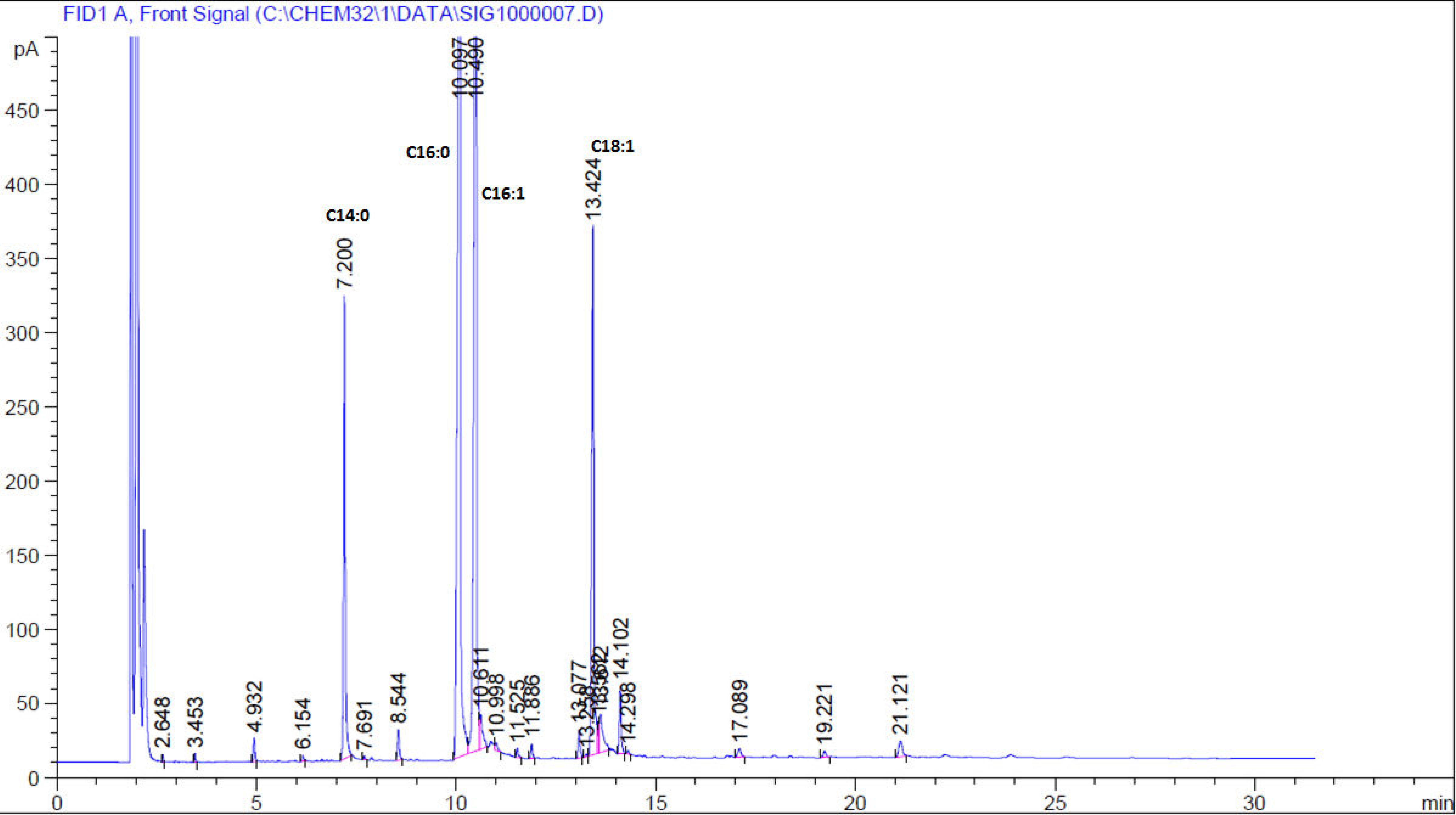
$C_{18}H_{32}O_2$



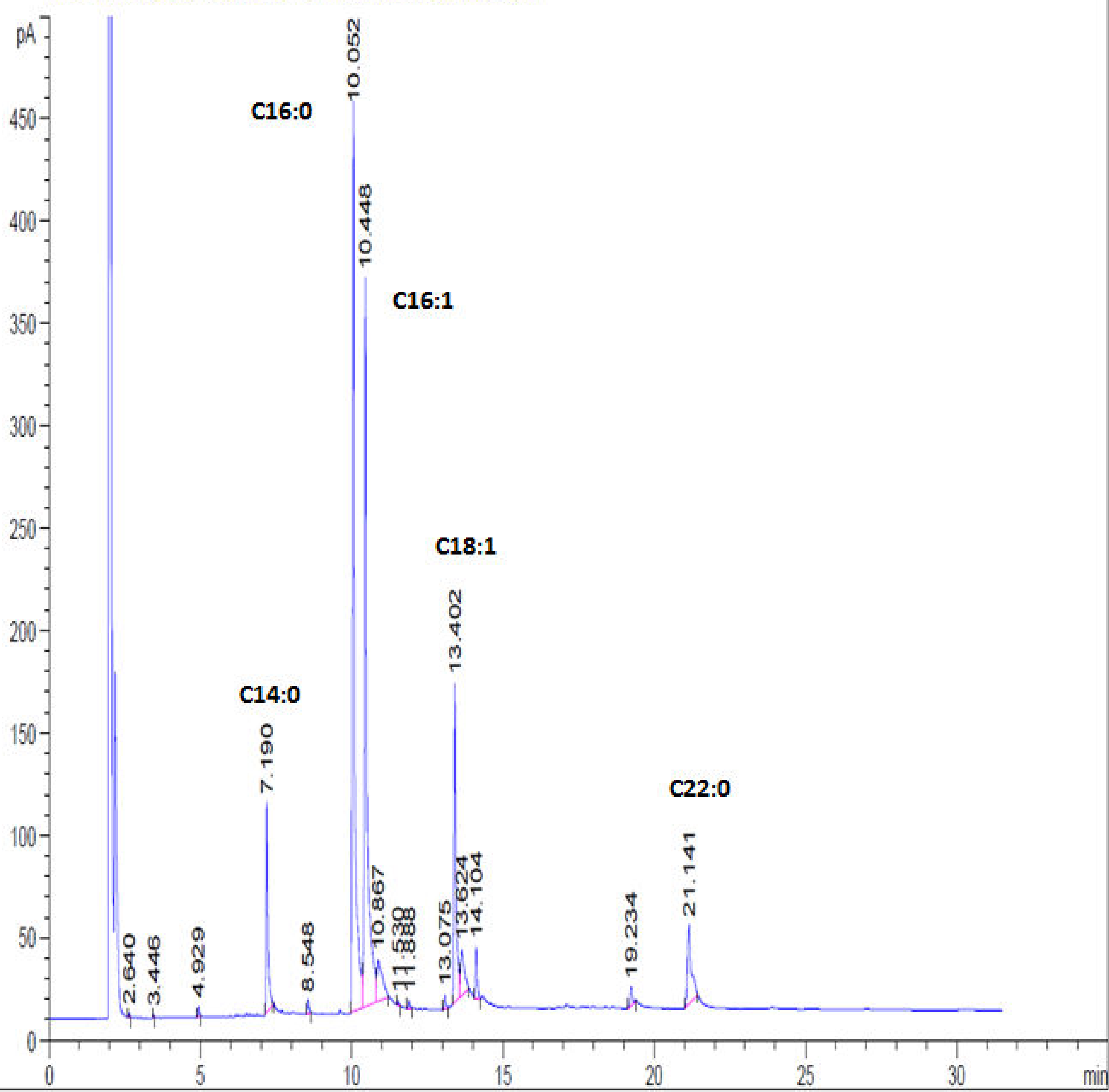
Figure


35°C, 5.9MPa
FID1 A, Front Signal (C:\CHEM32\1\DATA\SIG1000009.D)
pA
450
400
350
300
250
200
150
100
50
0
0
5
10
15
20
25
30
min
2.787
4.959
10.001
10.400
13.380
13.862
14.626
17.700
23.916
25.332
C18:3
C20:5
C22:5
C22:6
35°C, 7.6MPa
FID1 A, Front Signal (C:\CHEM32\1\DATA\SIG1000010.D)
pA
450
400
350
300
250
200
150
100
50
0
0
5
10
15
20
25
30
min
2.750
4.936
9.994
10.401
13.378
13.853
14.576
17.696
23.912
25.329
C18:3
C20:5
C22:5
C22:6
55°C, 7.6MPa
FID1 A, Front Signal (C:\CHEM32\1\DATA\SIG1000008.D)
pA
450
400
350
300
250
200
150
100
50
0
0
5
10
15
20
25
30
min
2.731
4.923
7.161
9.993
10.399
13.376
13.863
17.706
23.912
25.335
C20:5
C22:5
C22:6

Figure


FID1 A, Front Signal (C:\CHEM32\1\DATA\SIG1000007.D)
pA
0
50
100
150
200
250
300
350
400
450
C14:0
C16:0
C16:1
C18:1
2.648
3.453
4.932
6.154
7.200
7.691
8.544
10.097
10.490
10.611
10.998
11.525
11.886
13.077
13.424
14.102
14.298
17.089
19.221
21.121
0
5
10
15
20
25
30
min

Figure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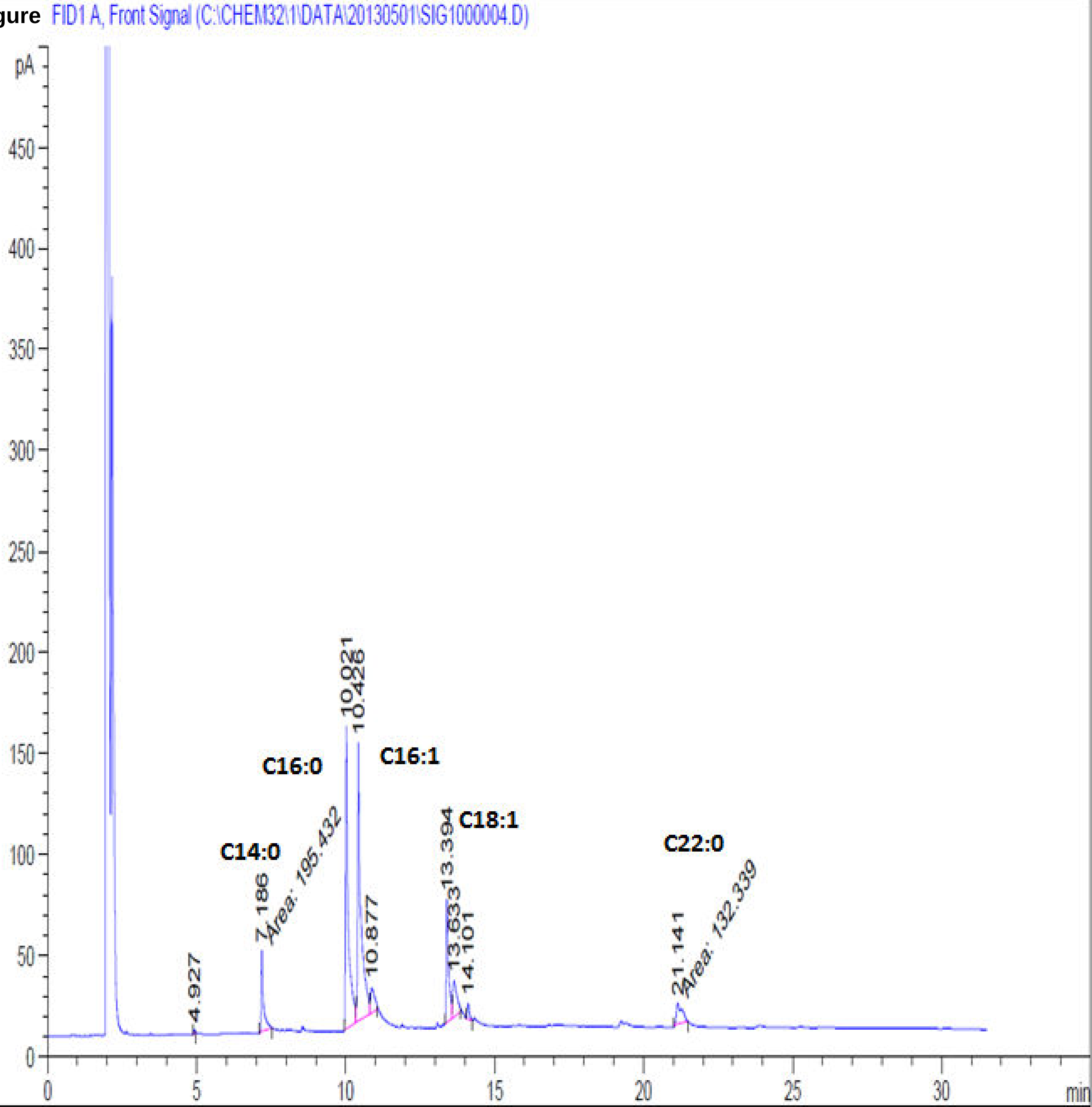
400
350
300
250
200
150
100
50
0
0
5
10
15
20
25
30
min
C14:0
C16:0
C16:1
C18:1
C22:0
2.640
3.446
4.929
7.190
8.548
10.052
10.448
10.867
11.530
11.888
13.075
13.402
13.624
14.104
19.234
21.141

Figure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pA
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400
350
300
250
200
150
100
50
0
0
5
10
15
20
25
30
min
4.927
C14:0
7.186
Area: 195.432
C16:0
10.021
10.428
C16:1
10.877
13.394
C18:1
13.633
14.101
C22:0
21.141
Area: 132.339

Species	SCF	Temp. (°C)	Pressure (MPa)	Reference
Botryococcus braunii	CO_2	40	30	Mendes et al. [41]
Chlorococcum sp.	CO_2	60	31	Halim et al. [40]
Scenedesmus dimorphus	CO_2	100	41	Soh and Zimmerman [2]
Nannochloropsis sp.	Methanol	260	8.3	Patil et al. [1]
Chlorella vulgaris	Water	250	NA	Levine et al. [42]

	Temperature (°C)	Pressure (MPa)	Solvent	Solvent:CO_2 volume ratio	Transesterification	Centrifugation
1	37	5.9	Hexane	1:3	Yes	Yes
2	37	7.6	Hexane	1:12.5	Yes	Yes
3	37	5.9	Methanol	1:2.5	No	Yes
4	37	7.6	Methanol	1:20	No	Yes
5	55	5.9	Methanol	1:2	No	Yes
6	55	7.6	Methanol	1:10	Yes	

Variable	p-value	F	F crit
Methanol pressure	0.36	1.14	10.13
Methanol temperature	0.40	0.97	10.13
Hexane	5.59 x10^{-2}	7.12	7.71