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REVIEW

Green Extraction of Bioactive Compounds from Microalgae

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Abstract
At present, there is an increasing demand for natural bioactive compounds able to provide health benefits when included and consumed in a functional food or in a nutraceutical. In this regard, microalgae are promising natural sources with great potential, not only considering that these organisms are largely underexplored, but also because microalgae can be produced at large scale and their chemical composition might be tuned to over-synthesize a particular target compound. The use of advanced sustainable extraction techniques to recover these bioactive compounds is a must nowadays. This work presents an overview on the use of compressed fluid-based extraction techniques to obtain bioactive compounds from microalgae that can be seen also as a first step towards its recovery at larger scale. When relevant, the description of the analytical procedure used to chemically characterize the bioactive compounds is also included.

Keywords Bioactives · Compressed fluids · Green extraction techniques · Microalgae · Pressurized liquids · Supercritical fluids

1 Introduction

The relevance of the extraction and characterization of bioactive compounds from natural sources as a research topic is beyond any doubt today. New bioactive compounds, able to provide with additional health benefits are constantly investigated; nowadays, there are a lot of examples of different types of natural bioactive compounds already included in some commercial products, including plant stanols and sterols, bioactive peptides, polyunsaturated fatty acids or polyphenols, just to mention a few. In an effort to find new potential sources for these kinds of compounds, different ongoing researches are exploring new vegetable materials, also including agro-food by-products [1, 2]. Moreover, other natural sources still underexplored are also receiving increasing attention. Among those, marine organisms have a good potential, as many of them have not been studied and there is a wealth of diversity to be explored [3].

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Microalgae are one of those important new sources of bioactive compounds; they possess some interesting characteristics such as: (1) the high amount of different strains and species that can be potentially screened to find interesting bioactive components that can be subsequently employed in commercial products; (2) the possibility of isolating them from marine environments or growing biomass using bioreactors; (3) the minimal use of arable land for their production since microalgal cultivation is not necessarily linked to coastal regions; (4) the possibility of growing them under controlled conditions that can be tuned and modified to foster the over-production of particular target compounds. However, in a first step, the different microalgal species have to be studied and chemically characterized to confirm their potential for the production of a particular bioactive component.

To this aim, at present, advanced environmentally friendly extraction techniques and processes are preferred. It is a fact that traditional extraction methods are characterized using great quantities of organic solvents as well as for having limited extraction efficiency. For this reason, the development of new processes avoiding the use of toxic solvents while increasing efficiency and sustainability are a must. This is closely related to the green chemistry principles, which are directed towards the more efficient use of energy and resources [4]. Derived from these principles, six specific

aspects related to the extraction of natural products were formulated [5], including the search for new renewable natural sources, use of alternative green solvents, mainly water, the reduction of energy consumption and unit operations, and the valorization of co-products instead of generating by-products. Compressed fluid-related extraction techniques are very well suited to comply with those requirements and, thus, are very interesting for the extraction of natural components from different natural sources such as, for example, microalgae. Consequently, the goal of this contribution is to show an overview of the latest advancements of green extraction techniques, based on the use of compressed fluids, suitable for the extraction of bioactive compounds present in microalgae. To this aim, the most important aspects related to those techniques are briefly described, and the most relevant recent applications and developments directed to the extraction of bioactives from those organisms critically commented. When relevant, the analytical procedure used to chemically characterize the bioactive compounds is also discussed.

2 Modern green extraction techniques: overview

Compressed fluid-based extraction techniques are among those that can be considered as green extraction techniques if appropriate conditions are selected. These have in common their higher extraction efficiency when compared to extraction procedures performed at ambient pressures. Moreover, the solvents employed are submitted to certain conditions that, in practice, significantly modify their physico-chemical properties in a way not attainable otherwise. Nowadays, supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) are the most widespread techniques based on the use of high pressures for the extraction of natural bioactive compounds.

2.1 Principles of supercritical fluid extraction (SFE)

SFE is characterized by the use of a particular solvent under pressures and temperatures above its critical point. At those conditions, the fluid suffers physico-chemical changes that impact on its properties as a solvent. Indeed, under supercritical conditions (high enough temperature and pressure) no phase separation is produced, obtaining a homogeneous supercritical fluid. This way, supercritical fluids possess viscosities similar to gases whereas their densities remain similar to liquids, with intermediate diffusivity values among them. These and other changes make supercritical fluids significantly different solvents than those found at ambient conditions. For the extraction of natural sources, including microalgae, carbon dioxide is the most-employed supercritical fluid. Some reasons for this include its mild critical temperature and pressure (31.2 °C and 73.8 bar) that are easily reachable, and it is GRAS for the food industry, cheap and safe. Besides, since CO_2 is co-produced from a variety of industrial processes, its use can be considered as environmentally friendly, as it allows the reuse of a by-product. Moreover, a final aspect that makes CO_2 a very interesting alternative is that it will remain a gas in a room pressure. This means that once the extraction procedure is finished the CO_2 from the extract can be directly evaporated, leaving a completely solvent-free extract. Another interesting characteristic of this technique is that supercritical CO_2 (sc-CO_2) can be a very selective solvent. Temperature and pressure are the most influential parameters during extraction, and their combination defines the density of the sc-CO_2, and thus, its ability to selectively extract some substances from the natural matrix.

On the other side, the most important shortcoming is related to the low polarity of sc-CO_2. This implies that some natural bioactive components, which are often relatively polar, cannot be extracted. In those situation, the use of a co-solvent or modifier, usually small percentages of ethanol, together with the sc-CO_2 allows a modification in the polarity of the resulting solvent, allowing the extraction of more polar compounds, and thus, increasing the range of bioactive compounds that can be targeted. For more detailed information regarding principles, operation and instrumentation for SFE, readers are referred to other recent reviews [1, 6].

2.2 Principles of pressurized liquids extraction (PLE)

Pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE) or pressurized fluid extraction (PFE) (or subcritical water extraction (SWE) or pressurized hot water extraction (PHWE) when water is employed as extracting solvent) is characterized by the use of high temperature (below the critical point) and pressures high enough to maintain the solvent in the liquid state. PLE usually employs smaller volumes of solvents compared to conventional techniques and provides faster extractions due to an improved mass transfer rate. In fact, thanks to the higher temperatures, solvents possess increased solubility and decreased viscosity which helps to increase mass transfer rates and penetration into the matrix. Moreover, in the particular case of water, when maintained liquid, an increase in temperature produces a marked decrease in dielectric constant (ε). This value is commonly considered as a measure of the polarity of a solvent. This way, whereas water at ambient conditions possesses a ε of approximately 80, when submitted to 250 °C under enough pressure to keep the liquid state, this value decreases to near 30, which is comparable to the dielectric constants of some organic solvents, such

as ethanol or methanol. This implies that under those conditions, the solvent characteristics of water could be very similar to those of these organic solvents. In this regard, the use of water as solvent (SWE) could be considered as the greenest alternative involving the use of pressurized liquids.

As can be deduced, temperature and pressure are important parameters in PLE, whereas pressure should be enough to maintain the solvents in the liquid state. In fact, increments on extraction pressure beyond that point have been repeatedly reported as having little or no effect on the extraction process [7]. However, solvent selection is of utmost importance and should be decided in agreement to the nature of the target compounds. Other available published works can be consulted for more fundamental information regarding principles and instrumentation for PLE [1, 6].

3 Bioactive compounds from microalgae

3.1 Extraction of lipids from microalgae

Traditionally, lipids have been related with negative effects such as obesity, cardiovascular diseases or metabolic syndrome. However, lipids are one of the essential components of the diet. As macronutrients, these molecules are responsible for important biological and structural functions in the human body as well as the main energy storage source. Moreover, nowadays the importance and the positive effects that some classes of lipids produce in the human health are well known, such as the anti-inflammatory effect and cardiovascular protection exerted by polyunsaturated fatty acids (PUFAs). For this reason, the interest in the search of new natural sources of bioactive lipids has increased not only for the consumer but also for the industry with the aim of obtaining bioactive lipid-enriched products.

Microalgae appear as an obvious alternative of the main source of bioactive lipids, i.e., fish. In this sense, microalgae are well known for their content in bioactive lipids that can reach up to 85% of the dry weight; in particular, their high concentration in PUFAs is one of their most appreciated characteristics. The two main PUFAs in microalgae are ω-6 (α-linoleic and arachidonic acid) and ω-3 [α-linolenic, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] fatty acids. These PUFAs are considered as essential for the human diet and several bioactivities have been related to them, for instance potential prevention and reduction of cardiovascular diseases, arthritis, autoimmune disorders, inflammation, psoriasis or cancer [8]. This is one of the main reasons why the development of functional foods consisting of products enriched with PUFAs, in particular ω-3, becomes more extensive. Lipids of microalgae are classified into two groups, neutral lipids, such as triglycerides (TG), fatty acids (FA) or sterols, and complex or polar lipids, mainly phospholipids (PL) and glycolipids. However, the comprehensive lipid composition of microalgae is very difficult to determine due to the huge variability between different microalgal varieties and the effect of the harvested conditions.

Conventional extraction methods for the recovery of lipids from fish or microalgae consist of solvent extraction, steam distillation or hydrodistillation. However, these methods present several disadvantages such as the use of high temperatures and long extraction times that can degrade and oxidize some of the interesting compounds of the oil, severely affecting the quality of the final product while modifying the original content of the lipid fraction. Besides, another important drawback is the use of toxic solvents (hexane, methanol, dichloromethane) [9]. Characterization of the lipid profile of the obtained product is often the next step after the lipid extraction from microalgae. The most common analytical tools employed for the chemical characterization of lipids are GC–MS for the identification of the fatty acid profile and LC coupled to evaporative light-scattering detector (ELSD) or MS for determining the composition in TG, PL or tocopherols. Determining the composition of the lipids in the extracts is essential to select the optimal extraction conditions or to control how these conditions are affecting the compounds of interest. Moreover, the analysis of the lipid composition of different algae may help in the selection of the most adequate species [9]. Additionally, thanks to the chemical characterization of the lipid extracts, the identification of the compounds responsible of the health-promoting effect can be determined [10]. Due to the health benefits of lipids present in microalgae, as well as their easy degradation and the difficulty to isolate and concentrate these compounds through conventional extraction methods, milder, safer and environmentally friendly extractions such as SFE and PLE are interesting alternatives.

3.1.1 Supercritical fluid extraction for microalgal lipid extraction

Due to the non-polar nature of CO_2, this solvent presents all the characteristics required for the extraction of neutral lipids such as TG and FA. For this reason, its use for the extraction of sensitive and valuable lipids from microalgae is gaining attention. SFE using CO_2 as an extraction solvent has showed to provide a better specificity than conventional extraction methods, resulting in a different composition of the obtained extracts. A comparison between solvent extraction and SFE for the extraction of lipids from *Crypthecodinium cohnii* reported a more selective extraction of long-chain FA, mainly DHA, employing optimal SFE conditions at 30 MPa and 50 °C [11]. To overcome the low polarity of CO_2, the use of polar co-solvents is a recurrent alternative. The use of polar solvents during SFE has an important

impact both in the composition and in the extraction yield of the obtained extracts [12, 13]. A biorefinery approach was developed by Gilbert-López et al. with the aim to provide an efficient use of the microalgal biomass using several green extraction steps to obtain different valuable fractions. In some of these steps, SFE was employed for the recovery of bioactive lipids. Using CO_2 alone as extraction solvent, the fraction obtained was composed solely by TG. However, in the following step, that used ethanol as co-solvent, medium polar compounds and polar lipids, such as mono- and diglycerols, phospholipids and free FA, were extracted [13].

As can be observed in Table 1, SFE for the recovery of lipids from microalgae requires high extraction pressures (higher than 30 MPa); the explanation proposed by Sovová et al. deals with the high adsorption capacity of microalgae; under low pressures, microalgae bind to the fraction of the

Table 1 Main recent applications related to the use of SFE for the extraction of microalgal lipids

SFE with sc-CO_2 as extraction solvent

Microalgae	Compounds	Temperature (°C)	Pressure (MPa)	Extraction time (min)	Yield and/or goal	References
Nannochloropsis oculata	FA and tocopherol	80	20.7	240	71% extraction yield	[14]
Tetraselmis suecica	FA and tocopherol	40	62	240	69.9% extraction yield	[14]
Dunaliela salina	SFA, MUFAs, PUFAS and volatile compounds	9.8	31.4	100	To maximized antioxidant activity	[10]
Isochrysis galbana	TG	51	30	60	Purified and rich-TG fraction	[13]
Crypthecodinium cohnii	DHA	50	30	180	Lipid extract with 72% DHA composition	[11]
Scenedesmus obliquus and *Scenedesmus obtusiusculus*	TG and PUFAs	20	12	540	Recovery of 92% of lipids with 59% of PUFAs	[15]

SFE with sc-CO_2 + co-solvent as extraction solvent

Microalgae	Compounds	Extraction solvent	Temperature (°C)	Pressure (MPa)	Extraction time (min)	Yield and/or goal	Ref
Isochrysis galbana	Mid and highly polar lipids	Sc-CO_2/ethanol	50	7.38	60	Polar lipid-purified fraction	[13]
Scenedesmus obliquus and *Chlorella protothecoides*	ALA	Sc-CO_2/ethanol	60	30	90	Low ω6:ω3 ratio	[9]
–	Polar lipids	Sc-CO_2/ethanol/DME	51.85	30	270	13.4/24.0% extraction yield	[16]

Cell wall pretreatment + SFE extraction

Microalgae	Compounds	Pretreatment; extraction solvent	Temperature (°C)	Pressure (MPa)	Extraction time (min)	Yield and/or goal	References
Chlorella vulgaris	SFA, MUFAs, PUFAs	MW-SFE; sc-CO_2	40	28	540	Extract with 80% of FA	[17]
Phaeodactylum tricornutum	TG-rich fraction	MW-SFE; sc-CO_2	45	35	125	7% extraction yield of a purified TG fraction	[18]
Schizochytrium limacinum	DHA	US; sc-CO_2/ethanol + urea purification	40	35	60	Fraction with 60.4% DHA	[19]
Chlorella vulgaris	Neutral and polar lipids	Bead milling-SFE; sc-CO_2/ethanol	60	60	90	53% extraction yield	[12]
Nannochlorpsis oculata	TG	Air-flow-SFE; sc-CO_2	60	40	15	Recovery of more than 90% of triglycerides	[20]

extracted lipids, whereas an increase of pressure decreases its adsorption and, therefore, extraction rates only depend on the oil solubility in the sc-CO_2 [21]. In this work, a mathematical model is proposed for the study and simulation of the effect of particle size, CO_2 flow rate, temperature and pressure on the mass transfer resistance, adsorption capability and partition coefficient. This model reveals that the adsorption of the lipids to the biomass is the most determining factor in the extraction rate of SFE of lipids from microalgae and, therefore, higher pressures during the SFE process than the usually employed for the extraction of oil from other natural sources should be employed. Other mathematical models have been also developed to study the SFE kinetics involved in process yield and FA composition in the lipid extracts [22]. Several studies reported the importance of the cross-over pressure that corresponds to the pressure above which the main factor that affects the extraction yield is the steam pressure instead of the sc-CO_2 density. That means that at pressures lower than the cross-over point, the extraction yield decreases with the increase of temperature (due to a decrease in density), while at higher pressures, the increase of temperature improves the kinetics of the extraction (due to an increase in the vapor pressure of the solute) [9]. The knowledge of the cross-over pressure helps to control the extraction in the region where the solubility is extremely sensitive to the pressure. For instance, Solana et al. employed this concept to control the supercritical fluid extraction of *Nannochloropsis salina* microalgae with the aim of producing an α-linoleic-rich oil with a healthier *ω*-6:*ω*-3 ratio. In this work, authors concluded that low temperatures and pressures together with short extraction times favor the extraction of *ω*-3 FA.

On the other side, in the extraction of bioactive compounds from microalgae, the extraction yield is strongly related to two important parameters: the water content of the biomass and the structure of microalgae, in particular, the rigid and thick cell walls composed by cellulose, hemicellulose, glucosamine, lipids, proteins, and ash may imply a barrier to recover the compounds of interest. In reference to the first parameter, there are two main processes for reducing the water content of microalgae: air-flow and freeze-drying. Crampon et al. evaluated the effect of these two drying processes on the SFE of lipids, observing that both pretreatments produced very similar extraction yields (ca. 90%), although the process employing microalgae treated with air-flow followed by SFE provided a faster extraction kinetics [20]. Nevertheless, the extraction of lipids with sc-CO_2 from wet biomass (up to 20% of water content) has been successfully achieved with good results both for extraction yield as well as for extraction kinetics [16, 20].

Regarding the microalgal cell wall, several works report cell disruption methods as pretreatments before the SFE to improve the accessibility to the intracellular bioactive lipids. These methods consist of mechanical treatments of the biomass such as bead milling. This pretreatment was employed for improving the SFE extraction yield of lipids from *Chlorella vulgaris*. Microscopy observation of the cells after the milling showed the rupture of the microalgal cell walls, which resulted in a better accessibility of the sc-CO_2 to the intracellular compartments giving rise to a reduction of the extraction time; in particular, maximum extraction yield was obtained in 90 min, whereas the extraction without pretreatment lasted 180 min [12]. Other pretreatments employed for the disruption of the cell wall consist of the combination of microwave or ultrasounds with SFE. Microwave as pretreatment has demonstrated to provide higher extraction yields as well as to increase the FA concentration of the SFE extracts [17]. Besides, microwaves have been tested in combination with deep eutectic solvents before the SFE of lipids from *Phaeodactylum tricornutum* showing a significant improvement in the extraction efficiency (20-folds greater), producing highly purified TG extracts [18]. Ultrasounds are also a successful pretreatment to improve the SFE kinetic of lipids, reducing by half the extraction time for obtaining a DHA-rich extract from microalgae [19].

The ultimate objective of the extraction of lipids from microalgae is to obtain a product rich in bioactive lipids and to study their impact on the human health. Mendiola et al. studied the antimicrobial activity of a supercritical fluid oil extract obtained from the microalgae *Dunaliella salina*, evaluating the effect of pressure and temperature. Extracts obtained at high pressure (31.4 MPa) and low temperature (9.8 °C) showed the maximum antimicrobial activity. Thanks to an exhaustive study of the chemical composition of this extract by GC–MS, palmitic, stearic, α-linolenic and oleic acids could be related to the antimicrobial activity since these FA represented around 80% of the total chromatographic area [10].

Besides TG and FA, another important lipid compound that has been extracted from microalgae is tocopherol or vitamin E, well known as a powerful lipophilic antioxidant related to important biological activities such as inhibition of the proinflammatory pathway and reduction of adverse inflammatory effects [23]. Bong et al. reported the extraction of FA and tocopherol using sc-CO_2 in two different microalgae: *Nannochloropsis oculata* and *Tetraselmis suecica*. With a conventional solvent extraction using dichloromethane/methanol and hexane as extraction solvents, only α-tocopherol was extracted from both algae, whereas using SFE, and α-, β- and γ-tocopherol were detected in the extracts from *N. oculata* and α- and β-tocopherol were extracted from *T. suecica* [14].

As mentioned previously, the optimization of SFE conditions for analytical purposes can be seen as a first step towards the scale up of these processes for industrial applications. As an example, Lorenzen et al. developed an industrial

process including an on-line purification step with bentonite as adsorbent [15], while Crampon et al. carried out the scaling up of an analytical SFE process evaluating the influence of the pressure, the sc-CO_2/microalgal mass ratio and the particle size in the yield and extraction kinetics. Employing readily applicable operating conditions (50 MPa, 60 °C, 0.5 mm particle size and a CO_2/biomass ratio lower than 30), they obtained extracts with similar composition to those achieved at analytical scale [20].

3.1.2 Pressurized liquid extraction for microalgal lipid extraction

PLE offers several advantages regarding lipid extraction from microalgae such as short extraction times, absence of oxygen and the possibility of carrying out a selective extraction of different lipid classes modifying the polarity of the extraction solvents. However, in spite of these advantages, the number of works that employed PLE for this purpose is much lower than those using SFE. Table 2 presents a summary of the most recent interesting applications on this topic.

PLE has been employed for the extraction of microalgal bioactive lipids with different biological activities. The antiviral activity of PLE extracts from *Haematococcus pluvialis* and *Dunaliella salina* has been reported [24]. In this study, different extraction solvents were tested, i.e., hexane, ethanol and water, with the aim of evaluating the antiviral activity of the different fractions against herpes simplex virus type 1 (HSV-1). The extract obtained using ethanol as extraction solvent showed the better antiviral activity producing an inhibition of the virus by 85%, whereas the inhibition of the water and hexane extracts was 75 and 50%, respectively. Comparing the bioactivity of both microalgae, *D. salina* resulted less effective than *H. pluvialis*. The GC–MS analysis of ethanolic PLE extracts of both microalgae could correlate the *H. pluvialis* activity with the presence of high amount of short-chain FA (propionic, lactic and butanoic acids) and hexadecatrienoic acid, while in *D. salina* other lipid compounds such as neophytadiene, palmitic acid and α-linolenic acid could be related to the antiviral effect.

Plaza et al. compared the antimicrobial activity of extracts obtained with PLE and with ultrasound-assisted extraction (UAE) using different extraction solvents. Authors demonstrated that temperature had a crucial effect in both, extraction yield and composition of the extracts; in fact, better extraction yields were obtained at the highest tested temperature (200 °C) due to the increase of solubility and the improvement of mass transfer at high temperatures. Both methods presented similar bioactivities, in particular the highest antimicrobial activity was provided by the ethanol extracts. However, PLE provided higher extraction yields and a more controlled, automatic and fast extraction. Palmitic, hexadecatrienoic, oleic, linoleic and α-linolenic acids were detected as the main FA in the ethanolic extracts and, therefore, these compounds were related to the observed antimicrobial activity [25].

The effect of solvents with a wide range of polarities (hexane, hexane/isopropanol (2:1, v/v) and ethanol) was evaluated by PLE to maximize the FA extraction from *Nannochloropsis oculata.* The major extraction yield, as well as the major FA yield (particularly EPA) were achieved using ethanol as extraction solvent [26]; the different yields obtained with each solvent can be observed in Fig. 1.

Table 2 Main recent applications related to the use of PLE for the extraction of microalgal lipids

Microalgae	Compounds	Extraction solvents	Temperature (°C)	Pressure (MPa)	Extraction time (min)	Yield and/or goal	References
Haematococcus pluvialis	Short-chain FA	Ethanol	100	10.34	20	Antiviral activity	[24]
Dunaliella salina	Short-chain FA	Ethanol	160	10.34	15	Antiviral activity	[24]
Chlorella vulgaris	HDA, phytol, tocopherol and phytosterols	Water	150–200	10.34	20	Antimicrobial and antioxidant activity	[25]
Nannochloropsis oculata	PUFAs	Ethanol	60	10–12	48	36.4% extraction yield	[26]
Arthrospira platensis (Spirulina), *Phormidium* sp, *Anabaena planctonica Stigeoclonium* sp	Medium and polar FA	Limonene/ethanol	200	20.7	15	Recovery of 70% of lipids	[27]
Haematococcus pluvialis	Short-chain FA and tocopherol	Water	200	10.34	20	Antimicrobial and antioxidant activity	[28]

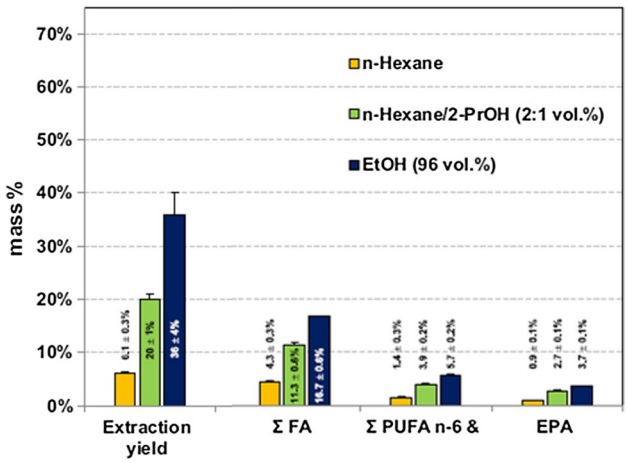

Fig. 1 Comparison of the extraction yield, total FA, total PUFAs and total EPA obtained using different solvent polarities in the PLE extraction of the microalgae *Nannochloropsis oculata*. Modified from [26] with permission from Elsevier

Limonene/ethanol mixtures have also shown promising results as solvent in PLE for the extraction of medium and polar lipids from *Arthrospira platensis* (*Spirulina*), *Phormidium* sp., *Anabaena planctonica* and *Stigeoclonium* sp. microalgae [27].

3.2 Extraction of carbohydrates from microalgae

Microalgae are a rich carbohydrate source; in particular, the carbohydrate fraction is mostly composed of sulfated polysaccharides. Nevertheless, little information is available about the classes of polysaccharides present in most microalgal species, with the exception of some β-glucans and spirulans [29, 30]. However, microalgal carbohydrates, and mainly their structure and physico-chemical properties, are responsible for the different and important health-promoting properties such as anti-inflammatory, antitumor, anti-adhesive, antiviral or antibacterial activities [30]. Besides, they have been considered as immunomodulatory and infection-preventive agents and have been described as a good antilipidemic and antiglycaemic compounds [30–32]. However, establishing the relationship between the chemical structure of the polysaccharides and their biological activity is not an easy task due to the huge complexity of microalgal carbohydrates, characterized by highly branched heteropolymers with a high diversity in their monosaccharide composition and distribution as well as in their glycoside bonds [30, 33]. Therefore, the exhaustive characterization of microalgal carbohydrates presents some analytical limitations making more complex their analysis as the size of the carbohydrates increases [29].

Regarding the extraction of these compounds, the great majority of the applications consist of a concentration step using low temperatures followed by precipitation with a variety of organic solvents. Membrane filtration techniques have recently been used for the recovery and purification of microalgal carbohydrates [33]. However, these conventional methods using low temperature are not completely effective for the extraction of carbohydrates from microalgae due to their complexity and the inherent tensile strength of the microalgal cell wall [34]. Therefore, extraction techniques that applied higher temperatures in combination with high pressures could improve the recovery of these bioactive compounds.

3.2.1 Pressurized liquid extraction for microalgal carbohydrates extraction

Among the pressurized extraction techniques, subcritical water extraction (SWE) is the most common technique for the extraction of carbohydrates from microalgae. Although its use is not widespread yet, this technique has shown promising results in some successful applications for the recovery of bioactive microalgal polysaccharides. The main parameters that affect the extraction yield and the composition of the extract in SWE are the temperature, the extraction time, the biomass loading and the particle size. Some of the works that apply SWE for the extraction of microalgal carbohydrates evaluate the effect of these extraction parameters [34, 35]. The results of these studies showed that the polysaccharide content on the final extract is greatly affected by the temperature. Carbohydrates have a good solubility in subcritical water when the temperature increases from 100 to 150 °C due to the reduction of the dielectric constant of water and, therefore, the maximum extraction yield is obtained at temperatures close to 200 °C. However, at higher temperature, the carbohydrate extraction yield decreases due to the degradation of the polysaccharides and besides, to the lower contribution of the reduction effect of the subcritical water dielectric constant.

Apart from high temperatures, Awaluddin et al. showed that long extraction times and high biomass loading negatively affected the carbohydrate recovery [35]. In other studies, the effect of SWE conditions was also evaluated in terms of chemical composition and bioactivity of the obtained extracts. Regarding the antioxidant activity of the SWE carbohydrate-rich extracts, the highest activity is obtained at lower temperatures, whereas it decreases when the temperature increases. SWE extraction and purification of carbohydrates from the microalgae *Haematococcus pluvialis* and *Dunaliella salina* was also investigated for their potential antiviral activity, and the purified fraction showed higher antiviral action than the original water extracts. Besides, the GC–MS analysis of these carbohydrate-rich fractions revealed two different profiles in both microalgae being glucose the main component of *D. salina* (94.34%),

whereas *H. pluvialis* presented mannose as major compound together with high quantities of glucose and galactose [24].

Interestingly, during the SWE procedure at high temperatures some reactions that involve carbohydrates are frequent, giving as result products which have been highlighted for their interesting antioxidant capacity such as the Maillard reaction products generated as a result of reactions between reductive sugars and amino acids and also related to caramelization reactions. For instance, extracts obtained by SWE at 200 °C from *Haematococcus pluvialis* showed a good antioxidant activity that could be associated to the occurrence of those neoformed compounds. In particular, this activity was associated with the caramelization of derivative compound α-D-fructofuranoside-1,2´:2,1´-β-D-fructopyranoside, identified by GC–MS [28].

Besides SWE, PLE with other solvents was employed in a biorefinery approach with the aim of recovering all the functional ingredients from *Isochrysis galbana* microalgae [13]. Different percentages of water/ethanol were studied to achieve PLE extracts with the maximum carbohydrate extraction yield as well as with potential antioxidant activity. Compared to extracts obtained with water, ethanol extracts showed an antioxidant activity twofold higher; this fact allowed to elucidate that the antioxidant activity was related to monomers and oligomers, since ethanol tends to precipitate highly polymerized carbohydrates [13].

3.3 Extraction and characterization of proteins from microalgae

By definition, proteins are three-dimensional macromolecules consisting of one or more polypeptide chains, which are, in turn, formed by chains of amino acids linked together by peptide bonds [36]. It is well known that proteins are very sensitive to many factors that trigger denaturation (non-reversible breakage of the three-dimensional structure), losing their chemical properties and their biological activity. These factors can be physical (i.e., temperature or pH), chemical (i.e., detergents, organic solvents or heavy metals) or biological (i.e., enzymes or contaminants) [36, 37]. Thus, it is difficult to isolate or purify proteins from any matrix without affecting their structure and bioactive properties.

Microalgae are considered a rich source of proteins, comprising between 50 and 70% of their composition. For that reason, there are many microalgae such as *Chlorella vulgaris, Spirulina platensis, Scenedesmus obliquus, Dunaliella* sp.*, Porphyridium* sp. or *Tetraselmis suecica* that have been used as a supplement in food, animal feed, or aquaculture due to their high protein levels [38]. Although proteins, in general, are not the most high-value products in microalgae, peptides and amino acids derived from parent proteins (i.e., released by proteolytic enzymes or fermentations) are being recently studied due to their bioactivities. Thus, some potential benefits such as antioxidative, antihypertensive, anticoagulant, antiproliferative or immune-stimulant activities have been related to some microalgal biopeptides or amino acids [39].

In relation to green processes, there is not much information about the specific isolation of proteins from microalgae. Instead, some authors have obtained protein-rich fractions (as co-products or residues) in some microalgae such as *Phaeodactylum tricornutum* and after a biorefinery process. In these studies, several extraction steps involving supercritical fluid extraction, gas-expanded liquids and pressurized liquid extraction were performed to obtain extracts from different bioactive compounds (such as carotenoids or lipids). Interestingly, they could not identify either microalgal proteins or sugars in these extracts, so these valuable compounds were mostly contained in residues, giving an interesting alternative to these by-products [40, 41]. In any case, all the developed applications are limited to the use of pressurized water, according to the chemical nature of proteins.

3.4 Extraction of minor components from microalgae

Within the chemical composition of microalgae, several minor components are present which may represent important targets from a bioactivity point of view. These components are considered as secondary metabolites and are produced by the cells in response to different growing conditions. For this reason, multiple approaches have been studied to achieve the over-production of certain compounds included in this category [42], which is one of the strongest points to be considered for the large-scale cultivation of microalgae.

3.4.1 Carotenoids

Carotenoids are lipophilic compounds that can be found in many natural sources as plants, seaweeds and microalgae. Their C40 structure is based on isoprene units which can contain oxygen, so they can be classified in two main groups: carotenes and xanthophylls [43]. There is an extensive list of potential benefits that have been attributed to these natural pigments, mainly due to their role within the cells as antioxidants or free-radical protectors. Moreover, carotenoids seem to play an important role reducing or preventing many human diseases such as cardiovascular disease (CVD), atherosclerosis or metabolic syndromes, and also enhancing immune responses to bacteria, virus and other infections [44]. Sathasivam and Ki properly reviewed recent investigations on health and cosmetic benefits associated with main carotenoids found in microalgae. Table 3 summarizes these biological activities [45].

Table 3 Summary of biological activities attributed to main microalgal carotenoids

Carotenoids	Biological activities
Astaxanthin	Cardioprotective, anti-cancer, anti-diabetes, anti-inflammatory, antioxidant, beauty-enhancing effect
Canthaxanthin	Anti-cancer
Capsanthin	Anti-cancer
α-carotene	Anti-cancer, anti-diabetes
β-carotene	Anti-angiogenic, anti-cancer, anti-diabetes, beauty-enhancing effect, age-related macular degeneration
Crocetin	Anti-cancer
β-cryptoxanthin	Anti-diabetes
Fucoxanthin	Anti-angiogenic, cardioprotective, anti-cancer, anti-diabetes, anti-obesity, beauty-enhancing effect, neuroprotective, osteo-protective, weight loss
Lutein	Anti-cancer, anti-diabetes, age-related macular degeneration
Lycopene	Anti-cancer, anti-diabetes, beauty-enhancing effect
Neoxanthin	Anti-cancer, anti-obesity
Phytoene	Anti-cancer
Zeaxanthin	Anti-cancer, anti-diabetes, age-related macular degeneration

As carotenoids are related to a great number of human health benefits, many authors have focused on obtaining carotenoid-rich extracts from microalgae using green and sustainable processes (PLE, SFE and gas-expanded liquids—GXL). In Table 4, a list of carotenoids extracted from different microalgae using PLE, SFE or GXL as well as the reported extraction conditions (solvent, time, temperature and pressure) is presented. In general, there is no single method to obtain carotenoids from microalgae, mainly because each microalga has a different composition. One important point to consider is the nature of the sample, so extraction conditions will change depending on growing conditions, moisture content of the sample, pretreatments before extraction, and so on. In this sense, Jaime et al. analyzed the chemical composition of PLE extracts from *Haematococcus pluvialis* grown under normal and stress conditions (green vegetative cells and red cysts, respectively) and found that extracts from both phases contained different carotenoid composition [46]. Figure 2 shows the chromatograms obtained from those ethanol extracts obtained at 100 °C as well as the identification of the main compounds. Thus, lutein was the major carotenoid in green-phase cells, whereas astaxanthin-derived compounds were the main pigments in red-phase cells of *H. pluvialis* (up to 90% of total compounds). Furthermore, they reported that these differences in carotenoid composition were closely related to their antioxidant activities. In that application, pressurized ethanol was employed as extracting solvent, whereas the chemical characterization was performed by combining HPLC and TLC methods.

It is important to remark that carotenoids cover a wide range of polarities, so the solvents or the method used will be different depending on the target compound. For instance, for β-carotene extraction, a pressurized liquid extraction using ethanol could be favorably used [40, 41].

One interesting green-process design was reported by Gilbert-López et al. [13]. In that work, a biorefinery process was developed consisting of four sequential steps (including SFE, GXL and PLE) to obtain a fractionation of different valuable compounds from *Isochrysis galbana*. They found that carbon dioxide-expanded ethanol (CXE) containing 45% ethanol, operated at 70 bar and 50 °C was the most suitable solvent to maximize the extraction of the major carotenoid (fucoxanthin).

Another similar biorefinery approach is illustrated in Fig. 3, where a combined process was devised to obtain lutein and other bioactive compounds from *Scenedesmus obliquus* [41]. Again, the CO_2-expanded ethanol step (using 75% EtOH, 70 bar and 50 °C) turned out to be the most effective method to obtain the highest possible concentration of carotenoids.

3.4.2 Phenolic compounds

Phenolic compounds are a wide group of secondary metabolites. Their basic structure consists of one or more hydroxyl groups bound to an aromatic ring, making them polar compounds. Phenols are mainly synthesized by plants, mostly as esters or glycosides rather than as free compounds, playing an important role in a variety of functions such as growth, development and defense. These hydrophilic compounds can be also found in bacteria, fungi and algae [65, 66].

As carotenoids, polyphenols in microalgae play an important role in UV protection and antioxidation processes. However, there are other biological activities related to these components such as antiproliferative, antibiotic, antidiabetic,

Table 4 Most-relevant recent applications devoted to the extraction of carotenoids from microalgae using green processes (pressurized liquid extraction *PLE*, supercritical fluid extraction *SFE* or gas-expanded liquids *GXL*) published during the period 2008–2018

Main carotenoid	Microalgae	Other carotenoids extracted	Methods	Extraction solvents	T (°C)/P (MPa)	Extraction time (min)	References
Astaxanthin	*Haematococcus pluvialis*	Lutein, neoxanthin, β-carotene	PLE	Ethanol	50/10.3	20	[46]
			SFE	Ethanol as modifier	50/31	20	[47]
			SFE	Ethanol as modifier	65/43.5	210	[48]
β-carotene	*Phormidium sp*	Lutein, violaxanthin, neoxanthin	PLE	Ethanol	150/10.3	20	[49]
	Synechocystis sp	Zeaxanthin, myxoxanthophyll, echinenone	PLE	Ethanol	100/10.3	20	[50]
	Chlorococcum littorale	Zeaxanthin, violaxanthin, neoxanthin, antheraxanthin, lutein	SFE	Ethanol as modifier (10%)	60/30	180	[51]
	Dunaliella salina		SFE		40–60/20		[52]
Canthaxanthin	*Chlorella vulgaris*	Astaxanthin	SFE	Sc-CO_2	40–55/10–35		[52]
Fucoxanthin	*Phaeodactylum tricornutum*	Zeaxanthin, β-carotin	PLE	Ethanol	100/10	20	[53]
		Diatoxanthin	PLE	Ethanol	50/10	20	[40]
			PLE	Ethanol	100/10.3	30	[54]
	Isochrysis galbana	Diadinoxanthin	PLE	Ethanol/Water	80/10	30	[13]
			GXL	45% Ethanol and 55% sc-CO_2	50/7	30	
			SFE	Sc-CO_2	50/30		
Fucoxanthin and zeaxanthin	*Himanthalia elongata*		PLE	Ethanol	100/0.3	20	[50]
Lutein	*Neochloris oleoabundans*	β-carotene, violaxanthin, zeaxanthin, cantaxanthin, astaxanthin derivates	PLE	Ethanol	112/10.3	20	[55]
Lutein	*Scenedesmus sp*	Astaxanthin, β-carotene, neoxanthin, violaxanthin, zeaxanthin	SFE	Sc-CO_2	60/30	60	[56]
		Astaxanthin, β-carotene, neoxanthin, zeaxanthin	SFE	Ethanol as modifier (10%)			
			SFE	Ethanol as modifier (30 mol %)	70/40	60	[57]
	Scenedesmus obliquus	β-carotene, violaxanthin, zeaxanthin	PLE	Water	50/10	45	[41]
		β-carotene, violaxanthin, neoxanthin, zeaxanthin	GXL	75% Ethanol and 25% sc-CO_2	50/7	150	
		β-carotene, violaxanthin, luteoxanthin	SFE	Sc-CO_2	50/36	120	
	Scenedesmus almeriensis	β-carotene	SFE	Sc-CO_2	60/40	300	[58]
	Chlorella vulgaris	Neoxanthin, violaxanthin, lutein, α-carotene, β-carotene	PLE	Water, ethanol, acetone	50–200/10.3	20	[25]
		β -carotene	SFE	Ethanol as modifier	40/40		[59]
				Ethanol as modifier (7.5%)	80/50	300	[60]

Table 4 (continued)

Main carotenoid	Microalgae	Other carotenoids extracted	Methods	Extraction solvents	T (°C)/P (MPa)	Extraction time (min)	References
Vaucheraxanthin	*Nannochloropsis sp.*	Violaxanthin/neoxanthin, astaxanthin, canthaxanthin, β-carotene lutein/zeaxanthin,	SFE	Ethanol as modifier (20%)	40/30	140	[61]
Zeaxanthin	*Chlorella ellipsoidea*	Violaxanthin, β-carotene	PLE	Ethanol	115.4/10.3	23.3	[62]
	Nannochloropsis oculata	Fucoxanthin, neoxanthin, lutein, β-cryptoxanthin, β-carotene, β-carotenin	SFE	Ethanol as modifier	50/10		[63]
			SFE	Ethanol as modifier (16.7%)	50/35		[64]

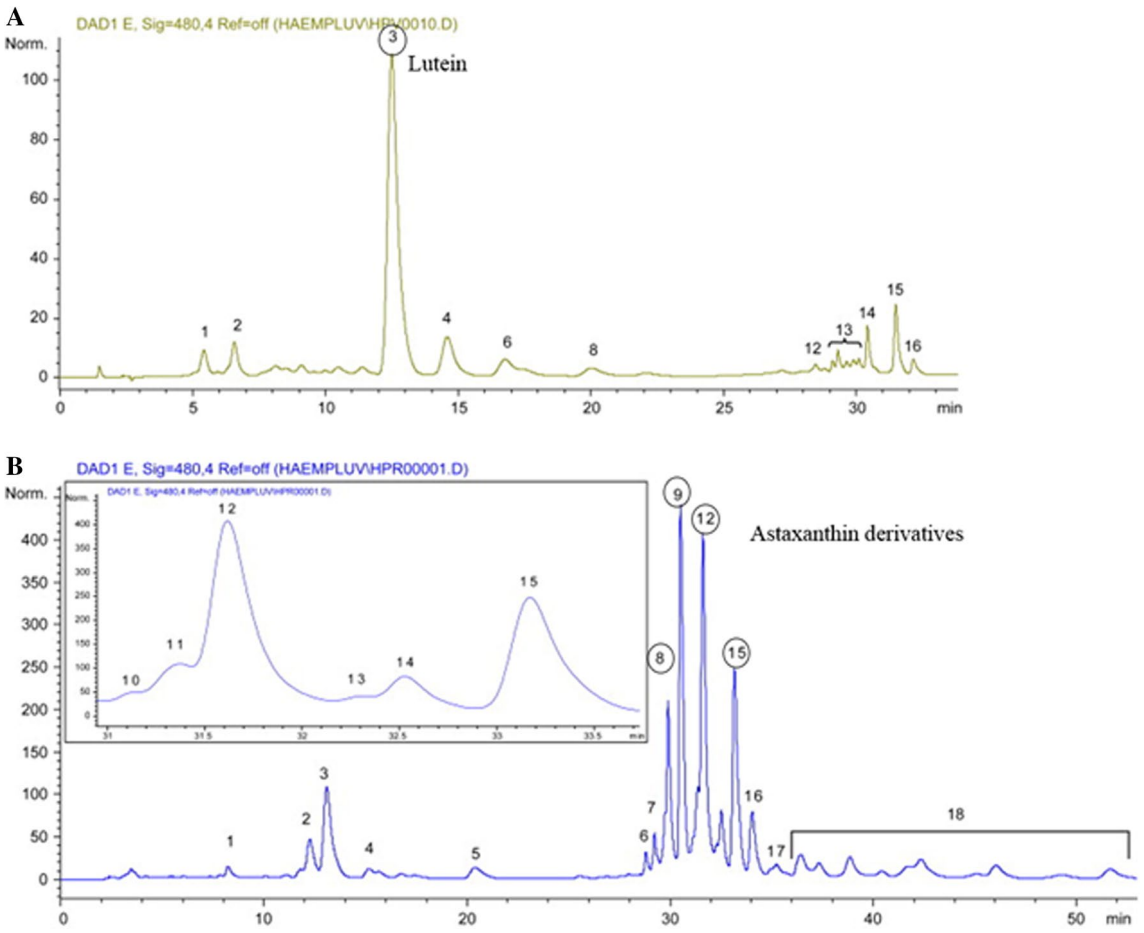

Fig. 2 HPLC chromatograms (480 nm) of an ethanol extract obtained at 100 °C of *Haematococcus pluvialis* green phase (**a**) and red phase (**b**) and identification of main pigments Modified from [46] with permission from Elsevier

anti-HIV, antiallergic, and anti-inflammatory activities, that have been described [67].

These hydrophilic compounds can be easily isolated or fractionated using conventional protocols by extraction with

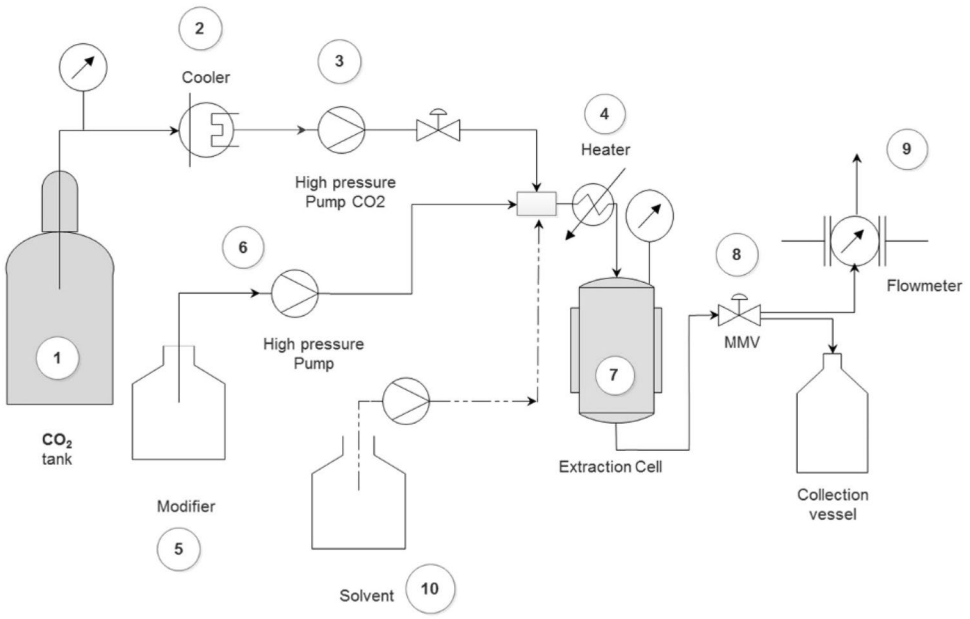

Fig. 3 Scheme of the supercritical fluid extractor capable to perform SFE, GXL and PLE sequential processes for the extraction of valuable components from *Scenedesmus obliquus*. Dashed lines indicate the flow path of the solvent in the PLE step. Reprinted from [41] with permission from Elsevier

solvents such as acetone, methanol or water [68]. In terms of green processes, there are a few researches in which a deep characterization of microalgal extracts is performed. For instance, Klejdus et al. combined a solid-phase extraction step and a supercritical fluid extraction procedure (SPE/SFE) to obtain mostly hydroxy benzaldehydes and benzoic acid derivatives from *Spongiochloris spongiosa* [69]. Using this combined method, and adding the defined extraction solvents sequentially, recoveries of ca. 94% of identified phenols, including p-hydroxybenzoic, protocatechuic, vanillic, syringic, caffeic and chlorogenic acids and 4-hydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde, among others, were reported. Another approach was also optimized for the extraction and determination of isoflavones from the same microalgal strain, using SFE in combination with ultrasounds. Interestingly, eight isoflavones were described for the first time in *Spongiochloris spongiosa* (mainly genistin), and also in *Scenedesmus* (mainly daidzein and ononin, in this case) [70]. Derived from their previous work, Klejdus et al. proposed the use of this combined SPE/SFE method to obtain rich-phenolic fractions from different algal species, so their experimental design could be used for the evaluation and screening of phenolic compounds in algal samples [71].

Another green method reported to obtain phenols from microalgae is SWE, which was used by Rodríguez-Meizoso et al. for *Haematococcus pluvialis* [28]. The effect of the extraction temperature was closely studied and the obtained results demonstrated that this parameter had a positive influence in the extraction yield (up to 30%) and antioxidant activity (TEAC value: 1.974 ± 0.053) up to extraction temperatures of 200 °C. This result is very interesting since higher temperatures are commonly associated with higher degradation of phenolic compounds; in these applications, authors showed that the highest content on phenolic compounds was reached using the highest tested temperature.

However, as phenolic compounds are not major compounds in microalgae, most of investigations are not actually focused on the specific identification of phenols. Instead, more often, total phenolic contents (TPC) are reported after the use of green extractions at different conditions. For instance, Gilbert-López et al. compared different PLE extracts from *Phaeodactylum tricornutum,* obtained under different conditions, in terms of TPC showing that ethanol at 50 °C was the optimal condition to reach the highest TPC value (ca. 40 mg gallic acid equivalents/g extract) [40]. In contrast, Cha et al. used pressurized liquid extraction to obtain relatively high TPC values from *Chlorella vulgaris* extracts using 90% ethanol at 160 °C (15 mg of GAE/g of dry extract) [72]. This fact clearly supports the idea to carrying out specific optimization protocols for each different microalgal species.

4 Conclusions and future perspectives

The main conclusion that can be pointed out from the information shown in this review is that the use of compressed fluid-based extraction techniques coupled with advanced analytical tools is widely extended for the extraction and characterization of bioactive compounds from microalgae. However, there is still room for new applications, as microalgal species remain largely underexplored and unknown, including the development of new processes able to improve

the yield and/or the selectivity of the extraction of bioactive compounds from these natural sources.

Although SFE using carbon dioxide will continue in the future, plenty of new solvents could be introduced for their use in PLE processes. Environmentally-green food-grade solvents such as ethyl lactate or D-limonene could displace to some extent the use of low polarity solvents. Moreover, ionic liquids, and more importantly, deep eutectic solvents (DES) will surely open new possibilities for the extraction of bioactive natural molecules.

Besides, the coupling of these extraction techniques under intensified or integrated processes will probably be extended in the future. Even more important will be the adoption of biorefinery approaches, so that, not only the target bioactive compounds will be obtained from the microalgae, but also a complete group of valorized fractions will be obtained thus reducing or eliminating wastes while maximizing efficiency [13].

Thus, a great amount of new research can be expected in this area in the coming years, as the search for new natural understudied sources for bioactive compounds will intensify.

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