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Use of supercritical CO_2 to obtain extracts with antimicrobial activity from *Chaetoceros muelleri* microalga. A correlation with their lipidic content.

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Abstract

Supercritical CO_2 extracts of the marine diatom *Chaetoceros muelleri* (gracilis) have been investigated for their potential use as food preservatives, namely, as antimicrobials. A screening of different pressures and temperatures for supercritical CO_2 extraction was assayed in order to determine the main factors controlling the yield and antimicrobial activity of the extracts. Since the potential antimicrobial activity of these CO_2 extracts is mainly induced by the lipidic fraction, HPLC with evaporative light scattering detection (HPLC-ELSD) and GC with flame ionization detection (GC-FID) were used to identify lipid families and fatty acids, respectively. Antimicrobial activity of the extracts was measured against *Staphyloccocus aureus*, *Escherichia coli* and *Candida albicans*. Possible correlations between antimicrobial activity of extracts and their chemical composition were investigated, concluding that the total triglycerides and the DPA content seem to be the main parameters controlling the antimicrobial activity of the studied extracts.

Keywords:

Chaetoceros muelleri; diatom microalgae; supercritical CO_2; SFE; antimicrobial activity; HPLC-ELSD; fatty acids; correlation.

1.- Introduction

Diatom is a basic component of marine hatchery operations because it serves as alternative natural source of polyunsaturated fatty acids [1]. The diatom *Chaetoceros muelleri* is considered one of the most popular strains used for feeding shrimp larvae depending its composition mainly on the cultivation method. Triglycerides, polar lipids and free fatty acids are the main components of the lipid fraction when this *Chaetoceros muelleri* microalgae is cultured in batch [2].

The ability of fatty acids to interfere with bacterial growth and survival has been known for several decades [3]. Structure–function relationship studies on free fatty acids against human pathogenic bacteria indicate that antimicrobial activity can depend on both the chain length and the degree of unsaturation [4]. It has also been demonstrated that compounds, such as cholesterol, can antagonize the antimicrobial properties of fatty acids [5]. Consequently, both composition and concentration of free lipids can influence antimicrobial properties [3].

Supercritical fluid extraction is a well known technique to extract different types of lipids [6; 7], furthermore, extraction of polar lipids and free fatty acids can be improved by adding small quantities of ethanol [8].

The goal of the present investigation was the screening of the potential antimicrobial activity of supercritical extracts of *Chaetoceros muelleri* obtained under several pressure/temperature extraction conditions. The effect of different temperatures and pressures on the supercritical CO_2 yield was investigated. All extractions were done considering algae at the optimum cultivation conditions and the extracts were analyzed to determine the composition and concentrations of free lipids trying to correlate it with their antimicrobial properties.

2.- Experimental

2.1.- Cell culture

The microalgae *Chaetoceros muelleri* (CCMP1316) (CHGRA) was grown in batch cultures in f/2 medium [9] with addition of silicates. The culture was previously synchronized with three periods of four days each. Lighting (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was applied by Phillips tubes fluorescent in a 12:12 light-dark cycle, temperature and salinity were maintained at 24 ± 1°C and 35 PSU. A continuous airflow was supplied to the cultures and pH remained between 7.5 and 8.2 by adding CO_2. In these conditions growth curve were previously determined for each microalgae by counting cells with a Neubauer chamber. Cells were harvested at late logarithmic phase of each treatment by centrifugation at 7000 rpm/min for 10 min, freeze-dried and maintained at –20 °C until analyzed.

2.2.- Supercritical fluid extraction

A Suprex PrepMaster (Suprex, Pittsburgh, PA, USA) supercritical fluid extractor was used for all the experiments. Sample (1 g of Chaetoceros dry weight basis mixed with 0.2 mL of ethanol, 99.5% Panreac, Barcelona, Spain) was placed into a 5 mL stainless-steel extraction cell. The supercritical CO_2 (N38 quality, AL, Air liquide España, Madrid, Spain) flow rate was controlled using a needle valve as variable restrictor. Total extraction time was 60 minutes; during the first 15 minutes extraction was static followed by 45 minutes of dynamic extraction. Extracts were collected in a glass vessel cooled by ice.

Different extraction pressures and temperatures were selected as variables to study the effect of the experimental conditions on the extraction yield obtained. Pressure was selected between 200 and 400 atm and temperature between 40 and 80°C. The different combinations (pressure-temperature of CO_2) provided extraction densities from 0.6 to 0.96 g/mL.

2.3.- Lipid composition analysis

Lipid fraction of extracts was analyzed using two chromatographic techniques. Liquid chromatography coupled to evaporative light scattering detector (HPLC-ELSD), to identify the different lipid classes, and gas chromatography coupled to flame ionization detector (GC-FID) to identify the free and esterified fatty acids.

2.3.1.- HPLC-ELSD

The analyses were done on a Kromasil silica 60 column (250mm×4.6 mm, Análisis Vínicos, Tomelloso, Spain) coupled to a CTO 10A VP 2 oven, a LC-10AD VP pump, a gradient module FCV-10AL VP, a DGU-14A degasser, and a evaporative light scattering detector ELSD-LT from Shimadzu (IZASA, Spain). Details of the chromatographic method used to analyze the products of the extraction are described elsewhere [10]. All HPLC solvents were HPLC purity from Labscan (Dublin, Ireland).

2.3.2.- GC-FID

To prepare ethyl esters of free and esterified fatty acids, samples were mixed with chloroform/ethanol 2/1 (v/v) and ethylated by addition of 1 mL of a solution of sulfuric acid in ethanol (5 %, v/v). This mixture was allowed to stand overnight at 50 °C. After addition of 200 μL miliQ water, the resulting mixture was extracted with two 1 mL portions of n-hexane and the final extract was then dried with sodium sulfate.

One μL of derivatized sample was injected into a Perkin-Elmer autosystem XL (Wellesley, MA, USA) gas chromatograph with a 30 m BTR-Carbowax column (0,25 mm i.d.). Injector and detector temperatures were set at 220 °C and 230 °C respectively. The temperature program was as follows: starting at 100 °C and then heating to 180 °C at 20 °C/min; followed by heating from 180 to 220 °C at 15 °C/min. The final temperature (220 °C) was held for 30

minutes. Identification of the ethyl esters of the various fatty acids was based on a menhaden oil fish standard (#4-7085) obtained from Supelco (Bellefonte, PA).

2.4.- Antimicrobial Activity measurement

2.4.1.- Microbial strains

The extracts were individually tested against a panel of microorganisms including two bacteria (*Staphyloccocus aureus* ATCC 25923 and *Escherichia coli* ATCC 11775) and one yeast (*Candida albicans* ATCC 60193). *Staphyloccocus aureus* and *Escherichia coli* strains stock cultures were kept on nutrient agar at 4 ºC. *Candida albicans* was kept on Sabouraud dextrose agar at 4 ºC.

2.4.2.- Determination of minimum inhibitory concentration (MIC) and minimal bactericidal and fungicidal concentration (MBC).

A broth microdilution method was used, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS), for determination of the minimum inhibitory concentration [11]. All tests were performed in Mueller-Hinton broth supplemented with 0.5% tween 20 (Fluka, Germany), with the exception of yeasts (Sabouraud dextrose broth + 0.5% tween 20). The inocula of bacterial strains were prepared from overnight Mueller-Hinton broth cultures at 37 ºC. Yeasts were cultured overnight at 25 ºC in Sabouraud dextrose broth. Test strains were suspended in Muller-Hinton (bacteria) or Sabouraud dextrose (yeasts) broth to give a final density 10^7 cfu/ml. The *Chaetoceros muelleri* extracts dilutions in DMSO ranging from 200 mg/ml to 10 mg/ml.

The 96-microwell plates were prepared by dispensing into each well 165 µl of culture broth, 5 µl of inoculums and 30 µl of different extracts dilutions. The final volume of each well was 200 µl. Plates were incubated at 37 ºC for 24 h for bacteria and at 24 ºC for 48 h for yeasts.

Negative controls were prepared using 30 μl of DMSO, the solvent used to dissolve microalgal extracts. Chloranphenicol and amphotericin B (Sigma, Madrid) were used as positive reference standards to determine the sensitivity of the microbial species used. After incubation, the MIC of each extract was determined by visual inspection of the wells bottom (bacterial growth was indicated by the presence of a white "pellet" on the well bottom). The lowest concentration of the extract that inhibited growth of the microorganism, as detected as lack of the white "pellet", was designated the minimum inhibitory concentration. The minimum bactericidal and fungicidal concentration was determined by making subcultures from the clear wells that did not show any growth. Each test was performed in triplicate and repeated twice.

2.5.- Statistical analysis

The statistical methods used for the data analysis were: cluster analysis (Average Linkage method from standardized variables) to discover natural groupings of the analyzed variables and principal component analysis from standardized variables to examine the relationship among the analyzed variables. Statgraphics program for Windows release 5.1 (StatPoint Inc., Virginia USA) was used for data processing.

3.- Results

3.1.- Supercritical fluid extraction

Different extraction conditions have been studied in the present work to obtain extracts with antimicrobial activity. The experimental conditions using supercritical CO_2 and their

158 corresponding extraction yields are shown in Table 1. As can be seen, yields were ranging
from 1.8 to 3.9 ‰ under these conditions. Although different pre-treatments of the microalga
160 using ultrasounds and microwaves were tested to increase these extraction yields, no
significant improvements were obtained. A possible explanation for these low yields can be
162 related to the siliceous cell wall of *Chaetoceros muelleri* [12] that, in fact, can preclude the
diffusion of the supercritical extractant inside the cell. Even considering these low yields, an
164 initial trend can be observed; extract 3 (obtained at 200 atm and 80ºC) show the lowest yield
while extract 2 (obtained at 400 atm and 40ºC) gave the highest; these values correlate with
166 the minimum and maximum CO_2 density, respectively, as can be seen in Table 1. These
results are also in agreement with data obtained by other authors extracting different
168 components from microalgal matrices [13; 14]. In spite of these low yields, the antimicrobial
activity of these extracts was studied based on the huge interest that nowadays exists on new
170 natural sources of food preservatives and nutraceuticals.

172 **3.2.- Antimicrobial activity**

Different microbial species, including a gram negative bacteria (*Escherichia coli)*, a gram
174 positive bacteria (*Staphylococcus aureus*) and a yeast (*Candida albicans*), were used to screen
the potential antimicrobial activity of supercritical extracts from *Chaetoceros muelleri*. Their
176 antimicrobial activity was quantified measuring their minimum inhibitory concentration
(MIC) and minimal bactericidal and fungicidal concentration (MBC). Results obtained are
178 given in Table 2, showing that *Candida albicans* was the most sensitive microorganism to
these extracts, with the lowest MBC values, whereas *Escherichia coli* and *Staphylococcus*
180 *aureus* were less sensitive. Comparing the results obtained for the different extracts, the most
active ones were 2, 3 and 5, followed by 1 and 4 (see Table 3). These results show that the use
182 of medium values of pressure and temperature provides higher extraction of antimicrobial

compounds. Moreover, the use of extreme pressure and temperature values (400 atm and 80ºC) gave less active extracts (experiment 1 in Table 3). Interestingly, the results of Table 3 also show that, in general these extracts possess a good antimicrobial activity and that they could be useful for the food industry.

A liquid-liquid extract of raw microalga using DMSO was obtained for comparing its antimicrobial activity with those obtained using supercritical CO_2. The results show (see Table 3) that the antimicrobial activity of the DMSO extract was about 3-fold lower than the obtained with the supercritical CO_2 extracts. This indicates that the use of supercritical CO_2 is more suitable to extract compounds with antimicrobial activity from *Chaetoceros*. Besides, some other additional advantages of using CO_2 have to be taken into account; namely, supercritical CO_2 extraction is an environmental-friendly procedure and the achieved extracts can be directly used by the food industry since no toxic solvents are present (in opposition to classical liquid-liquid extraction).

3.3.- Lipid composition of extracts

In an attempt to identify compounds responsibles of the antimicrobial activity of these *Chaetoceros muelleri* supercritical extracts and based on the well-known ability of different fatty acids to inactivate microoganisms [3], different families of lipids were analyzed [10]. Figure 1 shows a typical HPLC-ELSD chromatogram of one of the supercritical extracts of *Chaetoceros muelleri* (experiment 3 in Table 1, 200 atm, 80°C). As can be seen, a nice separation of the different lipid families found in the *Chaetoceros* extract is obtained, in which compounds as triglycerides (TAG), diglycerides (DAG), monoglycerides (MAG), sterols, free fatty acids (FFA) and hydrocarbons were detected.

208 A comparison of the lipid composition of the five CO_2 extracts of Table 1 and a raw microalgae extract obtained using classical liquid-liquid extraction is shown in Figure 2. As 210 can be observed, free fatty acids (FFA) were, in general, the main components accounting, in some extracts, for more than 75% of the total lipids. In general terms, free fatty acids were the 212 most abundant family after sterols followed by DAGs, TAGs and MAGs. Analysis of sterol fraction allowed identifying cholesterol as the main product; the presence of cholesterol had 214 been previously suggested by other authors in Chaetoceros [15; 2]. As can be seen in Figure 2, the relative contribution of the different families to the total lipid fraction mainly depends 216 on the extraction conditions, corroborating the specificity that the use of supercritical CO_2 as extractant provides (e.g., compare in Figure 2 extracts 4 and 5 in terms of MAG, FFA and 218 TAG contents).

220 A deeper analysis of the free fatty acids content of the *Chaetoceros muelleri* extracts was performed by GC-FID using the conditions described under experimental. This analysis was 222 required based on the fact that antimicrobial activity has already been linked to fatty acids content [16; 17]. Figure 3 shows the GC chromatogram of the extract 3 (obtained with CO_2 at 224 200 atm and 80°C), the one that has provided the best antimicrobial activity. The chromatogram is divided in zones including the fatty acids with the same carbon chain length. 226 As can be seen, C16 fatty acids (C16:0, C16:2, C16:3) are the most abundant family of fatty acids in this extract, being also remarkable the presence of long chain polyunsaturated fatty 228 acids (EPA, DPA and DHA) whose presence has also been related to antimicrobial activity [3]. Figure 4 shows the distribution of the different fatty acids detected in the five 230 supercritical extracts compared to the raw extract. As can be seen, big differences can be found among the extracts which also could be associated to the extraction conditions used, 232 corroborating the specificity of the extraction procedure used in this work.

Cluster analysis has been employed to establish some correlation between antimicrobial activity and the lipid composition of the extracts, including TAG, DAG, MAG, FFA, sterols, hydrocarbons content and the relative composition of fatty acids (as % mol). Figure 5 shows the dendrogram of standarized variable data obtained using the Pearson's correlation coefficient (absolute value) as measure of similarity among two variables and the average linkage as linkage rule between groups. In this dendrogram, a significant relationship can be observed between the antimicrobial activities against all the microorganisms tested and the triglycerides (TAGs) and DPA content. There exist a negative correlation between the antimicrobial activity and the TAGs (with Pearson correlation coefficients ranging from –0.745 to –0.796) and a positive correlation with DPA (Pearson correlation coefficient for antimicrobial activity against *Staphylococcus aureus* equal to 0.839), meaning that an increase of DPA imply an increase in the value of Minimal Bactericidal Concentration (MBC) and, therefore, a decrease in the effective antimicrobial activity. The opposite is observed with TAGs, meaning that an increase in its relative contribution to the composition of the sample implies a higher antimicrobial activity (or lower MBC concentration). Other variables grouped together but were not related with the antimicrobial activity. Even though free fatty acids have been strongly associated to the antimicrobial activity of different extracts [4; 18; 19], in this particular case, no correlation was observed either individually or as a sum of fatty acids. Moreover, the total content on fatty acids of recognized antimicrobial activity (such as C16:1, C18:2 and C18:3) [3; 20] did not give any correlation with the observed antimicrobial activities suggesting a more complex behaviour, a strongest contribution of other lipids and/or an inhibition of the fatty acids antimicrobial effects due to the cholesterol concentration in the extracts [5], as mentioned in the introduction.

Principal component analysis was also applied to establish relationships among antimicrobial activity and lipid composition. Five principal components were obtained that explained 97.6% of the total variance of data. Rotation of the five principal components (through Varimax method) provided the following results: the first principal component, which explained 30% of the total variance, was negatively correlated with C18 (-0.95), C18:1 (-0.95), C16 (-0.91) and C12 (-0.85) while the second component (which explained 25.2% of the variance) was strongly correlated positively with the antimicrobial activity against *Staphylococcus aureus* (0.978), anti *Escherichia coli* (0.939), anti *Candida albicans* (0.939) and DPA (0.822) and negatively correlated with TAGs (-0.821) what really confirms the results obtained by cluster analysis.

4.- Conclusions

The present study has demonstrated the interest of using supercritical CO_2 to obtain extracts of *Chaetoceros muelleri* with antimicrobial activity. The strong influence of the supercritical extraction conditions in both, the lipid composition of the extracts and, consequently, in the antimicrobial activity has also been shown. Statistical analysis of all the data, considering lipid composition and relative concentration of fatty acids in the different extracts suggested an important relationship among antimicrobial activity and triglyceride content (TAG) and DPA. The present study is presented as a first step to optimize the green extraction of antimicrobials from *Chaetoceros muelleri* that could be used as food preservatives.

Acknowledgements

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338 **Figure Captions**

Figure 1.- HPLC-ELSD chromatogram of the supercritical CO_2 extract obtained at 200 atm
340 and 80ºC (extract 3). See experimental for rest of conditions.

342 Figure 2.- Lipid class composition of the five supercritical fluid extracts of Table 1 and a raw *Chaetoceros muelleri* extract using liquid-liquid extraction. (TAG: Trygliceride; FFA: Free
344 Fatty Acids; 1,3-DAG's: 1,3-Diacylglicerides; 1,2-DAG's: 1,2-Diacylglicerides; MAG's: Monoglycerides).

346

Figure 3.- GC-FID chromatogram of of the supercritical CO_2 extract obtained at 200 atm and
348 80ºC (extract 3). Squares delimit the zones with the same length in fatty acid carbon chain. See experimental for rest of conditions.

350

Figure 4.- Fatty acid composition of the five supercritical fluid extracts of Table 1 and a raw
352 *Chaetoceros muelleri* extract using liquid-liquid extraction.

354 Figure 5.- Dendrogram of the 26 variables using the average linkage rule.

356

Table 1.- Experimental conditions for the supercritical extracts studied in this work and their corresponding yields from *Chaetoceros muelleri.*

Extract Number	Extraction Pressure (atm)	Extraction Temperature (ºC)	CO_2 density (g/mL)	Yield ‰
1	400	80	0.83	3.4
2	400	40	0.96	3.9
3	200	80	0.60	1.8
4	200	40	0.84	2.6
5	300	60	0.83	2.2

362 Table 2.- Minimum bactericidal concentration (MBC, given as mg extract/ml and μg of antibiotic/ml) of different *Chaetoceros Muelleri* extracts against the three microorganisms
364 used in this work.

Extract Number	*Escherichia coli*	*Staphylococcus aureus*	*Candida albicans*
1	15	15	9
2	12	12	7
3	12	12	7
4	15	17	9
5	12	12	7
DMSO extract	25	25	22
Reference Antibiotic	10	10	100

366

368

Figure 1

370

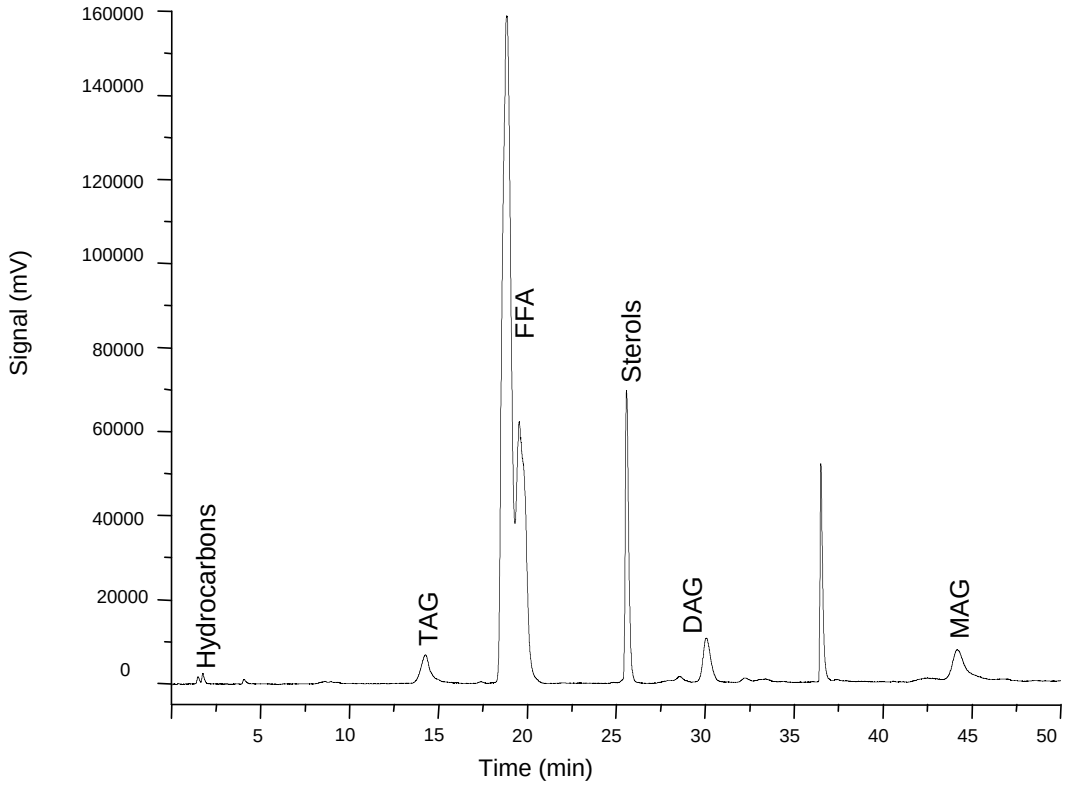

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Figure 2

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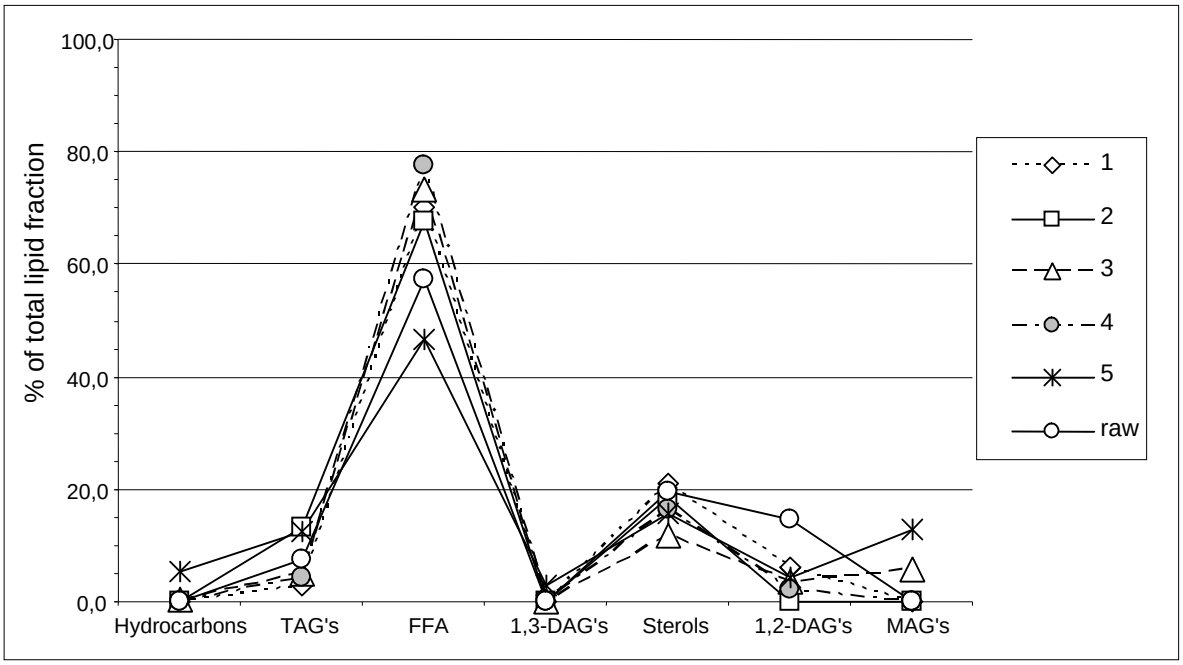

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Figure 3

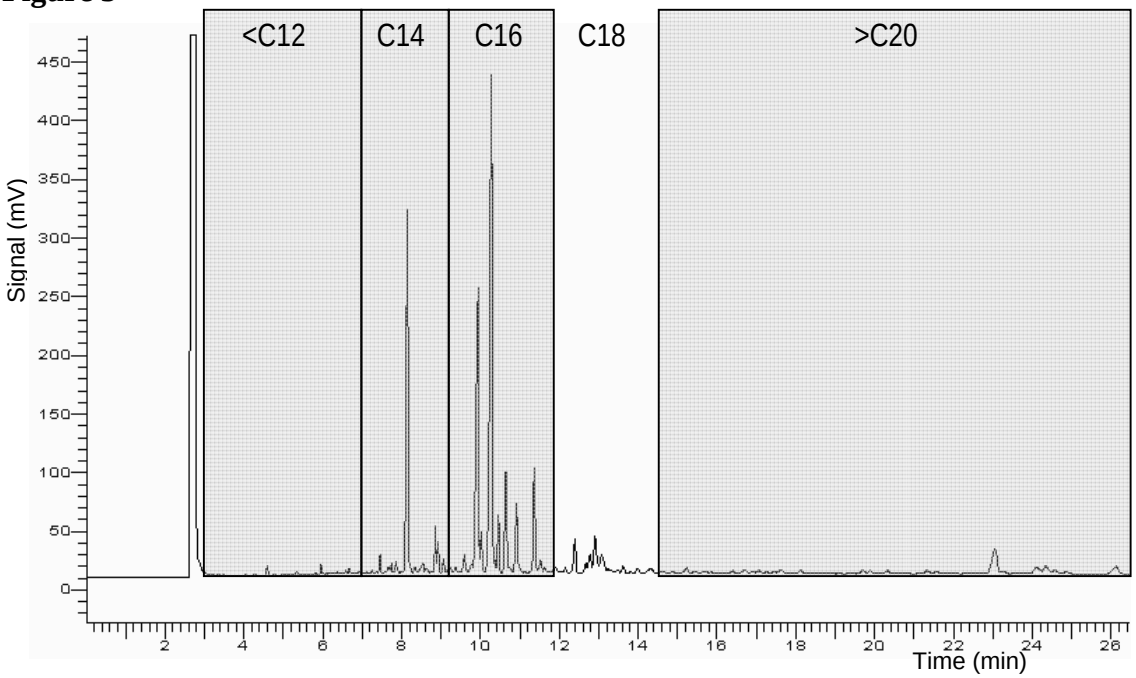

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Figure 4

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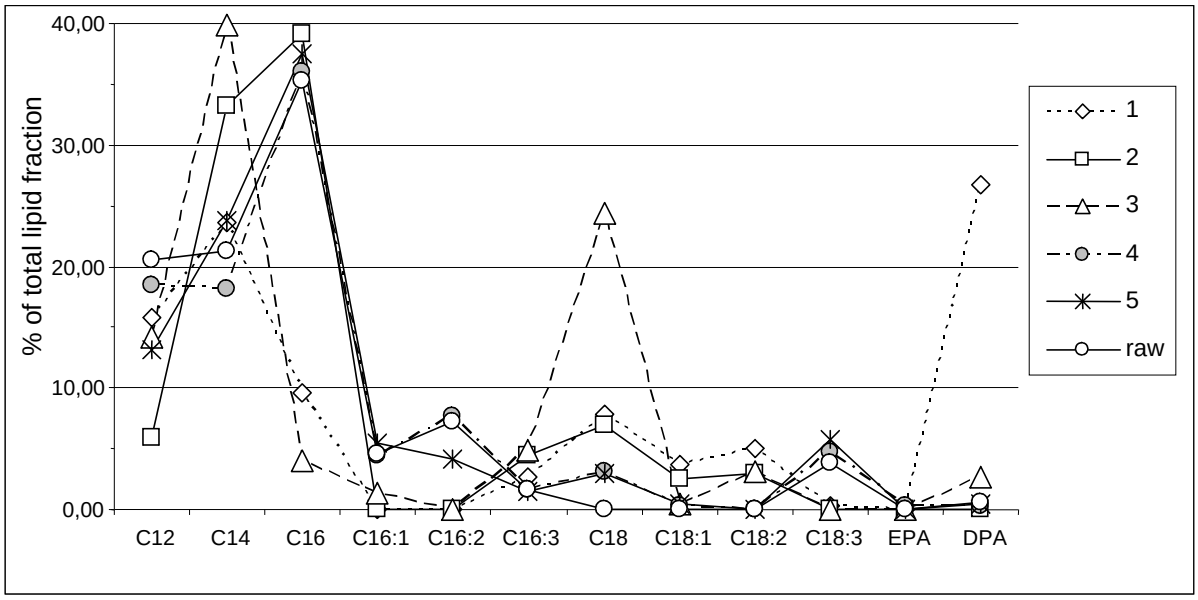

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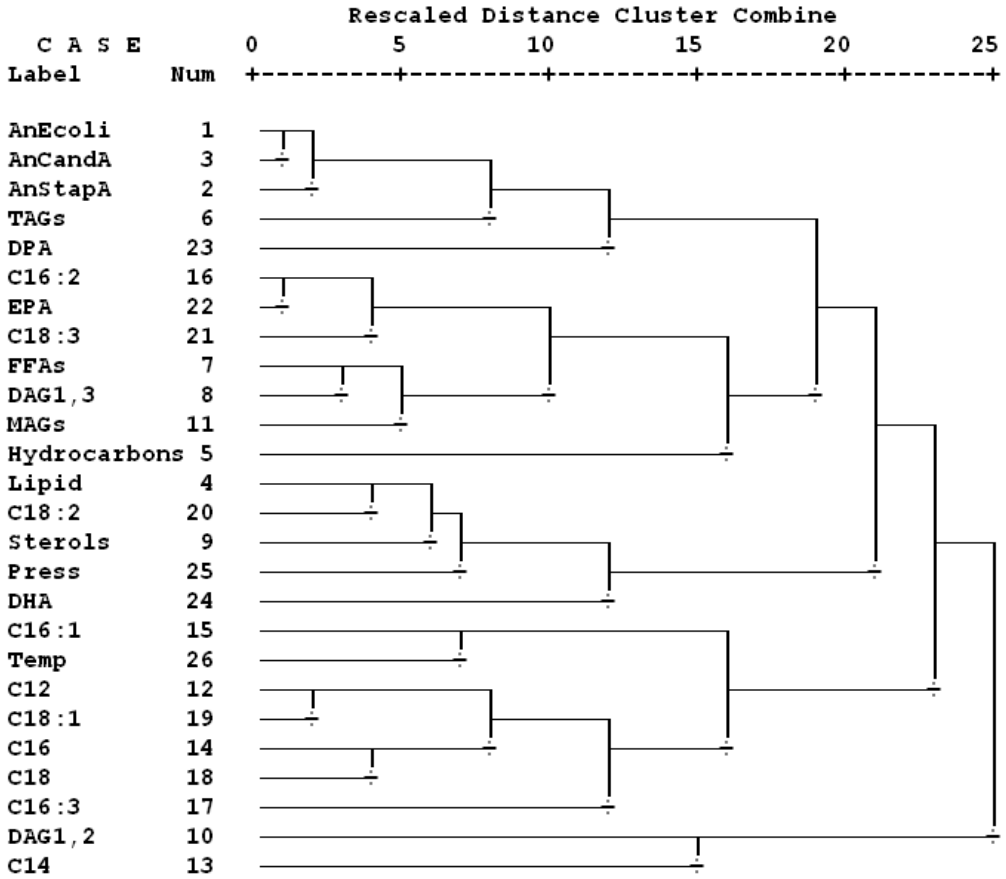
388 **Figure 5**



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