Cite as:
Golmakani, M. T.; Mendiola, J. A.; Rezaei, K.; Ibáñez, Elena "Pressurized limonene as an alternative bio-solvent for the extraction of lipids from marine microorganisms" (2014) Journal of Supercritical Fluids 92: 1-7
http://dx.doi.org/10.1016/j.supflu.2014.05.001

Pressurized limonene as an alternative bio-solvent for the extraction of lipids from marine microorganisms

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Abstract:

A fast and green process for the isolation of high value lipids from different marine microorganisms is presented involving the use of limonene, a green biodegradable solvent, as an alternative to traditional hexane extraction. The optimized process is based on pressurized liquid extraction (PLE) at 200°C for 15 min using limonene:ethanol (1:1, v/v) as extracting solvent. Under these conditions, lipids were extracted from different microalgae such as Spirulina, *Phormidium*, *Anabaena* and *Stigeoclonium* and their composition in terms of fatty acids were studied by using a Fast-GC-MS method and compared with the original content in the raw material. The extraction method provided the best results in terms of extraction yield for Spirulina, meanwhile the highest amount of ω-3 fatty acids were obtained from *Stigeoclonium*.

Keywords:

PLE, limonene, Spirulina, Anabaena, *Stigeoclonium*, *Phormidium*, γ-linolenic acid, PUFAs

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1- Introduction

Solvent extraction could be defined as a process for transporting materials from one phase to another for the purpose of separating one or more compounds from their sources. Hexane, which is normally obtained by the refining of crude petroleum oil, has traditionally been used as the major solvent in oil extraction. Other than its adverse effects on the environment, it has several toxicological effects, both in short and long term expositions, mainly related with neuropathies [1]. Therefore, considering such toxicological and environmental concerns and also due to its fire hazard as well as the occasional scarcities, finding alternative solvents is a top priority for the extraction industry [2]. Among the solvents that can be used to replace hexane and halogenated hydrocarbons, limonene has been suggested as a valuable green alternative. Limonene possesses a dielectric constant very close to that of hexane [3] and has been employed for the extraction of rice bran oil [4, 5], oil from olive residues [6], carotenoids from tomatoes [7] or algae [8] and, recently, for the extraction of algal lipids from wet biomass [9]. Limonene is a major by-product of the citrus fruits industry, being the major component of essential oils extracted from citrus peels [10].

Microalgae can be an interesting source of lipids since depending on the culture conditions, lipid content can be increased to values appropriate for biofuel production thus becoming a sustainable source of renewable energy and biofuels [11]. On the other hand, high-added value lipids such as γ-linolenic acid (GLnA) [12], an ω6-polyunsaturated fatty acid (PUFA) with antimicrobial, anti-inflammatory and anti-proliferative properties [13] can also be produced from microalgae such as cyanobacteria. In fact, GLnA has been found in Spirulina at concentrations ranging from 18 to 21%, w/w [14, 15].

In the present study, the cyanobacteria *Arthrospira platensis* (Spirulina), *Phormidium sp.* and *Anabaena planctonica*, together with the fresh water microalga *Stigeoclonium sp.* from the class

Chlorophyceae (Chlorophyta) have been studied due to their interesting lipid profile, being both a promising feedstock source for biodiesel production [16] and a valuable source of PUFAs. In the search for alternative media for the extraction of microalgae lipids, supercritical CO_2 [15, 17] and pressurized liquid extraction (PLE) [18, 19] have been practiced quite extensively. The use of compressed fluids can result in less solvent consumption and shorter extraction times, when compared to traditional Soxhlet extraction. In a previous work, current authors suggested the employment of expanded ethanol with CO_2 and pressurized ethyl lactate as green processes to obtain lipid fractions enriched with GLnA from Spirulina [20]. With the aim of seeking an alternative bio-solvent with physicochemical properties close to those of hexane, in the current study limonene was investigated in combination with ethanol under pressurized conditions and compared to hexane for the extraction of valuable lipids from different marine organisms.

2- Experimental

2.1- Samples and chemicals

Spray-dried Spirulina was purchased from Algamar S.A. (Pontevedra, Spain) and stored under dry and dark conditions until used. *Phormidium sp.*, *Anabaena planctonica* and *Stigeoclonium sp.* were kindly donated by the Spanish Bank of Algae (BEA) at the University of Las Palmas de Gran Canaria, Spain (http://bea.marinebiotechnology.org). Ethanol 99% and washed sea sand (0.25-0.30 mm in diameter) were supplied by Panreac Quimica S.A. (Barcelona, Spain). D-Limonene (food-grade and kosher), acetyl chloride (98%), GLnA (99%), heptadecanoic acid (98%), butylated hydroxytoluene (BHT, 99%) and PUFA standards of marine source (PUFA No. 1) were purchased from Sigma-Aldrich (St. Louis, MO). *n*-Hexane 95% was purchased from Labscan (Dublin, Ireland). Helium for GC-MS (premier quality 99.998%) and nitrogen (technical quality 99%) were obtained from Carburos

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Metalicos (Air Products Group, Madrid, Spain). Deionized water was obtained using a Milli-Q system from Millipore (Molsheim, France).

2.2- Pressurized liquid extraction (PLE)

PLE extraction experiments were performed using an Accelerated Solvent Extractor™ system (ASE 200, Dionex Corporation, Sunnyvale, CA) equipped with a solvent controller. Two g of microalgae and 4.0 g of sea sand were mixed and loaded into an 11-mL-volume extraction cell. The extraction cell was fitted with cellulose filter at both sides (from the inlet and outlet). In the first step, the extraction cell was filled with solvent and the pressure was increased to the desired level. Initial heat-up time was then applied depending on the extraction temperature. The heat-up time was automatically adjusted by the equipment. After the static stage of the extraction, the cell and the tubing were rinsed (with 60% of the cell volume) using fresh extraction solvent. Then, all the solvent present in the system was purged using N_2 gas. The extract from this stage was collected in a vial and then pressure was released from the unit. The extracts were subjected to solvent removal using a rotary evaporator (Rotavapor R-210, Buchi Labortechnik AG, Flawil, Switzerland) for ethanol and hexane removal and an N_2 stream at 100 °C for limonene removal.

2.3- Solvent selection and temperature optimization

To select the extracting solvent, four conditions were tested using *n*-hexane, *n*-hexane:ethanol (1:1, v/v), limonene, and limonene:ethanol (1:1, v/v) as solvents using a fixed pressure (20.7 MPa) and a constant temperature (180 ºC) and a total extraction time of 15 min. For temperature optimization, extractions were performed using limonene:ethanol (1:1, v/v) as solvent applying different temperatures of 50, 100, 150, and 200 ºC using a fixed pressure of 20.7 MPa and a constant extraction

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time of 15 min.

2.4- Fatty acid analysis

To determine the fatty acid contents of the lipid extracts, 30 mg of dried extract from each sample was treated with 3.0 mL of ethanol-acetyl chloride (95:5, v/v) solution. As an internal standard, 2.0 mg heptadecanoic acid was added to the mixture and sealed in a 20 mL PTFE-lined vial under a nitrogen atmosphere and maintained at 85 °C for 1 h. The temperature of the vial was then reduced to ambient conditions and after adding 1.0 mL water, it was shaken (vigorously) for 1 min and 3.0 mL hexane (containing 0.01% BHT to prevent the oxidation of double bonds during the isolation procedure) was added and produced ethyl ester derivatives were extracted. The hexane layer (upper phase) from this stage was transferred into a clean vial and injected into the GC-MS for qualitative and quantitative determination of fatty acid ethyl esters (FAEE) using a Shimadzu GC 2010 gas chromatography system (Kyoto, Japan) equipped with a Shimadzu AOC-20i autosampler and a split/splitless injector coupled to a QP-2010*Plus* single quadrupole mass spectrometer. The column was a 007-CW Carbowax, 12 m × 0.1 mm i.d. fused silica capillary column with a 0.1 μm film thickness (Quadrex, Woodbridge, CT, USA). The temperature levels in the injector, interface and ionization chamber were maintained at 220, 240, and 230 °C, respectively. A gradient oven temperature programming starting at 100 °C with a ramp of 20 °C/min to 160 °C and another ramp of 15 °C/min to 220 °C with an 8-min hold at 220 °C was applied for the separation of FAEE. A 0.5-µL sample was injected into the GC-MS with the injector in the split mode (split ratio at 1:10 level). Helium was used as the carrier gas. A solvent delay of 1.5 min was selected for the MS. A Shimadzu GC Solution software was used to process the data. Compounds were primarily identified by mass spectrometry in the SCAN mode using a mass interval ranging from 40 to 400 m/z. They were then identified by comparing the obtained

retention times with those of standards and also by comparing their mass spectra with those of Wiley library. Quantitative determinations of the fatty acids were carried out using heptadecanoic acid as internal standard as mentioned earlier. The absence of heptadecanoic acid in the lipids was confirmed prior to the study. For each FAEE, a working curve was drawn using the weights and area ratios with respect to those of the internal standard.

2.5- Statistical analysis

Data were presented as means ± standard deviations of at least two determinations. A general linear model (GLM) procedure from Statistical Analysis Software (SAS) version 9.1 (SAS Institute Inc., Cary, NC) was used to compare the mean values amongst the treatments at $P < 0.05$. Multiple comparisons of means were carried out by using the LSD (least significant difference) test.

3- Results and discussion

3.1. Selection of extraction solvent

In a previous work [20], the possibilities of using pressurized ethyl lactate as an alternative green solvent for lipid extraction from Spirulina was investigated. A maximum extraction yield of 20.7% (w/w) and a GLnA recovery of 68.3% were obtained under PLE optimized conditions (ethanol:ethyl lactate, 1:1, v/v; 20.7 MPa pressure; 180 °C temperatures and 15 min run time). Considering the common use of hexane in lipid extraction and the interest in finding green solvents that can be considered as alternate solvents to toxic organic solvents, limonene was chosen in the present study due to its similarities to hexane in terms of polarity. In order to be able to compare the results of this study with those of the previous study using ethyl lactate as extracting solvent [20], the above-mentioned optimized conditions were applied in the present study as starting point to examine limonene and limonene:ethanol mixture (1:1, v/v) to extract lipids from Spirulina and to obtain GLnA-enriched fractions. Table 1 shows the results obtained in terms of total extraction yield, lipid

concentration in the extract, GLnA concentration in the extract, lipid enrichment, lipid recovery and GLnA recovery, determined by using the following equations:

Total yield (%, w/w) = [weight of the extract (g)/weight of Spirulina (g)] × 100 *(1)*

Lipid concentration in the extract (%, w/w) = [weight of the extracted lipids (g)/weight of the extract (g)] × 100 *(2)*

GLnA concentration in the extract = [weight of GLnA (g)/weight of the extract (g)] × 100 *(3)*

Lipid enrichment = lipid concentration in the extract (w/w)/lipid concentration in the untreated Spirulina (w/w) *(4)*

Lipid recovery = [Total yield of extract (%, w/w) × lipid concentration in the extract (%, w/w)]/lipid concentration in the untreated Spirulina (%, w/w) *(5)*

GLnA recovery = [Total yield of extract (%, w/w) × GLnA concentration in the extract (%, w/w)]/GLnA concentration in the untreated Spirulina (%, w/w) *(6)*

Lipid enrichment is the ratio of the final concentration of the lipid in the extract to its primary concentration in the untreated Spirulina (8.6%, w/w) while lipid recovery is defined as the ratio of the amount of lipid in the extract to its amount in the untreated Spirulina. Similarly, GLnA recovery is defined as the ratio of the amount of GLnA in the extract to its amount in the untreated Spirulina (1.8%, w/w) taking into consideration the total extraction yield. These parameters were compared with those achieved by employing hexane and hexane:ethanol as extraction solvents under the above-mentioned optimized conditions.

Under given extraction conditions, different solvents from the current study showed completely different behaviours in terms of extraction yield. For instance, extractions using pure limonene resulted in a yield somewhat higher than that found for pure hexane (8.1 % compared to 5.6%, respectively). However, in comparison with the yield obtained with ethanol as co-solvent for limonene or hexane, it was almost half (around 14% for both). This finding can be related to the levels of polar lipids in Spirulina, namely, phospholipids, glycolipids and other cell membrane lipids [21]. In terms of lipid concentrations in the extracts, the quantities varied significantly from 31.4% (when using pure

limonene) to 38.9% (when using hexane:ethanol, 1:1, v/v). On the other hand, the levels of GLnA were similar for all the conditions studied, with no significant differences among them. Recoveries for GLnA ranged from 28.5% when using hexane to 67.8% when using hexane:ethanol (1:1, v/v) as the solvents for lipid extraction. The recovery for the latter case was not significantly different from the one achieved when using limonene:ethanol (1:1, v/v) as solvent. Such finding indicates that the selectivity of GLnA extraction can be improved simply by using a more appropriate solvent such as hexane:ethanol or limonene:ethanol mixtures. This shows that limonene behaves, as expected, very close to hexane for lipid extraction [3]. Figure 1 shows the chromatograms of the fatty acid profiles obtained by using the different solvents. Fatty acid profiles for different extracts were found at very similar quantities thus indicating that the extraction solvent has no significant impact on the fatty acid composition. It is worth mentioning that fatty acid compositions of extracts obtained in the present study were similar to those reported in literature using hexane [22, 23] or supercritical CO_2 extraction [15, 17, 24]. When comparing the pressurized limonene with the pressurized hexane for the extraction of lipids from Spirulina, it was found that limonene provided higher lipid extraction yields than did pure hexane. In fact, that could be related to the higher density of limonene. Therefore, it can be concluded that hexane can easily be replaced by limonene and that the use of this solvent in PLE has several advantages in terms of yield, recoveries and environmental impact.

3.2. Solvent temperature optimization

It is well known that one of the main factors that influences the PLE process in terms of yield and selectivity is the extraction temperature [19, 25], which was also confirmed for GLnA extraction from Spirulina using PLE with ethyl lactate as extraction solvent [20]. Therefore, once the extracting solvent was selected for achieving the maximum lipids and GLnA recoveries (limonene:ethanol, 1:1, v/v),

temperature was studied as the main parameter for the optimization of the PLE process. A wide range of temperatures was selected: 50, 100, 150, and 200 ºC. Extraction pressure and time were kept constant as those of the previous experiments; that is, 20.7 MPa and 15 min, respectively. Table 2 shows the results obtained by using limonene:ethanol (1:1, v/v) at different temperatures. As can be seen, the extraction yield increases from 7.5% (at 50°C) up to 17.6% (at 200°C). While at the same time quantities of lipids and GLnA concentration decreased significantly. Considering the higher yields, the global effect is that higher lipid and GLnA recoveries can be achieved at the highest temperature examined in the present study (almost 70.0% of lipid recovery and 73.6% GLnA recovery at 200°C). By analysing these results, we can conclude that the best conditions to obtain valuable lipid extracts from Spirulina within the tested ranges were: limonene:ethanol (1:1, v/v) as solvent, 200 °C of extraction temperature; 20.7 MPa as extraction pressure and a total of 15 min as extraction time. Results obtained were similar to those achieved using ethanol:ethyl lactate (1:1, v/v) as extraction solvent [20] at 180°C, 20.7 MPa and 15 min as extraction conditions providing an extraction yield of 20.0% and GLnA recovery of 68.0%.

3.3. Application of the PLE process using limonene:ethanol as extracting solvent to other cyanobacteria and microalgae

As mentioned, 2 different cyanobacteria, namely *Anabaena planctonica* and *Phormidium*, and one green microalgae (*Stigeoclonium*) were selected to test the efficacy of the PLE process using limonene:ethanol towards the extraction of lipids and, more specifically, towards the differential extraction of fatty acids (GLnA; saturated fatty acids, SAFA; monounsaturated fatty acids, MUFA and PUFA). Table 3 shows the total extraction yield, lipid and GLnA concentrations and recoveries, and lipid enrichment of the different cyanobacteria and microalgae studied. As can be observed, results

were very much dependent on the type of microorganisms, with total yields ranging from 3.1% (for *Anabaena*) to 16.7% (for *Stigeoclonium*). In terms of percentage of lipids in the extracts obtained, these ranged from 6.0% (for *Anabaena*) to almost 32.0% for *Stigeoclonium*, being this value really close to the one obtained for Spirulina. The wide variability on the lipid yield depends mainly on the microorganism and the culture conditions, as reported in other studies [26, 27]. The best results in terms of extraction of GLnA were obtained using *Stigeoclonium*, where this fatty acid was more than 38 %, w/w, implying a 96.4% of recovery as referred to the value of the untreated organism. As can be seen in Table 4, the fatty acid profiles of the other cyanobacteria were quite different and therefore the results obtained also differed among different algae studied here. Comparing the results for raw and treated microalgae in Table 4 indicates that the selectivity of the extraction process depends mainly on the solvent used (in this case limonene:ethanol, 1:1, v/v) and type of the fatty acids that are concentrated (i.e., polar, nonpolar or medium-polarity lipids). The concentrations of several fatty acids are clearly increased after the extraction process. For instance, the high levels of fatty acids in *Stigeoclonium* extract could be related to the presence of phosphatase [28], which could be responsible for the degradation of phospholipids in the *Stigeoclonium* cell wall, and therefore, the extraction rates are enhanced. Moreover, the levels of PUFAs from the C18 series (C18:2 and C18:3) are clearly increased after the extraction with limonene:ethanol indicating that these fatty acids can be mainly contained in medium-polarity lipids such as monogalactosyldiglycerides. Therefore, depending on the final use of the extracts, one can tune the selectivity of the process to concentrate unsaturated fatty acids or saturated ones (Figure 2). A good example is the case of *Stigeoclonium* that, as mentioned by Praveenkumar et al [16], might be a promising feedstock source for biodiesel production since it possessed fatty acids from the C16 and C18 series. But, in order to fulfil the requirements for biodiesel production according to the European standard EN14214, it should not contain C18:3 at a level higher than 12%. Therefore, if the final goal is to concentrate GLnA, the developed process in this study is

preferred. But, to use these microalgae for biodiesel production, different solvent compositions should be obtained. On the other hand, the strong cell wall present in *Anabaena* can be the reason for the low lipid concentration in its extract. This cell wall is especially strong in some of their cells called heterocysts, which are composed of several layers to reduce oxygen permeability [29]. For *Anabaena*, the amount of lipids extracted was similar to the original composition of the raw sample and only a small increase in the unsaturated fatty acids was observed (Table 4 and Figure 2). Due to the higher levels of C16:0 in *Anabaena* cyanobacteria, they have been suggested as potential sources for biodiesel production since proportion of SAFA and MUFA is preferred for increasing energy yield and oxidative stability [30].

Meanwhile, the extraction of lipids from *Phormidium* using this method was more selective (Fig. 3), since FA levels in the extracts were higher. This fact could be due to the high concentration of free fatty acids in *Phormidium*, which was previously reported by Rodriguez-Meizoso et al [31] and El Semary [32].

4- Conclusion

It can be concluded that the proposed process involving the use of limonene:ethanol (1:1, v/v) as extraction solvent at 200 °C and 20.7 MPa for 15 min of extraction can be an interesting option to be used as a selective process to obtain lipid extracts enriched in valuable fatty acids, mainly those present in the medium-polarity lipids, in a short extraction time. Extracts obtained with this method can be directly used in food, pharmaceutical or cosmetic preparations or can be employed as a new source for biodiesel production, depending on the species used.

5- Acknowledgement

This work has been financed by the Spanish Ministry of Science (Project AGL2011-29857-C03-01). M.T. Golmakani wishes to thank Iranian Ministry of Science, Research, and Technology (# 42/4/52566) and Research council of the University of Tehran for supporting his stay in CIAL-CSIC, Spain. Authors would like to thank Spanish Bank of Algae (BEA) at University of Las Palmas de Gran Canaria, Spain (http://bea.marinebiotechnology.org) for the donation of samples.

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Cite as:
Golmakani, M. T.; Mendiola, J. A.; Rezaei, K.; Ibáñez, Elena "Pressurized limonene as an alternative bio-solvent for the extraction of lipids from marine microorganisms" (2014) Journal of Supercritical Fluids 92: 1-7
http://dx.doi.org/10.1016/j.supflu.2014.05.001

368 Figure captions

369

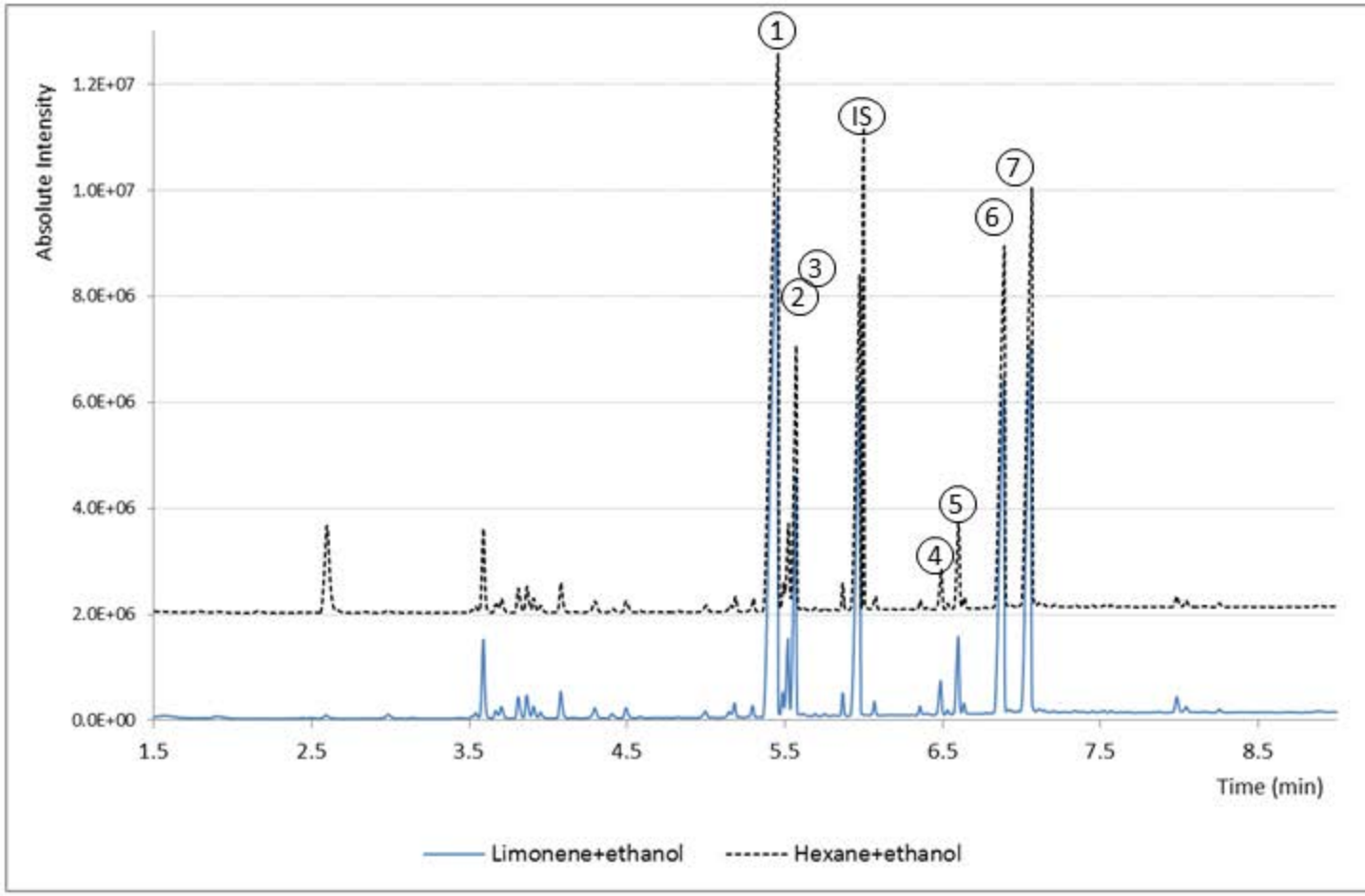
370

371 Figure 1. Changes in the fatty acid profile of Spirulina extracted with different solvents compared to untreated Spirulina
372 (1=C16:0: Palmitic acid; 2=C16:1: Palmitoleic acid; 3=C18:0: Stearic acid; 4=C18:1: Oleic acid; 5=C18:2: Linoleic acid;
373 C18:3: 6=γ-Linolenic acid).

374

375 Figure 2. Comparison of fatty acid profiles of raw and extracted samples. Data shown are in % (w/w). SAFA, Saturated
376 fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids.

377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1.0E+07
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4.0E+06
2.0E+06
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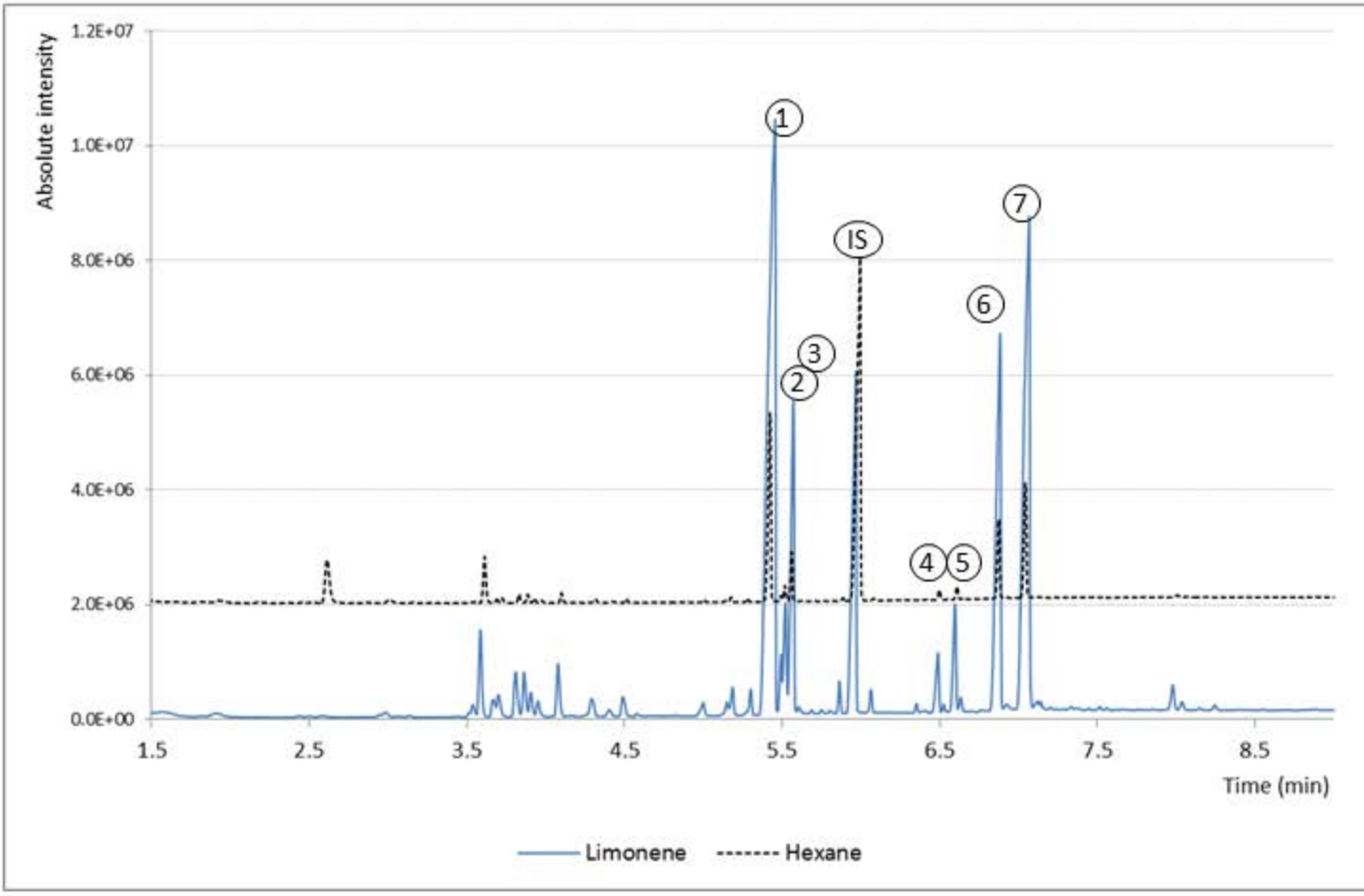
1
IS
7
6
3
2
5
4
1.5
2.5
3.5
4.5
5.5
6.5
7.5
8.5
Time (min)
Limonene+ethanol
Hexane+ethanol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4.5
5.5
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7.5
8.5
Time (min)
Limonene
Hexane

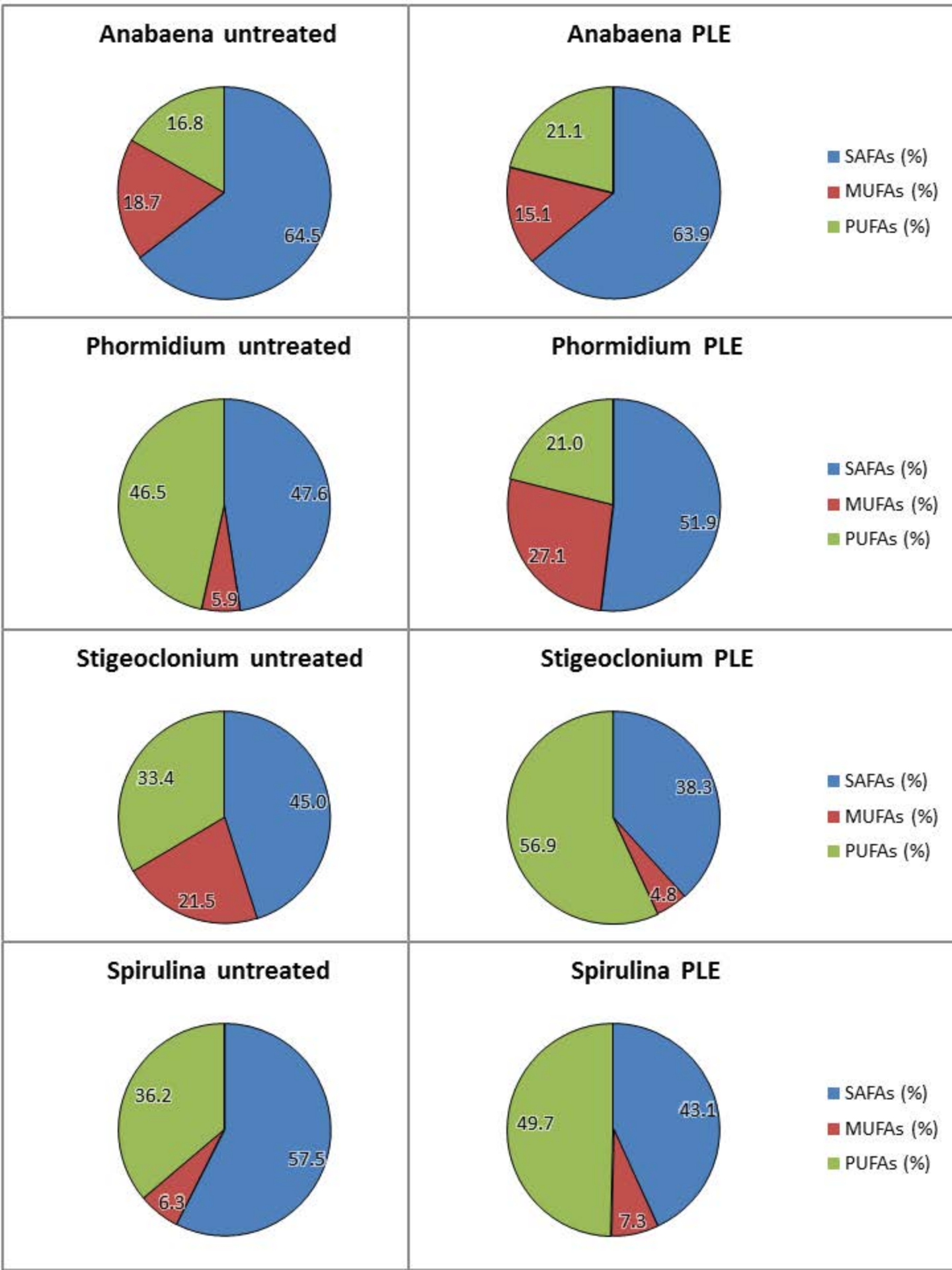
Anabaena untreated
16.8
18.7
64.5
Anabaena PLE
21.1
15.1
63.9
SAFAs (%)
MUFAs (%)
PUFAs (%)
Phormidium untreated
46.5
47.6
5.9
Phormidium PLE
21.0
51.9
27.1
Stigeoclonium untreated
33.4
45.0
21.5
Stigeoclonium PLE
38.3
56.9
4.8
Spirulina untreated
36.2
57.5
6.3
Spirulina PLE
49.7
43.1
7.3

Table 1. Total extraction yield, lipid concentration and concentration of γ-linolenic acid (GLnA) in Spirulina applying pressurized liquid extraction at 20.7 MPa and 180 °C for 15 min using different solvents

Solvent	Total extraction yield (%, w/w)[◊]	Lipid concentration in the extract (%, w/w)	GLnA concentration in the extract (%, w/w)	Lipid enrichment[†]	Lipid recovery[†]	GLnArecovery[♠]
Limonene	8.1 ± 0.6[b‡]	31.4 ± 2.2[b]	8.3 ± 0.6[a]	3.7 ± 0.3[a]	29.6 ± 2.1[b]	38.4 ± 2.7[b]
Limonene:Ethanol (1:1, v/v)	14.4 ± 1.0[a]	34.7 ± 2.5[ab]	7.7 ± 0.5[a]	4.0 ± 0.3[a]	58.1 ± 4.1[a]	63.0 ± 4.5[a]
Hexane	5.6 ± 0.4[c]	35.8 ± 2.5[ab]	8.9 ± 0.6[a]	4.2 ± 0.3[a]	23.3 ± 1.6[b]	28.5 ± 2.0[b]
Hexane:Ethanol (1:1, v/v)	13.2 ± 0.9[a]	38.9 ± 2.8[a]	9.0 ± 0.6[a]	4.5 ± 0.3[a]	60.1 ± 4.3[a]	67.8 ± 4.8[a]

[◊]Yield expressed as g of dry extract/100 g Spirulina (w/w)

[†]Values relative to total lipid content in untreated Spirulina

[♠]Values relative to total GLnA content in untreated Spirulina

[‡]Mean ± SD (n = 2); in each column, means identified with the same letter are not significantly different (p>0.05).

Table 2. Total extraction yield, lipid concentration and concentration of γ-linolenic acid (GLnA) in Spirulina applying pressurized liquid extraction at 20.7 MPa, 15 min, using limonene:ethanol (1:1, v/v) as extraction solvent at different temperatures

Temperature (°C)	Total extraction yield (%, w/w)[◊]	Lipids concentration in the extract (%, w/w)	GLnA concentration in the extract (%, w/w)	Lipid enrichment[†]	Lipid recovery[†]	GLnA recovery[♠]
50	7.5 ± 0.2[d‡]	42.9 ± 3.0[a]	10.2 ± 0.7[a]	5.0 ± 0.4[a]	37.6 ± 2.7[c]	43.6 ± 3.1[c]
100	10.4 ± 0.1[c]	39.5 ± 2.8[ab]	8.9 ± 0.6[ab]	4.6 ± 0.3[ab]	47.7 ± 3.4[bc]	52.7 ± 3.7[bc]
150	12.1 ± 0.1[b]	39.5 ± 2.8[ab]	8.7 ± 0.6[ab]	4.6 ± 0.3[ab]	56.1 ± 4.0[b]	60.4 ± 4.3[b]
200	17.6 ± 0.2[a]	33.7 ± 2.4[b]	7.3 ± 0.5[b]	3.9 ± 0.3[b]	69.6 ± 4.9[a]	73.6 ± 5.2[a]

[◊]Yield expressed as g of dry extract/100 g Spirulina (w/w)

[†]Values relative to total lipid content in untreated Spirulina

[♠]Values relative to total GLnA content in untreated Spirulina

[‡]Mean ± SD (n = 2); in each column, means with different letters are significantly different ($p<0.05$).

Table 3.- Total extraction yield, lipid concentration and concentration of γ-linolenic acid (GLnA) in the different microalgae applying pressurized liquid extraction at the optimized extraction conditions (limonene:ethanol, 1:1, v/v; 200 ºC; 20.7 MPa and 15 min).

	Total extraction yield (%, w/w)[◊]	Lipid concentration in the extract (%, w/w)	GLA concentration in the extract (%, w/w)	Lipid enrichment[†]	Lipid recovery[†]	GLnA recovery[♠]
Anabaena	3.1 ± 0.5	6.0± 1.1	1.3± 0.4	3.1± 0.1	9.7± 2.0	17.4± 1.2
Phormidium	6.8± 1.4	13.0± 1.3	--	7.1± 1.2	48.3± 5.5	--
Stigeoclonium	16.7± 0.1	31.9± 1.7	6.9± 0.8	5.9± 0.1	98.6± 3.2	96.4± 3.8

[◊] Yield expressed as g of dry extract/100 g of raw material (w/w)

[†] Values relative to lipid contents in the untreated microalgae

[♠] Values relative to total GLnA contents in the untreated microalgae

Table 4. Individual contribution (%, w/w) of different fatty acids detected in the samples compared to the total lipid content

Type of microorganism	Type of fatty acid♠						
	C16:0	C16:1	C16:2	C18:0	C18:1	C18:2	C18:3
Anabaena untreated	59.53 ± 6.09	14.75 ± 1.94	-	4.99 ± 0.63	3.95 ± 0.18	4.69 ± 0.33	12.09 ± 0.08
Anabaena extract◊	47.49 ± 3.79	9.68 ± 0.21	-	16.37 ± 1.10	5.39 ± 0.47	4.96 ± 0.71	16.11 ± 0.56
Phormidium untreated	35.92 ± 1.71	5.93 ± 0.76	46.52 ± 5.50	11.63 ± 1.31	-	-	-
Phormidium extract◊	46.96 ± 1.12	27.09 ± 2.23	21.03 ± 0.13	4.93 ± 0.03	-	-	-
Stigeoclonium untreated	39.21 ± 3.20	1.58 ± 0.02	2.78 ± 0.41	5.83 ± 0.35	19.94 ± 1.36	8.50 ± 0.49	22.17 ± 1.39
Stigeoclonium extract◊	36.87 ± 0.13	2.52 ± 0.09	1.59 ± 0.02	1.43 ± 0.20	2.28 ± 0.25	17.03 ± 0.68	38.29 ± 3.29
Spirulina untreated	36.82 ± 5.38	17.39 ± 1.98	1.29 ± 0.01	1.17 ± 0.03	4.59 ± 0.19	20.12 ± 1.43	19.92 ± 1.12
Spirulina extract◊	41.97 ± 5.28	11.53 ± 0.77	1.48 ± 0.07	1.28 ± 0.05	2.66 ± 0.18	17.83 ± 0.68	23.25 ± 1.57

◊ PLE extracts done with limonene:ethanol (1:1, v/v) as extraction solvent, 200 °C, 20.7 MPa, and 15 min of extraction time

♠ C16:0: Palmitic acid; C16:1: Palmitoleic acid; C16:2: Hexadecadienoic acid; C18:0: Stearic acid; C18:1: Oleic acid; C18:2: Linoleic acid; C18:3: γ-Linolenic acid