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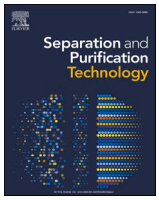

Deep eutectic solvents for the extraction of fatty acids from microalgae biomass: Recovery of omega-3 eicosapentaenoic acid

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ABSTRACT

Microalgae are a vast group of autotrophic microorganisms whose metabolic diversity makes them a natural source of valuable organic compounds such as lipids, carbohydrates, proteins, vitamins, and bioactive molecules. Several microalgae species contain notable amounts of polyunsaturated fatty acids, particularly eicosapentaenoic acid (EPA), which is an important alpha-linolenic acid derivative for human health. Conventional methods are considered effective at recovering total lipids from microalgae, however, they imply the use of large volumes of organic solvents such as methanol and chloroform, which are toxic and pose environmental risks. Thus, it is necessary to find new methods involving sustainable and green extracting phases. Deep eutectic solvents (DES) are renewable compounds often formed, but not exclusively, by quaternary ammonium salts and non-hydrated metal halides. Due to their availability, low cost, biodegradability, and environmental friendliness, DES are a promising alternative to organic solvents in extraction processes. This work assesses the efficiency of several DES phases for the extraction of fatty acids from the microalgae *Nannochloropsis gaditana* with a special interest in the recovery of EPA. The tested phases include mixtures containing choline chloride, lactic acid, ethylene glycol, and sodium acetate. Their performances were compared to those provided by conventional methods based on the use of organic solvents. Specifically, an *in-situ* transesterification process based on methanol with 10 %v/v of HCl was optimized in terms of temperature, time, and catalyst amount to be used as a reference. The results show that several of the tested eutectics such as choline chloride-ethylene glycol were capable of matching and even outperforming the best results obtained for EPA, with 104 % of extracted EPA methyl ester as the percentage of the mass obtained with HCl-methanol. The extraction capacity of DES was also improved by microalgae biomass pretreatment using ultrasonic and NaCl-based methods in a further stage. In the case of EPA extraction, and under optimal conditions, DES were capable of recovering over 18 % more quantity than the obtained with HCl-methanol. These results demonstrate that DES are effective at both recovering total fatty acids from pretreated biomass and at selectively recovering EPA using both unpretreated and pretreated biomass.

1. Introduction

The demand for resources is constantly growing to meet human needs due to the increase in the world population. The use of green sources of raw materials has become urgent for responsible economic production. In this sense, microalgae offer the possibility of obtaining high-value products for sustainable development [1]. Microalgae are a heterogeneous group of prokaryotic and eukaryotic microorganisms that can be found in seawater, freshwater, and soil environments. The unicellular and simple multicellular structure of microalgae facilitates their rapid growth, multiplying exponentially, even under severe conditions [2,3]. Microalgae can perform photosynthesis by fixing carbon dioxide and using water and sunlight due to cell pigments such as chlorophyll [4]. Because of their metabolic diversity, they are a natural and sustainable source of a broad range of compounds including carbohydrates, proteins, lipids, and bioactive molecules in significant amounts with applications in third-generation biofuel production [5], pharmaceutical uses [6], food industry [7] and fine chemistry [8], among others. Thus, the research of microalgae as a promising feedstock for a wide range of industrial ends and as a potential technology for CO_2 emission reduction

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and capture has greatly intensified in the last years. Another key advantage of microalgae is that they can be cultivated industrially in bioreactors with higher production rates per unit of area in comparison to vascular plants [9,10].

Microalgae cells contain between 30 and 80 % in lipids, representing an important reservoir of these compounds both for biofuel and bioactive compound production [11]. Part of these lipids are formed by glycerol and highly unsaturated fatty acids with chains of 12 or more carbon atoms. Fatty acids found in microalgae with chains between 14 and 20 carbon atoms are commonly employed for biodiesel production. On the other hand, lipids in microalgae with chains formed by 20 or more carbon atoms are mostly polyunsaturated fatty acids (PUFAs) that include omega-6 and omega-3 fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), respectively [12]. Omega-3 fatty acids are well known as bioactive compounds that provide health benefits to the population, especially for the prevention of cardiovascular diseases. As the human body is not capable of producing these compounds efficiently, they need to be supplied as food health supplements. Currently, PUFAs are mostly obtained from fish oils, which raises concerns about the depletion of marine resources. Thus, cultivated microalgae are a good alternative for the commercial production of bioactive fatty acids [13].

Nannochloropsis sp. such as *oculata, oceanica, salina,* and *gaditana* are recognized as promising feedstocks for lipid and fatty acids. Moreover, they contain significant amounts of EPA for the synthesis of omega-3-based products [14]. The extraction of the target compounds requires the disruption of the strong microalgae cell structure which is a relatively difficult process because of the complex composition of the cells, formed by proteins, polysaccharides, cellulose, and lipids, offering strong resistance to mechanical and chemical treatments. This can be achieved using organic solvents such as chloroform/methanol (2:1), acetone, and ethanol. After extraction, lipids are subjected to transesterification for their conversion into fatty acid methyl esters in contact with methanol and in the presence of an alkali or acid catalyst [15]. One-step *in-situ* transesterification processes have also been reported as a faster and simpler method to obtain FAMEs and produce omega-3 fatty acids using methanol and acid catalysts such as hydrochloric acid and sulfuric acid [16,17]. Alternative methods developed for lipid extraction from microalgae include microwave [18] and ultrasonic [19] assisted processes using organic solvents such as methanol and propanol, and supercritical fluid such as sc-CO_2 and enzyme-based methods [20].

The use of deep eutectic solvents (DES) as extract media for the recovery of natural products is a recent approach as an alternative to conventional organic solvents, which are flammable, volatile, poorly biodegradable, and toxic [14]. In contrast, DESs are considered environmentally friendly with similar thermodynamic properties to ionic liquids including thermal stability, low volatility, easy recyclability, and low cost synthesis [21,22]. The replacement of organics with these emerging solvents for the recovery of compounds from biomass could greatly contribute to the development of green and sustainable processes [23].

DES can be formed by the complexation of a hydrogen bond acceptor (HBA) like quaternary ammonium salts and a hydrogen bond donator (HBD) like ammine, carboxylic acids, or urea [24]. These solvents are mainly characterized by a significant decrease in melting points in comparison to both individual constituents. DES can be simply synthesized through the mixture of the primary constituents at temperatures between 50 and 80 °C under stirring until the appearance of a homogenous phase, and they can be readily eliminated since no chemical reaction occurs during the formation of DES, which results in asymmetric components. Thus, the interactions can be easily broken under sustainable conditions without the necessity of complex procedures [23,25]. In the case of DESs being formed by natural eutectic compounds from plant metabolites derivatives such as choline chloride, acetic acid, citric acid, and glucose, among others, they are named natural deep eutectic solvents [26,27]. This type of DES is highly biodegradable and presents almost zero toxicity. The resulting viscosity of DES is higher than those of conventional organic solvents and water. However, they are greatly tunable, and their viscosity can be reduced e.g. by the addition of water to enhance mass transfer and thus extraction yields [28].

Due to the high solubilization capacity of DESs, they can be used as solvents for organic, inorganic, and polymeric materials. Many studies reported in the literature on DES as extraction solvents have focused on the recovery of bioactive phytochemicals from plants or by-products from the food and agricultural industry, for instance, phenolic compounds [29], proteins [30], minerals [31], and essential oils [32]. Other applications include pretreatment of lignocellulosic materials [33], extraction and microextraction of chemicals [34], reaction media [35], enzymatic systems [36], and use in analytical techniques such as chromatography [37], among others.

The present work researches the extraction performance of several eutectic solvents by direct contact with microalgae biomass under stirring conditions at different times and temperatures. The eutectics choline chloride-lactic acid, choline chloride-ethylene glycol, choline chloride-glycerol, and sodium acetate-lactic acid were tested for the recovery of fatty acids from microalgae *Nannochloropsis gaditana*. The recovery of fatty acids and especially EPA was compared to that obtained with conventional organics such as methanol in the presence of a catalyst (HCl). Recovery rates were also improved by the pretreatment of microalgae biomass with ultrasonic and NaCl-based methods, respectively.

2. Materials and methods

2.1. Materials

Dried *Nannochloropsis Gaditana* strain was supplied by AlgaEnergy (Spain). Hexane (HPLC grade), methanol (HPLC grade), hydrochloric acid (37 % w/w), DL-lactic acid (80–85 %w/w), glycerol (99.5 % w/w), ethylene glycol (99 % w/w), choline chloride (98 % w/w) and sodium acetate anhydrous (99 % w/w) were purchased from Alfa Aesar (USA) and used as received.

2.2. Biomass pretreatment methods

Two pretreatment methods were tested to compare their influence on the lipid extraction yields. The first one was the osmotic shock using a 10 % NaCl solution. The microalgae biomass was mixed with the solution at 25 °C and stirred at 1000 rpm for 1 min, after that 25 mL of NaOH 0.5 M were added to enhance the microalgae settling. After 72 h, the mixture was filtered and washed with distilled water three times to remove NaCl traces and the recovered solid was dried at 80 °C until constant weight. The second pretreatment was the sonication of the microalgae using a Fisher Brand FB 11,205 ultrasonic bath at 32 Hz for 15 min.

2.3. In-situ extraction and transesterification with methanol-HCl solutions

In-situ extraction and transesterification were carried out by mixing 0.5 g of *Nannochloropsis Gaditana* with 45 mL of a methanol-HCl solution under continuous stirring and reflux, in which HCl acts as catalyst. Experiments were performed according to the experimental scheme shown in Table 1. Three key variables were studied at two levels to analyze their effect on extraction performance. Specifically, assays were carried out at temperatures of 60 °C and 70 °C, catalyst concentration (HCl) was analyzed at 5 %v/v and 10v/v in methanol, and time was fixed at 1.5 and 3 h, respectively. As a result of the combination of these values, a Box-Behnken factorial design with three levels and eight extraction scenarios was studied to find optimal conditions. Assays were performed in duplicate and mean values are reported.

Lipids recovery was performed by adding 5 mL of hexane over the

Table 1
Experimental scheme for the optimization of the *in-situ* extraction-transesterification experiments.

Run	Temperature (°C)	[HCl] (%v/v)	Time (h)
1	60	5	1.5
2	70	5	1.5
3	60	10	1.5
4	70	10	1.5
5	60	5	3.0
6	70	5	3.0
7	60	10	3.0
8	70	10	3.0

cool mixture, the procedure was repeated three times. The supernatants were collected and mixed in a 25 mL volumetric flask for further analysis.

2.4. Synthesis of deep eutectic solvents (DES)

A set of DES was synthesized according to the procedures previously reported (see last column of Table 2) to evaluate their extraction efficiency. Weighed samples were heated at 80 °C and stirred until a homogenous transparent liquid was formed. The deep eutectic solvents used in this study are summarized in Table 2.

Extraction experiments were carried out by mixing 0.5 g of *Nannochloropsis Gaditana* with 45 g of the eutectic. Extraction time and temperature were fixed according to the optimal variables achieved in Section 2.3. Extracted lipids were recovered using the procedure described in Section 2.3. Fatty acid methyl esters (FAMEs) were prepared by mixing the hexane extract with a methanol-10 %v/v HCl solution at 70 °C for 1.5 h under reflux and continuous stirring. After cooling down, FAMEs were recovered by adding hexane following the procedure described in Section 2.3.

2.5. Analysis of fatty acid methyl esters (FAME)

The hexane phase containing FAMEs was filtered through a 0.22 μm nylon membrane filter. A sample of 50 μL was transferred to a clean vial and analyzed by gas chromatography (Agilent Technologies 6890 N) equipped with a single quadrupole mass detector (Agilent Technologies 5975) and a DB-23 column (250 μm × 60 m × 0.25 μm). The injector temperature was set at 240 °C, and the source and quadrupole temperatures of the MS detector were set at 230 and 150 °C, respectively. The low and high mass interval was from 40 to 450 *m/z*. The temperature and time were programmed as follows: start at 50 °C (hold 1 min), and then elevated at a rate of 10 °C/min to 235 °C (hold 20 min). Helium was used as the carrier gas and 1 μL of the sample was injected in each run. FAMEs were identified and quantified by comparison with the retention times and mass fragments of a certified standard concentration which included the fatty acids displayed in Table S1. The fatty acids content in the standard were converted into FAMEs with a methanol-10 %v/v HCl solution at 70 °C for 1.5 h under reflux and continuous stirring.

Table 2
Synthesized deep eutectic solvents.

Hydrogen bond acceptor (HBAs)	Hydrogen bond donor (HBDs)	Molar ratio (HBD:HBA)	Code	Reference
Choline chloride	Lactic acid	(1:2)	ChCl-LA	[38]
Choline chloride	Ethylene glycol	(1:2)	ChCl-EG	[39]
Choline chloride	Glycerol	(1:2)	ChCl-Gl	[40]
Sodium acetate	Lactic acid	(1:3)	AcNa-LA	[41]

3. Results and discussion

3.1. In-situ transesterification with HCl-methanol

After the analyses of the extraction samples resulting from the *in-situ* transesterification method with HCl (10 %v/v) in methanol, seven out of the sixteen fatty FAMEs present in the standard were identified (Fig. 1). They include the methyl esters from dodecanoic acid (C12:0), tetradecanoic acid (C14:0), hexadecanoic acid (C16:0), cis 9-hexadecenoic acid (C16:1), oleic acid (C18:1), cis 9,12-octadecadienoic acid (C18:2), and EPA or eicosapentaenoic acid (C20:5). These FAMEs were identified in all the samples obtained under the extraction conditions described in Table 1. It is worth mentioning the natural presence of the polyunsaturated fatty acid EPA in *Nannochloropsis gaditana* due to its benefits to human health. The docosahexaenoic acid (DHA), which is another polyunsaturated fatty acid of interest, was not detected in this microalgae species in accordance with previously reported works [16].

Fig. 1 displays the amounts of individual fatty acids in the form of methyl esters obtained from microalgae *Nannochloropsis gaditana* under the eight tested experimental conditions. The results show that the highest quantities of FAMEs correspond to C16:1 and C16:0 methyl esters, respectively, followed by the amount of C20:5 (EPA) methyl ester in all assays. The extracted quantities of the rest of the fatty acids were significantly lower under all conditions, being C12:0 methyl ester present in the smallest amounts in the analyzed samples. The optimal conditions in terms of extractions rates were achieved at 70 °C and after 1.5 h of operation with a 10 %v/v of HCl (experiment 4). For these conditions, the amount of C16:1 methyl ester and C16:0 methyl esters were 36.46 mg and 25.78 mg per gram of microalgae, respectively. In the case of one of the target compounds, EPA, a total amount of 17.51 mg was obtained. The rest of the fatty acid recovery rates were 11.22 mg of C18:2 methyl ester, 9.83 mg of C18:1 methyl ester, and 9.36 mg of C14:0 methyl ester. For dodecanoic acid, the extraction yields were only between 1.41 and 1.09 per gram of microalgae in all assays, obtaining the highest value also in experiment 4. Although the conditions of experiment 4 were optimal considering the total amounts of FAMEs, the conditions of experiments 7 and 8 (60 and 70 °C, respectively, both at 10 %v/v of HCl and 3 h of operation), also yielded significant amounts of FAMEs. As a representative case, in experiments 7 and 8, the amount of C16:1 methyl ester was 91 % of that obtained in experiment 4. In the case of EPA methyl ester, this percentage decreases to 90 % (experiment 7) and 86 % (experiment 8).

In order to analyze the global contribution of each variable (temperature, time, and amount of catalyst, HCl) the main effects were obtained for each FAME type considering the factorial design. Main effects were calculated as the mean of the response (mg of FAMEs) at the high level of the variable minus the mean of the response at the low level of the variable. As seen in Table 1, for each variable level, there are four response values. As representative examples, Fig. 2 shows the response versus the level of each variable for the C16:1, C16:0, C18:1, and EPA (C20:5) methyl esters, respectively. It was found that both temperature and catalyst amount offered a positive effect on the response, while the time variable displayed near-zero (in the case of C16:1 and C16:0) o even negative effect (in the case of EPA). These results were also generally observed for the rest of the FAMEs. On the other hand, the effect of the catalyst amount was higher than that displayed by temperature. The net effect of HCl amount was 6.9 for C16:1 methyl ester, while the effect of temperature was 3.4. In the case of EPA methyl ester, the effect of HCl (4.1) was also more than double the effect displayed by the temperature (1.7). In the case of the time variable, 1.5 h can be considered sufficient for optimal extraction. The negative effects for EPA after 3 h of operation may be attributed to the possible degradation under thermal stress for such an extended period. According to Hădărugă et. al [42], PUFAs can display low oxidative and thermal

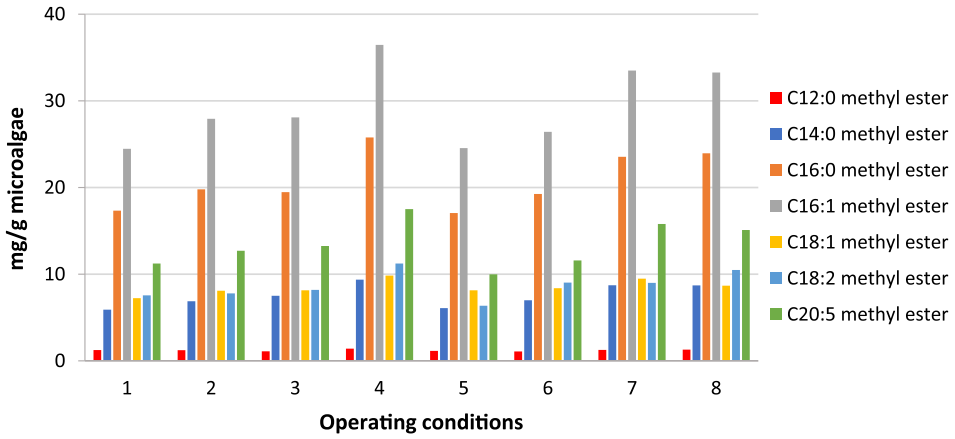

Fig. 1. FAME results via one-step *in-situ* transesterification with methanol (10 %v/v in HCl).

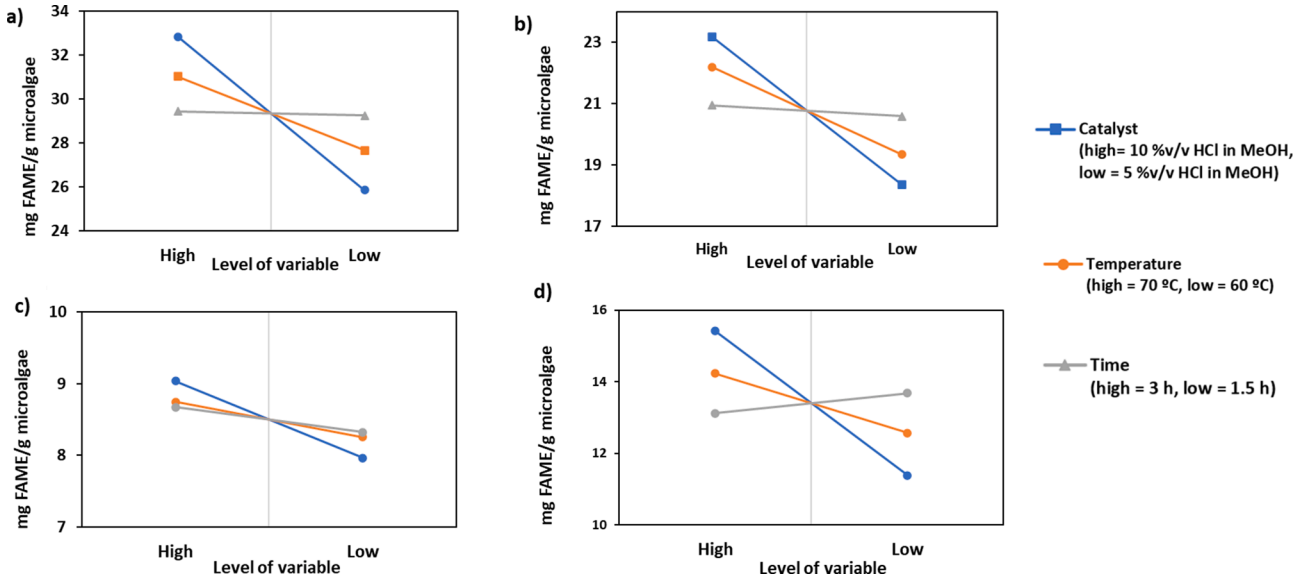

Fig. 2. Effects of variable levels on FAME recovery for a) cis 9-hexadecenoic acid (C16:1) methyl ester, b) hexadecanoic acid (C16:0) methyl ester, c) oleic acid (C18:1) methyl ester, and d) EPA or eicosapentaenoic acid (C20:5) methyl ester.

stability. Specifically, the omega-3 fatty acids EPA and DHA show a degradation rate higher than 60 % at temperatures ranged from 50 to 150 °C. Although the temperatures used in this study are not high enough to reach significant degradation of EPA, prolonged reaction times are likely to strenghten this effect.

3.2. Fatty acid extraction with deep eutectic solvents

The eutectic phases choline chloride-lactic acid, choline chloride-ethylene glycol, choline chloride-glycerol, and sodium acetate-lactic acid were tested as respective extract agents of fatty acids from dried

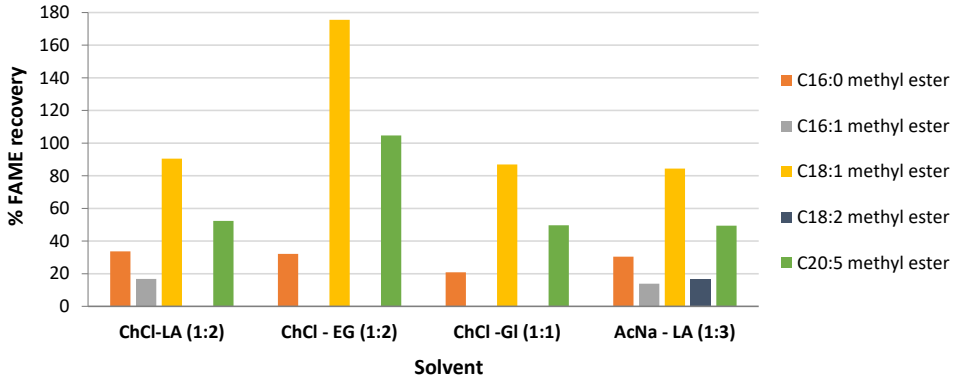

Fig. 3. FAME extraction with DES as a percentage of the amount extracted with HCl-methanol. ChCl-LA: choline chloride -lactic acid; ChCl-EG: choline chloride-ethylene glycol; ChCl-Gl: choline chloride -glycerol; AcNa-LA: sodium acetate-lactic acid.

microalgae biomass using the optimal conditions obtained from Section 3.1 to compare their performance to that offered by the HCl-methanol solution. Thus, extraction conditions were fixed at 70 °C and 1.5 h in the absence of a catalyst. Fig. 3 shows the profiles of FAMEs recovered as percentages of the amount of FAME mass extracted with the HCl-methanol solution. As seen, both the FAME types and their recovery percentages vary depending on the nature of the eutectics. Out of the seven FAME types extracted with methanol, only the C16:0, C16:1, C18:1, C18:2, and C20:5 methyl esters were found. In the case of the four eutectics, the highest recovery percentages were obtained for the methyl ester of cis 9,12-octadecadienoic acid or C18:1. In the case of the eutectic solvent choline chloride-ethylene glycol (1:2), the amount of C18:1 methyl ester was significantly high with a total of 175.5 % of the FAME mass obtained with HCl-methanol. In the rest of eutectics, the percentage was around 50 %. It is also worth noting that EPA (omega-3) was extracted with all the eutectic phases tested. Choline chloride-ethylene glycol could outperform the *in-situ* transesterification extraction process based on HCl-methanol, with a percentage of 104.7 % of the C20:5 methyl ester recovered with the latest. The other three eutectic solvents were only capable of extracting between 49.70 % and 52.52 % of the EPA methyl ester mass obtained with HCl-methanol.

These first results demonstrate the capacity of DES for the extraction of fatty acids contained in dry microalgae biomass by simple contact under mixing at 70 °C for 1.5 h. Remarkably, it was achieved a higher extraction rate for EPA than that measured for the *in-situ* transesterification process when employing the DES choline chloride-ethylene glycol with unpretreated microalgae. EPA has high affinity towards both hydrogen bond donor and acceptor molecules because it displays in its structure two hydrogen bond acceptors and one donor sites and, therefore, possibilities to form H-bonding with the DES molecules is likely to occur during extraction [43]. The nature of these bonds is expected to affect the extraction ability of DES towards EPA.

3.3. Fatty acid recovery with DES by biomass pretreatment

In order to improve the performance of the eutectics assessed in Section 3.2, the microalgae biomass was subjected to two respective pretreatments before the extraction process. These two pretreatments were based on the use of ultrasonics and NaCl solution (osmotic shock) for cell disruption. Fig. 4 shows the results obtained for ultrasonics. The total amount of FAMEs recovered was higher than in the absence of pretreatment. In comparison to non-pretreated biomass extractions, C16:1 methyl ester was found in all eutectic samples and C18:2 methyl ester was obtained in significant quantities when using the eutectics choline chloride-ethylene glycol and choline chloride-glycerol. The recovery of C18:1 also increased from 175.5 % up to 190.8 % (percentage of FAMEs over the amount recovered with HCl-methanol) when shifting from non-pretreated microalgae biomass to ultrasonic conditions. The amount of extracted EPA also increased significantly in all eutectic phases when employing ultrasonic pretreatment. The rise in the recovery percentage was especially notable in the first three phases tested, with percentages of 98.6 %, 118.0 %, and 75.9 % with choline chloride-acid lactic, choline chloride-ethylene glycol, and choline chloride-glycerol, respectively.

The extraction performance of DES was also improved when NaCl solutions were used as osmotic shock pretreatment of microalgae biomass in comparison to the use of untreated biomass microalgae. As in the case of the ultrasonic pretreatment method, choline chloride-ethylene glycol was the eutectic phase that enabled recovering the highest amount of fatty acids. The percentages over the quantities obtained with HCl-methanol were also significantly higher for the C18:1 and C18:2 methyl esters with values of 183.4 % and 295.1 %, respectively. EPA was found in all eutectic samples and the two phases choline chloride-ethylene glycol and choline chloride-glycerol outperformed the extraction capacity of the *in-situ* transesterification process based on HCl-methanol. Specifically, choline chloride-ethylene glycol offered the best results in terms of EPA extraction, since it extracted almost 11 % more of this fatty acid (in terms of FAME) than HCl-methanol.

Ultrasonics and osmotic shock have been researched in the literature for increasing lipid recovery from microalgae using organic solvents [44,45]. *Nannochloropsis* and *Chlorella* are among those microalgae whose wall is particularly difficult to break [46]. Although both ultrasonic pretreatment and osmotic shock increase the extraction of fatty acids with DES in comparison to unpretreated microalgae biomass, the use of ultrasound was more effective for cell disruption according to the results displayed in Figs. 4 and 5. While osmotic shock creates a hostile environment that causes damage to microalgae cell wall releasing the intracellular compounds, ultrasonication creates a series of successive compression and decompression waves which can create cavitation conditions inside the cell, producing heat, elevated pressure, free radicles, and shockwaves that destroy the cell walls [47] For comparison purposes, Fig. 6 summarizes the total amount of FAMEs obtained with the different solvents (methanol in the presence of HCl and eutectic phases) expressed as mass of FAMEs per gram of dry microalgae. The total quantity of FAMES obtained with the conventional solvent in the one-step extraction process amounts to 111.6 mg. It was only possible to recover higher amounts of fatty acids with the eutectic choline chloride-ethylene glycol when dry microalgae biomass was previously subjected to ultrasonic pretreatment, with a total of 130.9 mg of FAMEs, and to NaCl-based pretreatment, with a total of 123.86 mg. In the absence of biomass pretreatment, the total amount of FAMEs was clearly lower in the case of the eutectics in comparison to the use of HCl-methanol. However, looking at the individual FAMEs as commented before, the DES choline chloride-ethylene glycol could extract more EPA (C20:5) than the HCl-methanol, and also choline chloride-ethylene glycol obtained higher amounts of EPA when the dry biomass was pretreated with

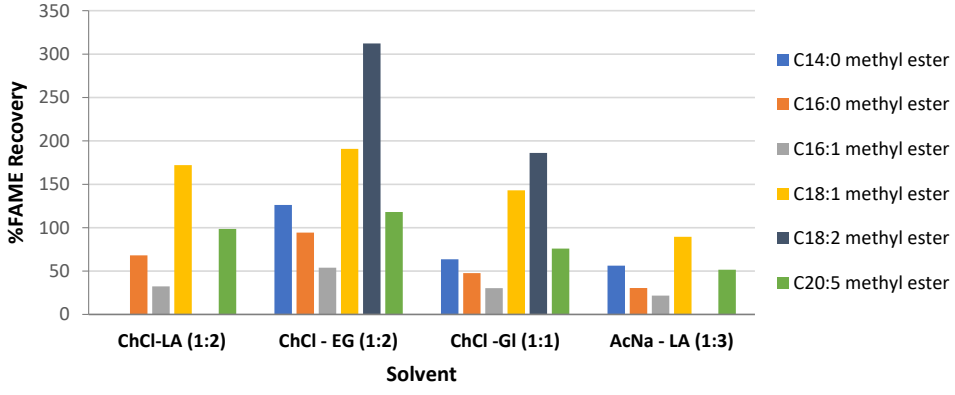

Fig. 4. FAME extraction from ultrasonic pretreated microalgae with DES expressed as a percentage of the amount extracted with HCl-methanol. ChCl-LA: choline chloride-lactic acid; ChCl-EG: choline chloride-ethylene glycol; ChCl-Gl: choline chloride -glycerol; AcNa-LA: sodium acetate-lactic acid.

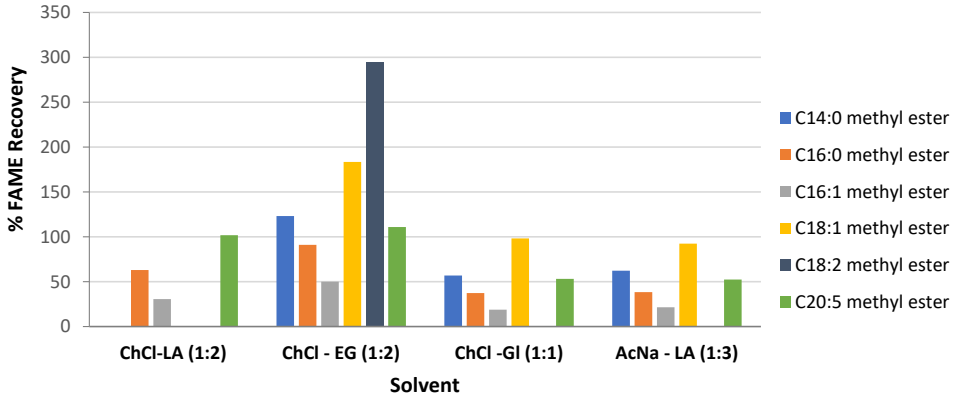

Fig. 5. FAME extraction from NaCl pretreated microalgae with DES expressed as a percentage of the amount extracted with HCl-methanol. ChCl-LA: choline chloride -lactic acid; ChCl-EG: choline chloride-ethylene glycol; ChCl-Gl: choline chloride -glycerol; AcNa-LA: sodium acetate-lactic acid.

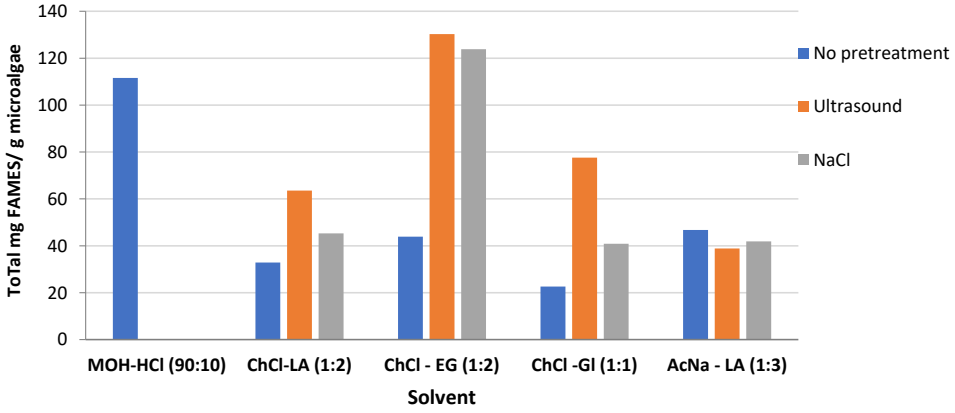

Fig. 6. Total amount of extracted FAMEs under tested conditions.

ultrasonic and NaCl methods. These results demonstrate that DES is effective at both recovering total fatty acids from pretreated biomass and at selectively recovering EPA using both unpretreated and pretreated biomass.

For analytical purposes, hexane was used for the re-extraction of the fatty acids content in the eutectic phases. For the development of a fully sustainable process, a re-extraction method should be developed in a next research stage. Nevertheless, according to the existing bibliography, several methods could be proposed for the recovery of target compounds from DES phases, including liquid–liquid extraction [48], application of antisolvents [49], and adsorption using microporous resin [50]. It is also worth mentioning that the addition of water to the DES phase could help to perform these re-extraction methods. After extraction, adding water to a DES can result into the weaknesses of the hydrogen-bond network in the DES as well as the interaction between the DES and the analytes [48]. On the other hand, supercritical CO_2 could be used for the recovery and purification of EPA from DES phases due to its solubility in this fluid [51].

4. Conclusions

Four deep eutectic solvents (DES) have been studied as extractive phases for fatty acids from microalgae biomass *Nannochloropsis gaditana* as alternatives to the use of organic solvents. Among the phases studied, choline chloride-ethylene glycol offered the best results in terms of fatty acid extraction, recovering 4 % more eicosapentaenoic acid (EPA) than the quantity obtained with *in-situ* transesterification process based on organic solvent methanol and the use of HCl as a catalyst using untreated microalgae biomass. The rest of the eutectics were capable of recovering 50 % out of the EPA extracted by this last method. These results demonstrate the capacity of DES in the extraction of fatty acids from raw microalgae. When microalgae were subjected to pretreatment by using ultrasonic methods or osmotic shock with NaCl, the amount of total fatty acids was higher with choline chloride-ethylene glycol than with the HCl-methanol process, followed by this order by choline chloride-glycerol, choline chloride-lactic acid and lactic acid-sodium acetate. With ultrasonic pretreated microalgae, the amount of EPA recovery by the choline chloride-ethylene glycol phase was 18 % higher than that obtained with HCl-methanol. Thus, eutectics were effective at recovering total fatty acids under pretreated conditions and offered high recovery rates of EPA both under unpretreated and pretreated conditions. Future works will delve into the optimal composition of the most satisfactory DES for the extraction of fatty acids as well as the optimal pretreatment conditions to maximize the recovery of fatty acids from microalgae feedstock.

CRediT authorship contribution statement

P. Moreno Martínez: Investigation, Writing – original draft. **V.M. Ortiz-Martínez:** Conceptualization, Writing – original draft, Supervision. **S. Sánchez Segado:** Conceptualization, Methodology, Writing – review & editing. **M.J. Salar-García:** Methodology, Validation. **A.P. de los Ríos:** Funding acquisition, Supervision. **F.J. Hernández Fernández:** Funding acquisition, Conceptualization, Supervision. **L.J. Lozano-Blanco:** Methodology, Writing – review & editing. **C. Godínez:** Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Antonia Perez de los Rios reports financial support was provided by Ministry of Science, Innovation, and Universities. Antonia Perez de los Rios reports financial support was provided by Seneca Foundation Science and Technology Agency of the Region of Murcia. Sergio Sanchez Segado reports financial support was provided by The Ministry of Science, Innovation, and Universities.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2022.121842.

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