Expanded ethanol with CO$_2$ and pressurized ethyl lactate:ethanol to obtain fractions enriched in γ-Linolenic Acid from *Arthrospira platensis* (Spirulina)

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Abstract:

In the present work, two extraction techniques and two green solvents have been tested to obtain γ-linolenic acid (GLnA)-enriched fractions from Arthrospira platensis (Spirulina). Expanded ethanol with supercritical CO₂ (Gas Expanded Liquid Extraction, GXL) and Pressurized liquid extraction (PLE) using mixtures of ethanol:ethyl lactate were tested and optimized chemometrically. Results obtained allow understanding the effects of the different factors involved in the tested processes and providing the optimum conditions to achieve the maximum total yield of lipids and also maximum GLnA recovery (% w/w). Total yields up to 20.7%, w/w, were obtained under optimum PLE conditions considering 20.7 MPa pressure, 180 °C temperature and 15 min extraction time and a solvent composition of ethanol:ethyl lactate (50:50, v/v), under which GLnA recoveries of 68.3% from the original amount was achieved. GXL provided total yields of 7.4% (w/w) and GLnA recovery of 35.3%, both of which were lower than those obtained by PLE.

Keywords: Ethyl lactate; Gas-expanded liquid; Green solvents; γ-linolenic acid; Near-critical fluids; Pressurized Liquid Extraction
Graphical Abstract
1. INTRODUCTION

For a long time, supercritical \( \text{CO}_2 \) (SC\( \text{CO}_2 \)) has demonstrated to be an effective and environmentally friendly solvent to obtain valuable products from natural sources [1-5]. However, low polarity of this solvent has been a major disadvantage for its use in extraction processes dealing with polar compounds. In recent years, alternative media for performing green extractions of polar bioactives have been the subject of many investigations [6-8]. Among those, lactate esters have demonstrated excellent solvent properties and low toxicity and are potential alternatives to many halogenated solvents. Lactate esters such as ethyl lactate have the ability to dissolve a wide range of chemicals; they can be used to remove greases, silicone oils, and adhesives in cleaning a variety of metal surfaces for fabrication and coating applications [9]. Due to its presence in beer, wine, and soy products, ethyl lactate has been approved by FDA for use in food industries [9]. Ethyl lactate is a colorless solvent produced from the fermentation of carbohydrate feedstock available from the corn and soybean industries. This solvent has a relatively high flashpoint and is considered environmentally friendly since it can be completely biodegradable into \( \text{CO}_2 \) and water [9, 10]. Because of its miscibility with both hydrophilic and hydrophobic compounds [10], ethyl lactate is considered as a suitable solvent for a diverse range of components including lycopene from tomato [10] and astaxanthin from the yeast *Xanthophyllomyces dendrorhous* [11]. Pressurized liquid extraction (PLE), which is also known as accelerated solvent extraction (ASE) is an alternative extraction method to supercritical fluid extraction (SFE) using a liquid at higher pressures (10.0-20.0 MPa) in a way that the solvent can maintain its liquid state when a temperature above that of its boiling point
(usually between 50 and 200 °C) is applied [12, 13]. Compared to SFE, the solvent in PLE is still below its critical conditions. Higher temperature applied for PLE accelerates the extraction kinetics and as a result a faster and more efficient extraction is achieved [12, 14][13].

In recent years, gas expanded liquids (GXLs) have emerged as promising media for performing extractions, reactions, and separations. As defined by Akien & Poliakoff [15], a GXL is a “mixture of a condensible gas and several other components in a way that at least 2 fluid phases or a single phase above the bubble point curve but below the critical composition exist”. Under such conditions, the properties of the liquid phase(s) are substantially different from those at atmospheric pressure [15]. In other words, a GXL is a liquid whose volume is increased when pressurized with a condensible gas such as carbon dioxide. CO₂-expanded liquids are the most commonly used classes of GXLs representing liquid media ranging from the neat organic solvent to supercritical-CO₂ (SC-CO₂) [16]. GXLs have been exploited in a variety of applications including separations, fine particle precipitation, polymer processing, and as reaction media for catalytic reactions [15, 16]. However, the possible use of GXLs for the extraction of highly valuable lipid components in foods or natural products has not been explored yet, but, the milder pressures required for working with GXL as compared to traditional systems also make the industrial applications of GXLs more commercially attractive [17].

The ω6-polyunsaturated C18:3 fatty acid γ-linolenic acid (GLnA) is the first intermediate product in the conversion of linoleic acid into arachidonic acid [18]. GLnA is found in human milk in small amounts and in a wide variety of common foods, notably organ meats. It is found in relatively high abundance in the lipids
of plant seed oils such as evening primrose (7–10%, w/w), blackcurrant (15–20%), borage (18–26%) and fungal oil (23–26%) [19-21]. Nowadays, the most used source of γ-linolenic acid is borage oil, but cyanobacteria have also demonstrated to have significant amounts of this compound. Among cyanobacteria, *Arthrospira platensis* (Spirulina) has several advantages in terms of large scale commercial cultivation and thus it is of great interest as an alternative source of GLA [22]. The level of GLnA ranged within 18–21%, w/w, of the total fatty acids in the lipids [18, 23].

The aim of the present work was to evaluate the use of novel extraction techniques and solvents to obtain GLnA-enriched fractions from Spirulina microalgae. Both ethyl lactate combined with ethanol under pressurized conditions (using pressurized liquid extraction, PLE) and GXLs (mixtures of \(\text{CO}_2\):ethanol under near critical conditions) were tested and a chemometric optimization was carried out for a better understanding of the factors that mostly influence important responses such as extraction yield, recovery and selectivity.

2. MATERIAL AND METHODS

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. Ethanol and washed sea sand (0.25-0.30 mm diameter) were supplied by Panreac Quimica S.A. (Barcelona, Spain). Ethyl lactate (food grade, kosher), acetyl chloride, GLnA, heptadecanoic acid, butylated hydroxytoluene (BHT) and polyunsaturated fatty acid (PUFA) standards of marine source (PUFA No. 1) were purchased from
Sigma-Aldrich (St. Louis, MO). n-Hexane was purchased from Labscan (Dublin, Ireland). He, CO₂ (both Premier quality) and N₂ (Technical quality) was obtained from Carburos 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)

Different PLE extraction experiments were performed using an accelerated solvent extractor system (ASE 200, Dionex Corporation, Sunnyvale, CA) equipped with a solvent controller to keep solvent ratios at desired level. A detailed description on this extraction technique and equipment has been previously published [24, 25]. Two g of Spirulina 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 the inlet and outlet to maintain the sample (Spirulina) in the extraction cell. In the first step, the extraction cell was filled with the selected 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 fixed by the equipment and corresponded to 5, 6, and 9 min for 60, 120, and 180 °C, respectively. A static extraction process was carried out with all valves shut closed for the specified extraction times (5, 10, or 15 min). After the static stage of extraction, the cell and the tubing were rinsed (with 60% of the cell volume) using the fresh extraction solvent. Then, all the solvent present in the system was purged using N₂ gas. The solvent from this stage was collected in a vial and pressure of the unit was released. A thorough rinse of the system was applied between the two successive extractions to avoid any carry-over from one experiment into the next. To avoid
the degradation and or loss of the extracted compounds, the vials containing
could be found at: http://dx.doi.org/10.1016/j.supflu.2011.11.026
wet extracts were stored at -20 °C. A Rotavapor R-210 (Buchi Labortechnik AG,
Flawil, Switzerland) was used in a later stage to remove ethanol from the above
samples. An N₂ stream at 100 °C was also used for the removal of ethyl lactate.
To assure the full removal of ethyl lactate, this procedure was continued until a
constant weight was achieved.

2.3. Gas Expanded Liquid Extractions
A Suprex PrepMaster (Suprex, Pittsburgh, PA) supercritical fluid extractor was
used for the GXL extractions, this extractor was equipped with a dual piston
pump for CO₂. Two g of Spirulina was mixed with 4.0 g of sea sand and the
mixture was loaded into a 20 ml stainless-steel extraction cell. The extraction
cell was fitted with glass wool at the inlet and outlet. Ethanol was pumped using
a Jasco PU2080 HPLC pump (Jasco Inc., Easton, PA) and mixed at high
pressure with supercritical CO₂ (SC-CO₂). Compressed fluid mixture was fed to
the heater prior to entering the extraction cell. The flow rate was controlled
using a needle valve as variable restrictor. Extracts were collected in a glass
vessel cooled by ice. To avoid sample degradation, the extracts were stored at -
20 °C protected from light until drying step, using a rotary evaporator.

2.4. Fatty acid analysis
To determine the fatty acid contents of Spirulina samples, 30 mg of dried extract
from each sample was treated with 3.0 mL of ethanol-acetyl chloride (95:5, v/v)
solution. Two mg heptadecanoic acid, whose absence in the sample was
previously verified before the analysis, was added as internal standard. The
mixture was sealed in a 20 mL PTFE-lined vial under a nitrogen atmosphere
and heated to 85 °C for 1 h. The vial was cooled and 1.0 mL double-distilled water was added and shaken (vigorously) for 1 min and extracted with 3.0 mL of hexane containing 0.01% BHT (to prevent the oxidation of double bonds during the isolation procedure). The extract obtained by this method contained mixtures of Fatty Acid Ethyl Esters (FAEE) in hexane (upper phase). Hexane layer was transferred into a clean vial and injected into the GC-MS for qualitative and quantitative determination of FAEE. FAEE were analyzed with a Shimadzu of GC 2010 gas chromatography system (Kyoto, Japan) equipped with a Shimadzu AOC-20i autosampler and a split/splitless injector coupled to a QP-2010Plus single quadrupole mass spectrometer. Data were acquired and processed using Shimadzu GC Solution software. 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 injector, interface and ionization chamber temperatures were maintained at 220, 240, and 230 °C, respectively. The oven temperature was programmed to start at 100 °C, heated to 160 °C at a rate of 20 °C/min and then increased to 220 °C at a rate of 15 °C/min, where it was held for 8 min. A volume of 0.5 µL sample was injected into the GC-MS with the injector in the split mode (split ratio: 1/10) and He was used as the carrier gas. A solvent delay of 1.5 min was selected for the MS. Compounds were primarily identified by mass spectrometry in the SCAN mode using a mass interval ranging from 40 to 400 m/z. FAEE were then identified by comparing their retention times with those of standards (PUFA No. 1, marine source; heptadecanoic acid and GLnA ethyl esters) and also by comparing their mass spectra with those of Wiley library [26]. Quantitative determination of the fatty acids was carried out using the internal standard as mentioned earlier. A
standard curve was plotted for each FAEE using the weight and area ratios with respect to those of the internal standard.

2.5. Chemometric optimization

Response surface methodology (RSM) concerning central composite design was employed for the statistical design of PLE data. As defined by Khuri [27], RSM consists of a group of mathematical and statistical techniques used in the development of an adequate functional relationship (a low-degree polynomial model) between a response of interest, $y$, and a number of associated input variables denoted by $x_1, x_2, \ldots, x_k$. Design-Expert version 8.0.3 (Stat-Ease Inc., Minneapolis, MN), fitted to a second-order polynomial equation, was employed for analyzing the relationship between the responses and process factors and also for prediction and verification of model equation. A four-factorial (extraction temperature, extraction pressure, extraction time and solvent composition), three-level central composite design consisting of 30 experimental runs was performed in the present work, including six replications at the central point. The process factors and their levels in the design (as applied in this study) are shown in Table 1. Experimental values ranged from 60 to 180 °C for temperature, from 3.4 to 20.7 MPa for pressure, from 5 to 15 min (one cycle) for time and from 100% ethanol to 100% ethyl lactate for the solvent composition. The GXL experiments were designed using Taguchi’s $L_9(3^4)$ orthogonal array that can deal with four factors at three levels each by performing nine experiments [28]. Design-Expert version 8.0.3 (Stat-Ease Inc., Minneapolis, MN) was employed for analyzing the relationship between the responses and process factors and also for the prediction and verification of model equation.
The process factors and their levels for the design used in this study are shown in Table 2. In this particular case, factors considered were those related to typical SCCO$_2$ extractions including extraction temperature, extraction pressure, extraction time and the fraction of organic solvent. Since GXLs are considered, higher percentages of ethanol are used, ranging from 10% to 50% (w/w). All of these conditions have in common the formation of a liquid phase saturated with supercritical carbon dioxide [29], that is, an expanded liquid phase.

3. RESULTS AND DISCUSSION

Tables 1 and 2 show both the experimental matrix of the different designs and the values obtained for the different responses considered. The equations used in this study to determine the different responses were as follows:

\[
\text{Total yield (\%w/w) = (Weight of the Extract (g) / Weight of Spirulina (g)) \times 100} \tag{1}
\]

\[
\text{Lipid \% in the extract = (Weight of the Extracted Lipids (g) / Weight of the Extract (g)) \times 100} \tag{2}
\]

\[
\text{GLnA \% in the extract = (Weight of GLnA (g) / Weight of Extract (g)) \times 100} \tag{3}
\]

\[
\text{GLnA recovery = ((GLnA \% in the extract / Total yield (\%, w/w))/ Total GLnA \% in Spirulina) \times 100} \tag{4}
\]

GLnA recovery indicates the total amount of extracted GLnA to the total amount of GLnA present in Spirulina (1.8\%, w/w), which was determined using the method described in section 2.4.

Since some ethyl esters could have been formed during the extraction steps (due to the presence of ethanol or ethyl lactate at high pressure and temperature), ethyl esters were selected instead of the commonly used methyl esters for analyzing the fatty acid profile.
Table 1 shows the experimental values (for the 4 factors considered in the design) and the 4 selected responses. These responses can be grouped in two types, those related to the amount of material extracted (total yield (% w/w) and GLnA recovery % w/w) and those accounting for the selectivity of the process (% of lipid and GLnA that can be found in the total extract, w/w). As can be seen, total yields (% w/w) obtained varied from 6.3 to 22.7 while GLnA recoveries reached a maximum of 74.7% of the total amount of GLnA present in Spirulina. In terms of lipid and GLnA% in the extract, maximum values of 47.9 and 11.4% were obtained, respectively.

Matrix of the experimental design for the Taguchi’s orthogonal array design considered for GXL extractions of GLnA are shown in Table 2. Maximum total yield achieved was 7.4%, w/w, with a maximum GLnA recovery of 35.3%. Lipids and GLnA% in the extract were obtained within 26.2-48.0% and 6.5-11.7%, respectively.

Comparing the results from Tables 1 and 2 indicates that total yields (% w/w) were up to 3 times higher in PLE than in GXL. The use of one extracting phase seems to favor the recovery of a higher amount of extract, in contrast to the use of two phases in GXL experiments (SCCO$_2$ and expanded liquid ethanol). Since ethanol is used at a level higher than that of its solubility level, the extra amount will be present as a second (liquid) phase. Critical conditions of ethanol (6.1 MPa and 240 °C) are always higher than the maximum conditions of pressure and temperature (30.0 MPa and 80 °C) applied in the SCCO2-expanded ethanol extractions of this study and as a result the additional ethanol in the system can be considered in its subcritical state [29]. These results are in agreement with those obtained previously in our research group concerning
Spirulina extraction using PLE and supercritical fluid extraction, SFE [30, 31]. The mean values of lipids in the extracts were 33.4% for PLE and 34.8% for GXL. While maximum values achieved were almost equal for both extraction processes (up to 48.0%), the values were clearly influenced by the operation conditions, as it will be discussed later. Although the average GLnA level in the extracts was a bit higher when using GXLs for the extraction (8.8% vs. 7.3% for PLE), but, as was the case with the total lipids, the maximum levels were the same (11.7%) in both processes. Therefore, the higher yields obtained in PLE resulted in higher recoveries of GLnA when using this extraction technique. Considering the GLnA recoveries (Tables 3 and 4), they were greatly influenced by the extraction conditions and ranged within 29.4-74.7% by modifying the extraction conditions for PLE and within 5.8-35.3% when working under GXL conditions (Table 2).

3.1. Statistical modeling

Multi-factor experiments are normally designed to evaluate multiple factors set at multiple levels. Extraction Time, extraction temperature and solvent composition have been identified as the most important factors controlling yield and selectivity in a pressurized extraction for different type of solvents [30, 32]. Therefore these factors were selected for PLE in the current study. Although pressure has not shown a strong influence on these types of extractions, it was also considered since no references were found concerning the use of ethyl lactate under PLE conditions. By using a factorial experimental design, it is possible to obtain information not only on individual factors but also on possible
interactions among the various factors of the study. Such data can provide clearer and more complete understanding of the processes taking place during the extraction. Moreover, an experimental design can lead to an equation (a model) to help predict the future responses by changing the value of the factors in the model [32]. Also, by using the optimal settings of the studied factors, it is possible to maximize a specific response such as extraction yield or the selectivity.

In the present study, the responses selected for the optimization (to maximize) were total yield (%, w/w) and GLnA recovery (Table 1). By studying the responses separately, only total yield (%, w/w) in the PLE extraction provided a quadratic model, while the other models obtained were polynomial (Table 3). For all responses considered, temperature was the main factor, in fact, the most significant one, as can also be observed in Figure 1 for total yield of the extraction. This can be explained by an increase in the solubility of the components at higher temperatures and also by a decrease in the liquid (solvent) viscosity favoring the mass transport diffusion from the matrix to the liquid solvent. On the contrary, the effect of temperature on the GLnA fraction in the extract is negative (as noted by the negative coefficients in Table 3). Such reduction in the GLnA fraction due to higher temperature is also expected by considering the increased number of the compounds (other than lipids) extracted at higher temperatures. The capability of prediction of the model ($R^2$ and Adjusted $R^2$) is very good for total yield (%, w/w) but poor for all other responses considered (Table 3). As can be deduced from Figure 1, pressure has a small effect on the extraction yield and therefore, it is not considered a significant factor in any of the responses studied here. For a Taguchi’s three-
level orthogonal array design, multilevel categoric factors are considered, where
the first coefficient is the difference of the level 1 from the overall average and
the second coefficient is the difference of the level 2 from the overall average
and so on. The negative sum of all the coefficients is the difference of the last
level from the overall average. In the Taguchi’s three-level experimental design,
the obtained models have two coefficients for each level (low, medium or high)
of the factors (Table 4).

Considering the GXL experiments, temperature was found the less significant
factor (Table 5). In fact, p values for the temperature were above 0.1 for all the
responses studied here and therefore it was not included in the final model. On
the other hand, the fraction of ethanol (% ethanol, w/w) and also the extraction
time were the main parameters (according to Table 5). Figure 2 shows the
effects of the main factors of this study on the total yield (%, w/w) of the extract.
Total extraction yield (%, w/w) for GXL increased by increasing both extraction
time and ethanol fraction (%) in the solvent. Such behavior in the extraction with
GXL is quite different from that of the traditional SFE, in which, temperature and
pressure play major roles in changing the solubility of the components and thus
the mass transfer and total extraction yield of the operation. In a GXL, the
physical behavior resembles that of a pressurized liquid, as can be seen when
approaching levels up to 50%. In fact, Figure 2 shows the important increase in
the total extraction yield (%, w/w) when increasing ethanol contribution from 10
to 30%, where the changes in the physical behavior of ethanol from a
supercritical (or near-critical) fluid to a GXL occurs. After such point (up to 50%),
the increase in the extraction yield was not as much as it did for the previous
step. Maximum values obtained at 50% ethanol and 90 min extraction time
were very close to those achieved with PLE at 100% ethanol under the pressure (experiment number 25, Table 1). Compared to that of supercritical conditions (experiment 3, Table 2), total yields were much higher with GXL, where better mass transfer properties and enhanced solubility is expected. On the other hand, under supercritical conditions, higher lipid purity (around 48.0% w/w) is achieved for the lipids in the extract. Although such fraction is also richer in GLnA, the low yield (0.9%) and a corresponding low GLnA recovery precludes its use at industrial level.

When a tuning is required in the experiment, the use of experimental designs can be helpful for optimizing the conditions to achieve the best results (considering the optimization of several responses at the same time). In the present work, although the main objective was to extract GLnA, considering a future scale-up of the process, the total yield should also be the maximum possible. Therefore, the selected responses to maximize were total yield (% w/w) and GLnA recovery. The optimal conditions predicted by the model are shown in Table 6. As expected, maximum temperature was proposed as the optimum conditions in PLE while maximum time and ethanol (%) was suggested as optimum conditions for GXL. These conditions were run experimentally (at least three times) to test the validity of the statistical models. As can be seen in Table 6, the prediction is pretty close to the mean real value proving the fitting of the mathematical models to the real extraction processes.

Regarding the fatty acid composition of the extracts, GC-MS analysis of ethyl esters showed little variation. Figure 3 shows the bar diagram comparing the values of the different fatty acids identified in the different PLE extracts compared to fatty acids found in raw Spirulina (red bar in each fatty acid). This
little variation could be associated with the lipid extracted whose composition was almost the same in all cases. The largest difference was observed, for instance in GLnA, whose concentration in raw Spirulina was around 20 while values up to 27 were obtained under GXL conditions.

4. CONCLUSIONS

In the present work the ability of two alternative extraction techniques (GXLs and pressurized ethyl lactate:ethanol) to obtain high-value lipids from natural products has been demonstrated. By using statistical models, it was possible to describe and predict future responses such as total yield and GLnA recovery. In fact, the use of experimental designs allowed optimizing several responses at the same time. This optimization demonstrated various extraction possibilities using ethyl lactate as alternate solvent for the food industry. Meanwhile GXLs, which was used in this work for the first time in food products, have demonstrated its performance as half-way between PLE and supercritical fluids for the extraction of is medium-polar compounds.

5. ACKNOWLEDGEMENTS

This work has been financed by the Ministry of Science and Innovation CSD2007-00063 FUN-CFOOD (Programa CONSOLIDER-INGENIO 2010) project and by Comunidad Autónoma de Madrid (2009/AGR-1469). M.-T. Golmakani wishes to thank Iran Ministry of Science, Research, and Technology (# 42/4/52566) for supporting his stay in CIAL-CSIC, Spain.
6. REFERENCES


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Table 1. Matrix of the experimental design for the central composite design considered in the pressurized liquid extraction of γ-linolenic acid (GLnA) from *Spirulina*

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<th>Run order</th>
<th>T † (°C)</th>
<th>P † (MPa)</th>
<th>Extraction time (min)</th>
<th>Ethyl lactate (% v/v)</th>
<th>Total yield of extract (% w/w)</th>
<th>Lipid % in the extract (w/w)</th>
<th>GLnA level in the extract (% w/w)</th>
<th>GLnA recovery</th>
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</table>

†T: Temperature, P: Pressure
Table 2. Matrix of the experimental design for the Taguchi’s orthogonal array design considered for the gas expanded liquid extractions of γ-linolenic acid (GLnA) from *Spirulina*

<table>
<thead>
<tr>
<th>Run order</th>
<th>T (°C)</th>
<th>P (MPa)</th>
<th>Extracton time (min)</th>
<th>Ethanol (% v/v)</th>
<th>Total yield of extract (%)</th>
<th>Lipid % in the extract (w/w)</th>
<th>GLnA level in the extract (%)</th>
<th>GLnA recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
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<td>90</td>
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<td>39.5</td>
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</tr>
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<td>34.7</td>
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<tr>
<td>9</td>
<td>40</td>
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<td>60</td>
<td>30</td>
<td>4.2</td>
<td>32.6</td>
<td>8.7</td>
<td>21.1</td>
</tr>
</tbody>
</table>

†T: Temperature, P: Pressure

Table 3. Equation models and linear adjustment for the experimental design of pressurized liquid extraction γ-linolenic acid (GLnA) from *Spirulina*

<table>
<thead>
<tr>
<th></th>
<th>Intercept</th>
<th>T (°C) †</th>
<th>P (bar) †</th>
<th>Time (min)</th>
<th>T × P †</th>
<th>T × T †</th>
<th>R²</th>
<th>Adjusted R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total yield (%, w/w)</td>
<td>12.6</td>
<td>6.091†</td>
<td>0.507◊</td>
<td>0.558‡</td>
<td>0.578‡</td>
<td>1.167†</td>
<td>0.9622</td>
<td>0.9543</td>
</tr>
<tr>
<td>Lipid% in the extract</td>
<td>33.5</td>
<td>-5.965†</td>
<td></td>
<td></td>
<td>0.578‡</td>
<td></td>
<td>0.5319</td>
<td>0.5152</td>
</tr>
<tr>
<td>GLnA% in the extract</td>
<td>7.3</td>
<td>-1.551†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5986</td>
<td>0.5842</td>
</tr>
<tr>
<td>GLnA recovery</td>
<td>51.6</td>
<td>12.289†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.6313</td>
<td>0.6182</td>
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</tbody>
</table>

†T: Temperature , P: Pressure, GLnA: γ-linolenic acid

Table 4. Factors coefficients for the three-level Taguchi’s orthogonal array design considered for the gas-expanded liquid extraction

<table>
<thead>
<tr>
<th>Different Levels of Each Factor</th>
<th>First coefficient (A1, B1, C1, and D1) †</th>
<th>Second coefficient (A2, B2, C2, and D2)</th>
</tr>
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<tbody>
<tr>
<td>Low Level of Each Factor</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Medium Level of Each Factor</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>High Level of Each Factor</td>
<td>-1</td>
<td>-1</td>
</tr>
</tbody>
</table>

†A: Temperature (°C); B: Pressure (bar); C: Ethanol (% v/v); D: Time (min)
Table 5. Equation models and linear adjustments for the experimental design used for the gas expanded liquid extraction of γ-linolenic acid (GLnA)

<table>
<thead>
<tr>
<th>Intercept</th>
<th>A1†</th>
<th>A2</th>
<th>B1</th>
<th>B2</th>
<th>C1</th>
<th>C2</th>
<th>D1</th>
<th>D2</th>
<th>R²</th>
<th>Adjusted R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total yield (%, w/w)</td>
<td>4.0</td>
<td>-1.776</td>
<td>0.156</td>
<td>-1.956</td>
<td>0.632</td>
<td>0.9153</td>
<td>0.8306</td>
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<td></td>
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</tr>
<tr>
<td>Lipid % in extract</td>
<td>34.7</td>
<td>2.864</td>
<td>-1.656</td>
<td>5.970</td>
<td>-1.724</td>
<td>3.545</td>
<td>0.417</td>
<td>0.9893</td>
<td>0.9574</td>
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</tr>
<tr>
<td>GLnA % in extract</td>
<td>8.8</td>
<td>0.521</td>
<td>-0.424</td>
<td>1.091</td>
<td>-0.010</td>
<td>1.121</td>
<td>0.202</td>
<td>0.9936</td>
<td>0.9745</td>
<td></td>
</tr>
<tr>
<td>GLnA recovery</td>
<td>5.0</td>
<td>0.297</td>
<td>-0.242</td>
<td>0.622</td>
<td>-0.006</td>
<td>0.640</td>
<td>0.115</td>
<td>0.9936</td>
<td>0.9745</td>
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</tr>
</tbody>
</table>

† p <0.01; ‡0.01 ≤ p <0.05; ◊0.05≤ p <0.10; 
† A: Temperature; B: Pressure; C: Time; D: Ethanol (%)

Table 6. Optimized pressurized liquid extraction (PLE) and gas expanded liquid (GXL) conditions predicted by software and experimental responses obtained for the extraction of γ-linolenic acid (GLnA)

<table>
<thead>
<tr>
<th>Extraction methods</th>
<th>Optimized conditions</th>
<th>Predicted responses</th>
<th>Experimental responses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T† (°C)</td>
<td>P† (bar)</td>
<td>Time (min)</td>
</tr>
<tr>
<td>PLE</td>
<td>180</td>
<td>207</td>
<td>15</td>
</tr>
<tr>
<td>GXL</td>
<td>40</td>
<td>300</td>
<td>90</td>
</tr>
</tbody>
</table>

† A: Temperature; B: Pressure
Figure captions

Figure 1. Effect of significant factors (temperature and pressure) on the total yield (% w/w) of pressurized liquid extraction (PLE).

Figure 2. Effect of significant factors (time and ethanol (%)) on the total yield (% w/w) of gas expanded liquid (GXL) extraction.

Figure 3. Bar diagram corresponding to the fatty acid profile (% total fatty acid) of Spirulina extracted by PLE; Line indicates the mean value for raw Spirulina.