

Chapter 2

Extraction and Characterization of Bioactive Compounds with Health Benefits from Marine Resources: Macro and Micro Algae, Cyanobacteria, and Invertebrates

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2.1 Bioactive Compounds from Marine Sources and Functional Foods

The occurrence and incidence of different diseases such as cancer, cardiovascular diseases, obesity, and diabetes may be related to the consumption of high caloric diets combined with sedentary lifestyles. The concept of functional foods first appeared in Japan where it was considered to be a tool to promote health and well-being. In 1992, the Japanese government established a policy of “Foods of Specific Health Uses” (FOSHU). This concept was further developed in Europe within the “Functional Food Science in Europe” (FUFOSE) project supported by the European Commission (EC) and co-ordinated by the International Life Sciences Institute (ILSI). Several interesting points were observed at the end of this project (Bellisle et al. 1998; Diplock et al. 1999), including a definition of a functional food as “a food which is demonstrated to positively affect one or more physiological functions, so that it is able to increase the well-being and/or to reduce the risk to suffer a disease” (Diplock et al. 1999). This definition implies that a functional food must maintain the shape of the food (thereby excluding pills and capsules) and that the functional food must impart a physiological effect following consumption that is above and beyond any observed nutritional effects.

One of the ways most often employed by food manufacturers in the production of novel functional foods is the addition of one or more interesting bioactive

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compounds to a traditional food. The added bioactive compounds are usually referred to as functional ingredients and they are responsible for the functional bioactivities that the new product might present. By using this strategy, several functional foods have already been developed and commercialized. For instance, products claiming antihypertensive activity, hypo-cholesterolemic effect, antioxidant properties, probiotic or prebiotic effects, or regulatory effects on the appetite, among others, are available on the market.

The marine environment, which contains a vast array of organisms with unique biological properties, is one of the most underutilized biological resources. To date, algae and microalgae are referenced in the literature as sources of bioactive compounds for use as functional food ingredients (Plaza et al. 2009; Plaza et al. 2008). The huge diversity in terms of the number of different species of macro- and microalgae that exist (as discussed in Chapter 1), coupled with the hostile environments in which these organisms live, make macro- and microalgae key targets for bioactive compound screening projects.

Algae comprise a complex and heterogeneous group of organisms characterized by their photosynthetic nature and their simple reproductive structures. According to their size, algae can be roughly divided into unicellular organisms, known as *microalgae* and multicellular organisms referred to as *macroalgae*. Algae frequently live in extreme environments of light, salinity, and temperature. In order to adapt to these extreme conditions, most algae produce a high variety of secondary metabolites that often have potent biological activities. Most algae are relatively easy to cultivate or produce at industrial scale. Thus, the production of algal-derived biologically active compounds may be tuned by the selection of appropriate cultivation conditions, making these algae true natural bioreactors. Bioactive compounds have also been isolated previously from other marine organisms including crustaceans, fish, and their by-products and this is reviewed in later chapters of this book. In some of these matrices, interesting functional compounds were isolated previously (Kadam and Prabhasankar 2010; Kim and Wijesekara 2010).

It is important to consider how functional ingredients are obtained from new matrices, such as micro- and macroalgae. In this regard, there is a need to combine appropriate, selective, cost-effective, and environmentally friendly extraction procedures with the legal requirements regarding the use of food-grade solvents and processes. Traditional extraction techniques such as soxhlet, solid–liquid extraction (SLE), or liquid–liquid extraction (LLE) are characterized by using high volumes of solvents and long extraction times. These techniques often produce low extraction yields of bioactives and present low selectivity. Furthermore, traditional extraction techniques are usually not automated procedures and their reproducibility can therefore be compromised. As described later in this chapter, the use of new state-of-the-art extraction techniques, such as supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), accelerated solvent extraction (ASE®), pressurized hot water extraction (PHWE), ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE) techniques, among others, may provide an effective alternative to the problems encountered with the use of traditional extraction procedures.

2.2 Screening of Bioactives from Marine Sources: An Integrated Approach

Considering the great biodiversity of marine species, the use of appropriate methodologies that can rapidly screen different marine sources for bioactive compounds is of great interest. To design this screening methodology, different parameters have to be considered. These parameters include the possible nature of the sought-after bioactive compounds (in terms of solubility, heat resistance, or molecular weight) and the bioactivity that is sought. Figure 2.1 proposes a screening methodology for the extraction and identification of bioactive compounds from marine sources. Initially, a suitable extraction technique should be selected. This selection must be carried out in accordance with the predicted nature of the expected/target bioactive compound(s). However, several extraction techniques could also be used to fully characterize the potential of the different natural sources, introducing different extraction selectivity.

The use of environmentally clean advanced extraction techniques allows for the attainment of the target compound(s) of interest with more efficient extraction procedures, while, at the same time, minimizing the use of organic toxic solvents. Depending on the extraction techniques selected, diverse extraction parameters should be tested in order to study the influence of solvents, temperatures, pressures, and other important parameters that might have a significant influence on the outcome of the extraction process employed. The different extracts, obtained using diverse conditions, must then be tested for biological bioactivities by performing the appropriate functional activity assay(s). The main aim of this step is to confirm that the obtained extracts from step one possess the sought-after bioactivity.

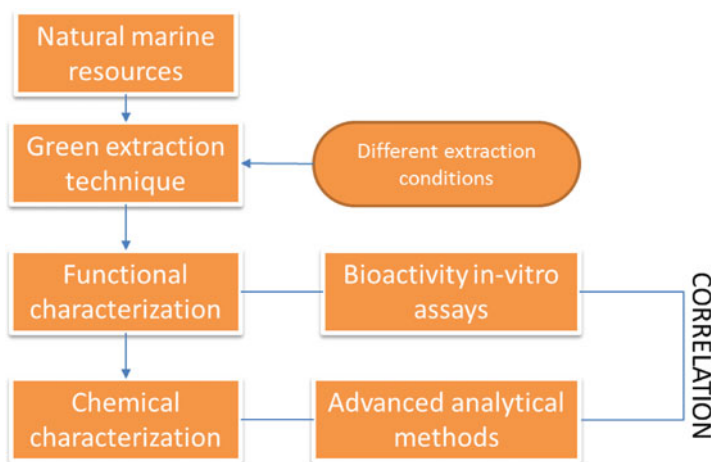


Fig. 2.1 Basic scheme showing the proposed workflow for the screening of bioactive compounds from marine sources

Functional characterization should be assessed through the application of fast *in vitro* assays directed to the confirmation of the sought biological properties, for instance, antioxidant capacity assays, antimicrobial activity assays, or antihypertensive activity assays.

Once the target biological activities have been confirmed, the next step involves chemical characterization of the bioactive components present in the initial extract, which may often be referred to as lead functional components (LFCs). Again, the analytical technique employed at this stage of the characterization process will depend on the nature of the initial extract or LFC in terms of its solubility, stability at different pH conditions, and heat stability, as well as the nature of the suspected bioactive compounds. In general, advanced analytical techniques are employed, even coupled, in order to maximize the identification potential. The final aim of this stage of the characterization process is the correlation between the chemical composition of the LFC and the bioactivities observed. Ideally, it will be possible to identify which compounds are responsible for the observed bioactivities. It is often necessary to return to the initial extraction method employed and to perform a fine-tuning of this extraction procedure in order to enrich the initial extract(s) with the target compounds, which has demonstrated biological activities and has a defined chemical structure. Further use of the information gathered using this integrated approach can be directed towards the design of upscale procedures for this extraction and characterization process and this is described later in this chapter (Sect. 2.4).

In the following section, a brief description of the main potential functional food ingredients that may be isolated from different marine sources is presented.

2.2.1 Antioxidants

The antioxidant capacity of compounds has been related to the prevention of several diseases including cancer, coronary heart diseases, inflammatory disorders, neurological degeneration, and aging (Wollgast and Anklam 2000; Madhavi et al. 1996). Polyphenolic compounds are among the interesting antioxidant compounds isolated previously from marine resources, including micro- and macroalgae. At least 8,000 different bioactive compounds are considered to be polyphenols (Bravo 1998). In general, phenolic compounds are divided into ten types, based on their structure. These ten groups are: simple phenols, phenolic acids, hydroxycinnamic acids, coumarins, naphthoquinones, xanthenes, stilbenes, anthraquinones, flavonoids, and lignins. Among them, flavonoids are the group known to have the greatest number of different structures, and at least 5,000 flavonoids have been characterized and referenced in the literature to date (Wollgast and Anklam 2000).

To date, most polyphenols isolated from marine sources and referenced in the literature are of macro- and microalgal origin. These phenolic compounds range from phenolic acids and other polyphenolic compounds with relatively simple chemical structures, to the more complex structures of phlorotannins, typically isolated from brown algae belonging to the Phaeophyceae class, and which consist of

polymeric structures made up of units of phloroglucinol (1,3,5-trihydroxybenzene). It is understood that the intensity of the antioxidant activity of these complex polyphenols is related to the degree of polymerization of the polyphenol. In general, lower degrees of polymerization result in greater antioxidant activities. Nevertheless, the main activity related to phenolic compounds is antioxidant activity (Li et al. 2009). In addition to their strong antioxidant activities, phlorotannins are known to possess other activities (Wijesekara et al. 2010) including antibacterial (Nagayama et al. 2002), chemopreventive (Kang et al. 2003), UV- protective (Artan et al. 2008), and antiproliferative effects (Kong et al. 2009). They are also known to act as detoxifying agents against heavy metals (Eide et al. 1980), and have myriad other bioactivities that could potentially be exploited for use in functional foods (Lee et al. 2009; Jung et al. 2006; Yoon et al. 2009). For example, the total antioxidant capacity of a *Polysiphoma urceolata* red algal extract is directly related to the total phenolic content (Duan et al. 2006). However, as occurs with other chemical constituents, the composition of phenolic compounds both qualitatively and quantitatively might vary depending on the species as well as on the environmental conditions and location of the seaweed.

In addition to polyphenolic compounds, other interesting antioxidants, such as carotenoids, can be found in marine resources. Carotenoids are a family of natural pigments that are widely distributed in nature. There are more than 600 known carotenoid structures described in the literature, and these natural pigments are often responsible for the color of the different natural matrices. The basic carotenoid structure is formed by eight isoprenoid units that constitute the symmetrical skeleton of the compound, along with a long chain with conjugated double bonds. This characteristic arrangement is responsible for the antioxidant activities of carotenoids. Carotenoids are synthesized by plants, algae, fungi, and other microorganisms but are not made by animals and need to be ingested in the diet if required. Carotenoids possess other bioactivities and are thought to be active agents for the prevention of cancer (De Spirt et al. 2010; Silberstein and Parsons 2010), cardiovascular diseases (Riccioni et al. 2008), and macular degeneration (Snodderly 1995).

Certain algal species in addition to crustaceans are known reservoirs for carotenoids. For instance, the microalga *Dunaliella salina* is able to accumulate large amounts of β -carotene when cultivated under certain conditions (Zhu and Jiang 2008). Other carotenoids are more typical from these organisms such as fucoxanthin or astaxanthin. Fucoxanthin is the main pigment found in brown algae. It has been observed that this oxygenated carotenoid is a very effective inhibitor of cellular growth and promotes apoptosis in human cancer cell lines (Kotate-Nara et al. 2001; Hosokawa et al. 1999). Moreover, this pigment possesses anti-inflammatory (Shiratori et al. 2005), antidiabetic (Maeda et al. 2007), and antioxidant activities (Sachindra et al. 2007). Astaxanthin has been found in different marine organisms but *Haematococcus pluvialis* is the major producer, being able to selectively accumulate this carotenoid in quantities that amount to approximately 5% of its dry weight (Yuan and Chen 2000). As a result of its color, this carotenoid is usually employed as an additive in aquaculture feed for salmon, trout, and shrimp. As occurs

with other carotenoids, the antioxidant activity associated with astaxanthin also influences other bioactivities and astaxanthin is thought to be active in the prevention of several diseases associated with oxidation (Higuera-Ciapara et al. 2006).

2.2.2 Lipids

Marine sources are widely regarded as possessing interesting lipid compositions, which make them attractive as a source for lipid extraction. The main polar lipids found in these substrates include monogalactosyl diacylglycerols (MGDG), digalactosyl diacylglycerols (DGDG), and phosphatidylglycerols (PG). These polar lipids possess several functional activities, but are mainly referenced in the literature for their anti-inflammatory activities (Bruno et al. 2005; Larsen et al. 2003). The lipid fraction consists primarily of the polyunsaturated fatty acids (PUFAs) that are well documented as essential for good human health. For instance, it has been widely studied how long chain ω -3 fatty acids, such as eicosapentaenoic (EPA, C_{20:5}) or docosahexaenoic acids (DHA, C_{22:6}) are useful in the prevention of cardiovascular diseases (Calzolari et al. 2009; Schuchardt et al. 2010; Zuliani et al. 2009). These fatty acids can be presented bound to polar lipids or even as triacylglycerols.

The composition and extraction of PUFAs from algae (Plaza et al. 2009), fish (Sahena et al. 2009), fish by-products (Wu and Bechtel 2008), and other marine sources (Juárez et al. 2010) have been studied previously. However, PUFAs are prone to lipid oxidation and this represents one drawback for their inclusion in functional foods. For this reason, PUFAs are often used in combination with other antioxidants in order to enhance their preservation.

Other attractive lipid bioactive compounds from marine sources include the group known as sterols. The composition of sterols isolated from macro- and microalgae (Cardozo et al. 2007) and other marine invertebrates (Kanazawa 2001) has been extensively studied. Sterols and some of their derivatives were found previously to play an important role in lowering LDL cholesterol levels in vivo (Francavilla et al. 2010). Other bioactivities are associated with sterols, include anti-inflammatory and antiaterogenic activity (Francavilla et al. 2010). In addition, phytosterols (C₂₈ and C₂₉ sterols) are important precursors of compounds including vitamins. For example, ergosterol is a precursor of vitamin D₂ and cortisone.

2.2.3 Carbohydrates

Macroalgae are regarded as a rich source of sulfated polysaccharides and the particular type of polysaccharide is different depending on the taxonomic group. Different carbohydrates including agar, carrageenan, or alginates are extracted from macroalgae and these carbohydrates are used widely in the food and pharmaceutical industries as functional ingredients such as stabilizers. For example, the alga

Chondrus crispus is traditionally employed for the extraction of carrageenan (also known as Irish moss), a highly sulfated polysaccharide. However, macroalgal polysaccharides also have potential for use as prebiotics as they are not digested in the human gut and, in addition, can be considered as a rich source of dietetic fiber. Among their associated bioactive properties, immunomodulating, anticancer, anti-inflammatory, antiviral, or antioxidant activities have been pointed out (Li et al. 2008). Other minor sulfated polysaccharides, typically from particular species, are also known, such as porphyrans produced by *Porphyra*. In general, these polysaccharides can significantly vary their composition, and therefore their related properties. For instance, the bioactivity might differ depending on the degree of sulfation, molecular weight, type of sugar found predominantly, and/or glycosidic branching (Qi et al. 2005).

Another carbohydrate-type product, abundant in different marine sources, is chitin. This compound is a polymer composed of B(1 → 4)-linked N-acetylglucosamine units. Chitin is one of the most extensive biopolymers in nature, and is found in different marine sources such as crustaceans where it is part of their exoskeleton. Chitosan is produced by the alkaline deacetylation of chitin, and is employed in a wide range of applications. Some interesting effects have also been associated with this polymer, including dietary fiber, lipid absorption reduction, and hypocholesterolemic or antidiabetic effects, among others (Ngo et al. 2011; Liu et al. 2007; Liao et al. 2007; Muzzarelli et al. 2007).

2.2.4 Peptides and Proteins

As discussed in the next chapter, proteins of marine origin have received attention recently due to their potential bioactive and functional properties. Phycobiliproteins are one of the most important groups of proteins from seaweed. These water-soluble proteins, mainly found in some blue-green and red algae, are characterized by possessing a tetrapyrrolic ring covalently attached to their structure. This pigment can be either phycocyanobilin (blue-green algae) or phycoerythrobilin (red algae) and it is partially responsible for the functional properties associated with these proteins, mainly hepatoprotective, anti-inflammatory, and antioxidant activities (Bhat et al. 1998; Romay et al. 2003; Bhat and Madyastha 2000). In fact, the extraction of these proteins from different algae has been extensively studied, considering their economic importance. Usually, the extraction method is the key factor for enhanced recovery of phycobiliproteins. The extraction protocols usually involve the selection of a suitable source followed by the disruption of the algae cells and release of proteins (Moraes et al. 2010; Patil et al. 2008).

However, interest in marine proteins might not be only directly correlated to the intact protein, but also to the possibility of generating bioactive peptides. In this sense, different peptides derived from marine proteins have been identified as having antioxidant activity (Byun et al. 2009) as well as antihypertensive, anticoagulant, or antimicrobial activities (Ngo et al. 2011). These peptides have been isolated from

diverse marine sources such as algae, crustaceans, and also different fish species. In general, bioactive peptides comprise relatively short chains of amino acids (from 3 to 20), which do not present any bioactivity prior to release from the intact parent protein. Nevertheless, upon digestion or hydrolysis *in vivo* or due to technological processes such as high pressure processing, these peptides are released demonstrating their bioactivities.

As shown in this section, marine-related products can be a good source of potential functional compounds that may be used as ingredients in the food industry. In the following sections, the application of different, environmentally clean extraction techniques to obtain these kinds of bioactive compounds from different marine sources are described.

2.3 Green Extraction Techniques for Bioactive Compound Isolation from Marine Resources

Since the early 1990s, the Green Chemistry movement has been exploring ways to reduce the risk of chemical exposure to humans and the environment. Simply stated, Green Chemistry reduces or eliminates the use or generation of hazardous substances from chemical products and processes and improves all types of chemical products and processes by reducing the impacts on human health and the environment (Fig. 2.2a). Green Chemistry technologies encompass all types of chemical processes, including synthesis, catalysis, reaction conditions, separations, analysis and monitoring. A Green Chemistry technology can involve implementing incremental improvements at any stage (Majors and Raynie 2011). In the 12 principles that rule Green Chemistry (Table 2.1) there are three main aspects that dominate: waste, hazard (health, environmental, and safety), and energy (Anastas and Warner 1998).

Green Engineering is the development and commercialization of industrial processes that are economically feasible and which reduce risk to human health and the environment. Both of these “Green concepts” are intimately related to sustainability (Fig. 2.2b), which means using methods, systems, and materials that will not deplete natural resources or harm natural cycles. The principles of Green Chemistry and Green Engineering (Table 2.1) provide a framework for scientists and engineers to use when designing new materials, products, processes, and systems (Anastas and Zimmerman 2003).

The discovery and development of marine bioactive compounds is a relatively new area when compared to the discovery of bioactive compounds from terrestrial sources. Therefore, in the development of this area, new, environmentally friendly, and sustainable trends should, if possible, be followed. All of the above-mentioned rules enable scientists and engineers to develop a compendium of novel protocols for the discovery of bioactive compounds for use in functional foods and pharmaceuticals. These new protocols should comply, as much as possible, with the principles of Green Chemistry and Green Engineering. Among the different advanced

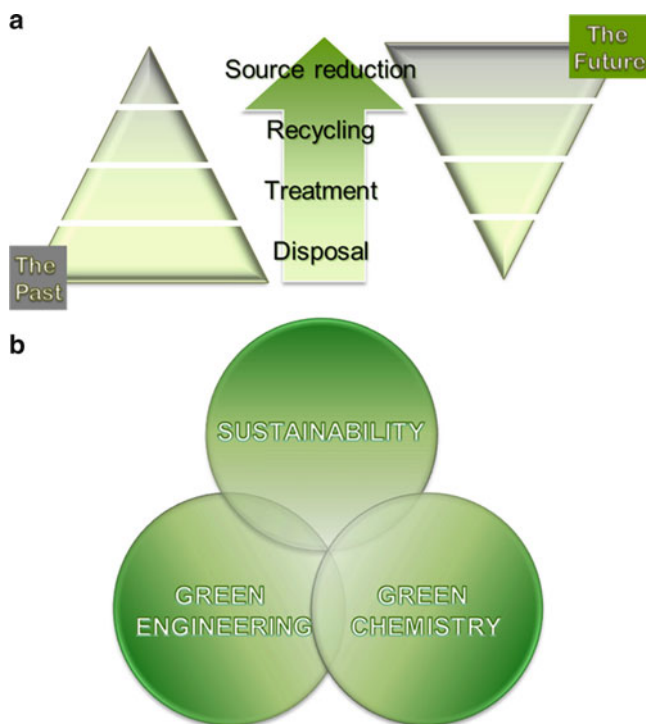


Fig. 2.2 Changes in waste prevention hierarchy (a) and environmental stewardship perspectives: green chemistry, green engineering and sustainability (b) (Adapted from “Pollution Prevention: The Basics” http://www.epa.state.oh.us/portals/41/P2basics_web.pdf (last accessed April 2011))

Table 2.1 Principles of green chemistry Anastas and Warner 1998 and green engineering Anastas and Zimmerman 2003

#	Green chemistry	Green engineering
1	Prevention It is better to prevent waste than to treat or clean up waste after it has been created	Inherent rather than circumstantial Designers need to strive to ensure that all materials and energy inputs and outputs are as inherently nonhazardous as possible
2	Atom economy Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product	Prevention instead of treatment It is better to prevent waste than to treat or clean up waste after it is formed
3	Less hazardous chemical syntheses Wherever practicable, synthetic methods should be designed to use and generate substances that possess little or no toxicity to human health and the environment	Design for separation Separation and purification operations should be designed to minimize energy consumption and materials use

(continued)

Table 2.1 (continued)

#	Green chemistry	Green engineering
4	Designing safer chemicals Chemical products should be designed to effect their desired function while minimizing their toxicity	Maximize efficiency Products, processes, and systems should be designed to maximize mass, energy, space, and time efficiency
5	Safer solvents and auxiliaries The use of auxiliary substances (e.g., solvents, separation agents, etc.) should be made unnecessary wherever possible and innocuous when used	Output-pulled versus input-pushed Products, processes, and systems should be “output pulled” rather than “input pushed” through the use of energy and materials
6	Design for Energy efficiency Energy requirements of chemical processes should be recognized for their environmental and economic impacts and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure	Conserve complexity Embedded entropy and complexity must be viewed as an investment when making design choices on recycle, reuse, or beneficial disposition
7	Use of renewable feedstocks A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable	Durability rather than immortality Targeted durability, not immortality, should be a design goal
8	Reduce derivatives Unnecessary derivatization (use of blocking groups, protection/ deprotection, temporary modification of physical/ chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste	Meet need, minimize excess Design for unnecessary capacity or capability (e.g., “one size fits all”) solutions should be considered a design flaw
9	Catalysis Catalytic reagents (as selective as possible) are superior to stoichiometric reagents	Minimize material diversity Material diversity in multicomponent products should be minimized to promote disassembly and value retention
10	Design for degradation Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment	Integrate material and energy flows Design of products, processes, and systems must include integration and interconnectivity with available energy and materials flows
11	Real-time analysis for pollution prevention Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances	Design for commercial “Afterlife” Products, processes, and systems should be designed for performance in a commercial “afterlife”
12	Inherently safer chemistry for accident prevention Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires	Renewable rather than depleting Material and energy inputs should be renewable rather than depleting

extraction techniques that fit within the remit of the fore-mentioned rules, supercritical fluid extraction, pressurized liquid extraction, ultrasound-assisted extraction and microwave-assisted extraction are the most promising for the isolation of marine-sourced bioactive compounds. In the following sections, detailed information about these techniques together with the necessary instrumentation and their application to different marine sources for the isolation of bioactive compounds is provided.

2.3.1 Supercritical Fluid Extraction (SFE)

Hannay and Hogarth first introduced supercritical fluid extraction as an alternative extraction method in 1879. However, it was not until circa 1960 that this extraction method started to be thoroughly investigated (Hosikian et al. 2010) as an alternative to conventional extraction methods, such as SLE and LLE, which utilize large amounts of hazardous chemicals such as chlorinated solvents. Supercritical fluid extraction is based on the use of solvents at temperatures and pressures above their critical points. This technique has been employed previously to extract a wide variety of interesting compounds from different food-related materials (Mendiola et al. 2007a), and algae are no exception (Herrero et al. 2006a).

One of the most valuable characteristics of SFE is the highly reduced (often to zero) employment of toxic organic solvents. Carbon dioxide (CO_2) is the solvent most commonly used to extract bioactive compounds from natural sources using SFE. In fact, CO_2 has a series of interesting properties for bioactive extraction: it is cost efficient, its critical conditions are easily attainable (30.9°C and 73.8 bars), and it is an environmentally friendly solvent that is Generally Recognized As Safe (GRAS) for use in the food industry. The main drawback of supercritical CO_2 is its low polarity, a problem that can be overcome by employing polar modifiers or cosolvents to change the polarity of the supercritical fluid and to increase its solvating power towards the analyte of interest. For example, the addition of relatively small percentages (1–10%) of methanol to carbon dioxide expands its extraction range to include more polar analytes. The modifiers can also reduce the analyte–matrix interactions, improving their quantitative extraction (Björklund et al. 2005). Other solvents have been proposed for SFE and these include propane, butane, and dimethyl ether but none of these fulfill all the principles of Green Chemistry and Green Engineering as well as CO_2 . Advantages of using CO_2 under supercritical conditions for the extraction of marine bioactive compounds include its high diffusivity and the relative ease in tuning the temperature and pressures applied, so that solvent strength and density can be modified easily. Another advantage of SFE when using CO_2 is the possibility of attaining solvent-free extracts. Once the extraction procedure is finished, the depressurization of the system converts CO_2 from a liquid to a gas making it easier to recover the extract. These properties are responsible for the extended use of supercritical CO_2 for extraction of bioactive compounds.

2.3.1.1 Instrumentation

Several parameters are involved in the extraction of bioactive compounds from marine resources by SFE. It is necessary to precisely control the effect of the extraction temperature, pressure, percentage, and type of modifier addition, amount of sample to be extracted, as well as its particle size and the use of dispersing agents. The first parameters are related to the solubility of the target compounds in the supercritical fluid, inasmuch as changes to the extraction temperature and pressure will have a strong influence on solvent properties, such as density. Although supercritical solvents have diffusivity in the matrix beyond those of liquids, a decrease in the sample particle size generally produces an increase in the extraction yield obtained. This is due mainly to the increase that results in the surface contact between sample and solvent, which enables an increase in mass transfer. In some applications, the use of dispersing agents such as sand, glass beads, and diatomaceous earth or a hydromatrix to absorb liquid from the sample can be useful.

A basic supercritical-fluid extractor (Fig. 2.3a) consists of a tank of the mobile phase, usually CO₂, a pump to pressurize the gas, an oven containing the extraction vessel, a restrictor to maintain the high pressure inside the system, and a trapping vessel. Extracts are trapped when the solute-containing supercritical fluid decompresses into an empty vial, through a solvent, or onto a solid or liquid material.

Extractions are done in dynamic mode, static mode, or a combination of both. In a dynamic extraction, the supercritical fluid continuously flows through the sample in the extraction vessel and out of the restrictor to the trapping vessel. In static mode, the supercritical fluid circulates in a loop contained in the extraction vessel for some period of time before being released through the restrictor to the trapping vessel. In combination mode, a static extraction is performed for some period of time, followed by a dynamic extraction.

Use of supercritical CO₂ also results in a “clean” extract when compared to other conventional extraction techniques. Indeed, the selectivity obtained through the use of supercritical CO₂ often results in the attainment of more purified extracts, reducing to a great extent the amount of interfering compounds extracted from the matrix. However, if the extraction of more polar compounds is targeted, other strategies have to be conceived. As mentioned, the main alternative in this case is the use of a given percentage of a modifier together with the supercritical fluid, which is normally CO₂ (Fig. 2.3b). When the modifier, usually a polar solvent, is added to the supercritical fluid, a change in the properties of the extracting mixture is produced, allowing the collection of more polar compounds, increasing the polarity of the solvent used for the extraction and also the range of applications of the SFE method.

With some simple engineering modifications, such as multiple extraction vessels connected in series that can be taken offline at any time, greater processing and economic efficiencies are produced (Fig. 2.3b). For example, at the end of the extraction period (for one of the vessels), carbon dioxide flow can be routed to another vessel (that has previously been filled with material to be extracted), and while the extraction process is continuing in the second vessel, the first vessel (containing the spent matrix) can be unloaded and loaded again with fresh sample.

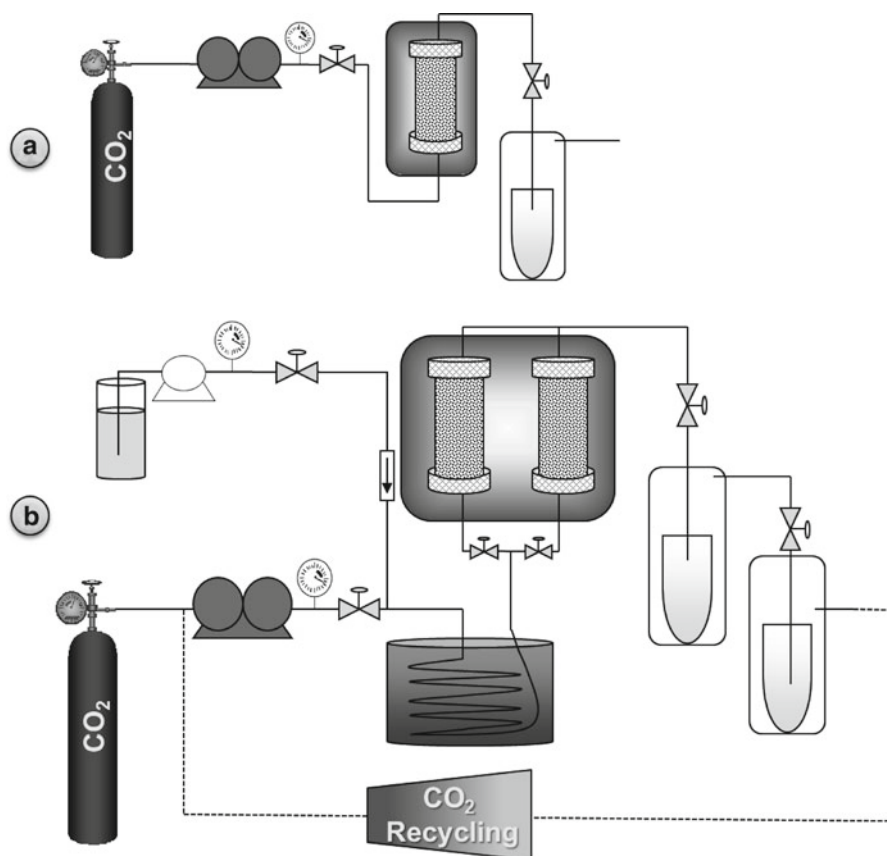


Fig. 2.3 Basic Scheme of supercritical fluid extractor (a) and typical scheme of industrial-scale supercritical fluid extractor including modifier circuit, CO_2 recycling circuit, extract fractionation, and dual extraction vessel and dual extraction vessel (b)

Instead of being vented to the atmosphere, the carbon dioxide present in the offline extractor at the end of the extraction period can be sent to the supply vessel and subsequently reused. The use of cascade depressurization allows fractionation of the extracts in different collectors depending on the solubility of the compounds at the selected depressurization conditions (Fig. 2.3b).

2.3.1.2 Application of SFE to Macroalgae, Microalgae, and Cyanobacteria

In spite of the potential of this technique, its usefulness will be related to the type of compounds to be extracted from the algae. Considering the low polarity of supercritical CO_2 , SFE is more suitable for the extraction of compounds with low polarity.

However, it is also notable that CO₂, when used at mild pressure and temperature conditions, allows obtaining volatile compounds without affecting its properties. In general, naturally produced volatile and semivolatile compounds play an essential role in the survival of organisms for chemical defense mechanisms and food gathering. When attacked by herbivores, land and marine plants can produce a variety of volatile compounds that attract carnivorous mutualists. One example of the extraction of volatile metabolites from marine sources includes the isolation of terpenoids and sulfur compounds from the brown alga *Dictyopteris membranacea* (El Hattab et al. 2007).

Microalgae also produce defensive volatile compounds. It is common in many microalgae to share their ecological niche with bacteria and other micro-organisms. Therefore, the defensive compounds secreted by microalgae possess antibacterial, antifungal, and often antiprotozoal activities. Our findings with the green microalga *Dunaliella salina* (Mendiola et al. 2008a) showed that an extract of this microalga isolated using SFE with CO₂ at 314 bar and 9.8°C displayed potent antimicrobial activity against the pathogens *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, and *Aspergillus niger*. This was probably due to the presence of indolic compounds, PUFAs, and compounds related to carotene metabolism, such as β -ionone and neophytadiene in the microalga extract.

In addition to volatile compounds, bioactive lipids, both saponifiable and unsaponifiable, are also preferentially extracted using SFE. Among saponifiable compounds, essential fatty acids can be easily extracted using supercritical CO₂. The effect of the extraction conditions in obtaining fatty acids from *Hypnea charoides* algae, using supercritical CO₂, was studied by Cheung in 1999 (Cheung 1999), who suggested the usefulness of this extraction technique for converting this alga species to a new source of omega-3 (ω -3) fatty acids. Temperatures from 40°C to 50°C and pressures from 241 to 379 bar were studied. In general, lipid recovery, as well as the ratio of unsaturated fatty acids, increased with extraction pressure and temperature. Extraction of ω -3 fatty acids was shown to depend on their chain length. The cyanobacterium *Spirulina platensis* was studied to determine the amount of lipids and GLA present in the microalgae (Qiuhui 1999). The maximum extraction yield was obtained at 350 bar, 40°C and a flow rate of 24 kg CO₂/h. Bioactive compounds from algal matter have been studied previously (vitamin E and carotenoids). For example, Mendiola et al. (2008b) optimized, by means of a central composite circumscribed design (CCCD), a process based on SFE at pilot scale, to obtain fractions highly enriched in vitamin E from *Spirulina platensis* (Mendiola et al. 2008c). The authors achieved a tocopherol enrichment of more than 12 times the initial concentration of tocopherol in the raw material by extracting with neat CO₂ at 361 bar and 83.3°C. In addition, SFE has previously been used to extract carotenoids from *Chlorella vulgaris* (Mendes et al. 1995), and *Spirulina plantesis* (Mendiola et al. 2008). In general, high pressures allowed high extraction yields and carotenoid extraction improved slightly by crushing the raw material. The addition of polar modifiers such as ethanol in the supercritical CO₂ allowed the extraction of more polar carotenoids, but also chlorophylls, thus decreasing the selectivity of the extraction process.

Other target bioactive compounds, such as diolefines, have been extracted from *Botryococcus braunii* cells by SFE (Mendes et al. 2003). This organism can store high amounts of long-chain hydrocarbons (i.e., 25–31 carbon atoms) that can be used as substitutes of paraffinic and natural waxes. Authors proved that the solubility of these types of compounds in CO₂ increased with pressure and found that 300 bar provided the optimum in terms of yield and extraction speed (Mendes et al. 2003).

One interesting area of research, which, is less studied, is the supercritical fluid extraction of phenolic compounds (simple phenols, flavonoids) from marine sources. Klejdus et al. (2010) developed a new hyphenated technique for the extraction and determination of isoflavones from sea macroalgae (*Sargassum muticum*, *Sargassum vulgare*, *Hypnea spinella*, *Porphyra* sp., *Undaria pinnatifida*, *Chondrus crispus*, and *Halopytis incurvus*), freshwater algae (*Spongiochloris spongiosa*), and cyanobacteria (*Scenedesmus* and *Nostoc* 17; Klejdus et al. 2010). The method involved sample pretreatment using sonication, followed by extraction using supercritical CO₂ modified with 3% (v/v) of MeOH/H₂O mixture (9:1, v/v) at 350 bar and 40°C for 60 min. This was followed by fast chromatography analysis and MS/MS determination. By using this approach, eight isoflavones were reported for the first time in different algae and cyanobacteria. Furthermore, Wang et al. (2010) reported the use of SC-CO₂ extraction to obtain antioxidants (flavonoids) from a novel microalga, *Chlorella vulgaris* C-C (Wang et al. 2010). Authors compared SC-CO₂ extraction at 310 bar, 50°C, using 50% aqueous ethanol as a modifier, to ultrasonic extraction with 50% aqueous ethanol and reported that flavonoid content obtained under SFE conditions was significantly higher than those obtained using UAE. This resulted in a higher antioxidant activity but also in better inhibition of lung cancer metastasis (Wang et al. 2010).

2.3.1.3 Applications to Invertebrates

Supercritical CO₂ extraction has not only been employed to extract bioactive compounds from macroalgae, microalgae, and cyanobacteria but also from marine invertebrates, such as crustacean (krill, crawfish, crab, or shrimp), squid, urchin, or starfish.

Crustacean shell wastes are a rich source of astaxanthin, which is the pigment responsible for their orange-pink coloration. Crustaceans are able to modify some carotenoids such as β -carotene and transform them into astaxanthin (Félix-Valenzuela et al. 2001). Yamaguchi et al. (1986) reported for the first time the application of SFE to crustacean waste. These authors extracted nonpolar lipids, mainly triglycerides, and astaxanthin (which is unstable at high pressures and temperatures around 80°C) from krill samples using a one-step extraction utilizing SC-CO₂ at 60°C and 245 bar (Yamaguchi et al. 1986). More recent work was focused on the extraction of astaxanthin from different crustacean shells using SC-CO₂ with 10% (w/w) ethanol as a cosolvent. After studying the experimental extraction conditions (in terms of

temperature, pressure, and sample load), extraction efficiency of astaxanthin was 57% from blue crab shells and was achieved using 45°C, 340 bar, and 17.5 g of sample load (Félix-Valenzuela et al. 2001). Charest et al. (2001) employed temperatures ranging from 50°C to 70°C and pressures from 138 to 310 bar to extract astaxanthin from crawfish shells (Charest et al. 2001). Results showed that 157.1 mg/kg of astaxanthin could be extracted at 60°C and 224 bar. Another interesting study compared the extraction efficiencies of *cis*- and *trans*-astaxanthin by supercritical CO₂ and solvent extraction from spear shrimp shells. The formation of *cis*- isomers or its derivatives may possess different biological activities to *trans*-astaxanthin (Lin et al. 2005). A SC–CO₂ extraction was carried out with pressures ranging from 250 to 400 bars, temperatures between 45°C and 75°C and with or without 10% ethanol as a modifier. Higher contents (µg/g) of *cis*- and *trans*-astaxanthin, as well as their esters, were achieved with ethanol, low temperature (45°C), and high pressure (400 bar). Compared to solvent extraction, which generates a greater content of *trans*-astaxanthin and astaxanthin ester, SC–CO₂ extraction could be used to produce slightly higher levels of 9-*cis*- and 13-*cis* astaxanthin.

Squid viscera, which is normally discarded during processing, and sea urchin (*Strongylocentrotus nudus*) gonads are rich in polyunsaturated fatty acids, which are essential nutrients for humans. Therefore, marine invertebrates can also be used as a potential source of high quality lipids. For instance, palmitic, oleic, eicosapentaenoic acid, and docosahexaenoic acid were the major fatty acids found in the oil extracted from waste squid viscera using SC–CO₂ with 1.5% (w/w) ethanol at temperatures between 25°C and 50°C and pressures ranging from 80 to 170 bar. Cholesterol (less than 54%) was also coextracted with the lipids (Kang et al. 2005a). From sea urchin gonad, lipids were extracted and compared to an enzyme-assisted aqueous method (papain, neutral protease, alkaline protease, and trypsin were tested) and a SC–CO₂ method (at 50°C and 280 bar). Lipid yields were similar for samples extracted using both methods (53.7% and from 61.5% to 82.0% for SC–CO₂ and the enzyme-assisted method, respectively). PUFAs were the main components, corresponding to approximately 35% of the total fatty acids extracted (Zhu et al. 2010). Recently, Chun et al. (2010) studied SFE with CO₂ for defatting, as an alternative to organic solvent use in defatting samples. The main goal of this work was to purify the phospholipase A₂ (PLA₂) from the defatted pyloric ceca of starfish (defatting conditions: SC–CO₂ at 40°C and 250 bar) to be used in the food industry (Chun et al. 2010).

2.3.1.4 Applications to Marine By-Products

Waste and by-products discharged by fisheries, such as fish heads, viscera, skin, tails, and blood often possess the highest concentration of bioactive compounds suitable for human health (Ferraro et al. 2010).

Marine organisms (fish, macroalgae, and microalgae) are an important natural source of Omega-3 PUFA. In fact, fish oil usually presents higher amounts of PUFA

than seed oils or microalgae. This makes fish oil a valuable product as a raw material for the generation of Omega-3 concentrates. SFE using CO_2 as the extracting solvent has been widely employed for fish oil extraction due to the good solubility of fish oil and its individual components in CO_2 (Rubio-Rodríguez et al. 2010). Dunford et al. (1997, 1998) investigated the effect of moisture content on mackerel oil extracted by SC-CO_2 at 345 bar and 35°C, concluding that the extraction yield increased when fish muscle moisture content decreased (Dunford et al. 1997). They also developed a mathematical model based on the interaction between oil and water in the fluid phase of SFE to explain the extraction behavior (Dunford et al. 1998). Esquivel et al. (1997) studied the SC-CO_2 extraction of highly polyunsaturated oil from sardine muscle. According to this study, at 40°C and 180 bar, it was possible to recover the oil (92% containing 209.7 and 136.7 g/kg oil EPA and DHA, respectively) without degradation of Omega-3 PUFA (Esquivel et al. 1997).

Fish by-products have also been employed as raw materials for Omega-3 rich oil SC-CO_2 extraction, demonstrating the applicability of SFE technology for valorizing waste products of the fish industry. For instance, oil rich in EPA and DHA was obtained by SC-CO_2 from freeze-dried sardine heads. A yield of 10.36% extracted oil containing 10.95% EPA and 13.01% DHA was achieved at 300 bar and 75°C (Létisse et al. 2006). From hake skin, more than 96% of the total oil contained in the raw material was extracted at 40°C and 250 bar in a semipilot SFE plant. The oil extracted contained a ratio of Omega-3/Omega-6 around 7 with a high content of EPA and DHA (6% and 14%, respectively, of the total fatty acid in the hake skin; Rubio-Rodríguez et al. 2008). Another study reported by Sahena et al. (2010) employed various SFC extraction techniques including continuous use of cosolvent (ethanol), soaking, and pressure swing of SC-CO_2 to extract oil from the skin of Indian mackerel. Soaking and pressure swing techniques (at 350 bar and 75°C) gave the highest yields of PUFA, especially arachidonic acid, EPA, and DHA. Considering extractability, CO_2 consumption, and recovery of EPA and DHA, pressure swing was deemed the more attractive technique for isolation of PUFA from Indian mackerel skins (Sahena et al. 2010). However, SC-CO_2 was also used previously for removal and deodorizing of lipids in tuna viscera before protein recovery. At optimum conditions (CO_2 + 3% ethanol, 124 bar and 35°C), 97% of the lipids (palmitic acid, heptadecanoic acid, and DHA) were extracted and the protein content found in the residue was about 50%, and consisted of L-Proline, taurine, and L-amino adipic acid (Kang et al. 2005b). Létisse and Comeau (2008) developed a fractionation process for fatty acid methyl esters (FAME) using SC-CO_2 to obtain extracts enriched in EPA and DHA from sardine and tails. Under optimized temperature (60°C) and pressure (264 bar) conditions, efficient fractionation according to chain length and degree of saturation was achieved, with high purity (up to 95% of FAME) and good yields (45% of EPA and DHA), (Létisse and Comeau 2008). Chang et al. (2008) carried out the concentration enhancement of Omega-3 fatty acids extracted from soft-shelled turtle fish oil bags using SC-CO_2 . They obtained the crude oil by top-down pressurized CO_2 at 60 bar and 55°C; then long-chain PUFAs were enriched from the esterified oil mixture using a countercurrent SC-CO_2 extractive distillation at 200 bar and 80°C (Chang et al. 2008).

Although fish oils are a natural source of Omega-3 fatty acids, the use of EPA and DHA as functional ingredients requires a concentration step into a chemical form that can be easily metabolized by humans. Hence, different processes to fractionate or concentrate PUFA from fish oil have been developed. Many of these methods refer to Omega-3 ethyl esters formed by the esterification or saponification of triglycerides with ethanol (Rubio-Rodríguez et al. 2010). Also, these methods often involve supercritical fluid fractionation to achieve higher yield and purity at lower cost. Thus, Fleck et al. (1998) carried out the fractionation of fatty acid ethyl ester (FAEE) from fish oil using SC-CO₂ (60°C, 145 bar) and an automated countercurrent column to obtain high separation efficiency (Fleck et al. 1998). Perretti et al. (2007) studied the fractionation of fish oil FAEE to concentrate the Omega-3 FAEEs with a suitable EPA/DHA ratio. Experiments were carried out using a pilot plant with three columns at 40°C, 50°C and 60°C, respectively, starting from the bottom and applying pressure changes from 100 to 150 bar and CO₂ flow rates of between 2.5 and 5 kg/h. Higher pressure (150 bar) and flow rates (5 kg/h) resulted in a higher EPA and DHA concentration and decreased the EPA/DHA ratio (Perretti et al. 2007). Moreover, Davarnejad et al. (2008) studied the solubility of menhaden fish oil in SC-CO₂ at temperatures from 40°C to 70°C and pressures of between 136 and 272 bar. The fractionated samples were converted to methyl esters by chemical esterification with methanol. The maximum solubility was achieved at 40°C and 272 bar (Davarnejad et al. 2008).

Following the fractionation of FAEE derived from fish oil according to their C-atom number using a phase equilibrium study with SC-CO₂ (Riha and Brunner 1999), a method to separate FAEE by countercurrent multistage extraction with SC-CO₂ in a pilot scale column was developed (Riha and Brunner 2000). Temperatures ranged from 40°C to 80°C and pressure from 65 to 195 bar. Low-molecular-weight components (C₁₄–C₁₈) were extracted as the top products and high-molecular-weight components (EPA- and DHA-rich esters) constituted the bottom products with 95% purity and 95% yield. Moreover, using a thermodynamic model, it was possible to determine the optimal conditions (145 bars and 60°C) for separation of FAEE by countercurrent SFE. This process, applied on an industrial scale, could produce EPA, DHA, and DPA (DHA and DPA in a mixture) at concentrations higher than 90% (Brunner 2000).

Other thermodynamic and mathematical models have been employed to optimize the fractionation of fish oil FAEE by SC-CO₂. For instance, Espinosa et al. (2002) developed a thermodynamic modeling, simulation, and optimization process for the supercritical fractionation of FAEE from fish oil based on Group Contribution Equation of State (GC-EOS), which provides reliable phase equilibrium predictions. They proposed a method for obtaining the maximum EPA and DHA recovery and purity from fish oil using a countercurrent system with three columns operating at similar temperatures (around 60°C) and pressures from 145 to 158 bar (Espinosa et al. 2002). Although recoveries of 80.34% of DHA (purity of 80.09 mol%) and 98.18% EPA (purity of 60.46 mol%) were obtained, the process needed a complex scheme to achieve complete fractionation, implying that high purity Omega-3 PUFA are associated with high extraction and purification costs. For that reason, the authors extended the thermodynamic model to include ethane–ester interaction parameters in order to use ethane as an alternative

supercritical solvent (Espinosa et al. 2008). Concentrations of 60% wt of EPA + DHA FAEE were obtained at optimal conditions (60°C and 84 bar), showing that the production of food additives containing EPA and DHA ester mixtures with moderate concentrations was possible at a relatively low cost.

Alternatively, a previous step based on urea complexation to increase the purity was also proposed. Gironi and Maschietti (2006) developed a thermodynamic model (assuming that the oil was composed of five ethyl esters) to represent high-pressure phase equilibria for the system. The method was validated with experimental data (temperature and pressure ranged from 42°C to 70°C and 101–172 bar, respectively) obtained in a semicontinuous single-stage fractionation process carried out on fish oil ethyl ester mixtures (Gironi and Maschietti 2006). Then the model was used to develop a simulation program of a continuous multistage fractionation process demonstrating that it is possible to produce a raffinate with 95% weight of EE-C₂₀ and EE-C₂₂, together with 95% recovery of these compounds. Martín and Cocero (2007) proposed a mathematical method, based on the differential mass and energy balances over the height of the extractor, to study theoretically the effect of operating conditions and extractor configuration in the fractionation of liquids with CO₂. They validated the model for the fractionation of fish oil ethyl ester mixtures and found that it was possible to predict the variation of the composition of the extract at the different operating parameters (Martín and Cocero 2007).

Catchpole et al. (2000) reported the countercurrent extraction and fractionation of different crude fish oils by SC-CO₂ and SC-CO₂ with ethanol. Vitamin A palmitate was recovered from cod liver oil ethyl ester/vitamin mixtures using pure CO₂ at 60°C and 200–300 bar. Under these conditions, vitamin A was preferentially recovered in the raffinate; then the cod liver oil ethyl esters virtually free from vitamin A could be used to obtain concentrates of DHA and EPA (Catchpole et al. 2007). In the same work, authors also investigated the use of mixtures CO₂ + ethanol to fractionate orange roughy (*Hoplostethus atlanticus*) and deep sea shark liver oils at a pilot scale. Peroxides, fatty acids, and odor compounds were removed from orange roughy oil and squalene was obtained from deep sea shark liver oil, at higher throughputs but lower selectivity using CO₂ with ethanol than using pure CO₂. An extraction process with pure CO₂ at a demonstration scale to produce squalene-rich extract and a diacylglycerylethers-rich raffinate (both compounds are used as health food supplements) was also developed.

Although fatty acids are usually converted into FAEE for oil fractionation, there are some references where Omega-3 PUFAs were obtained in their natural forms. For instance, Sarrade et al. (1998) combined SC-CO₂ extraction (310 bar and 40°C) with nanofiltration (using a multilayer composite nanofilter) for the fractionation and purification of fatty acids from fish oil, obtaining a significant concentration of EPA and DHA in the retentate stream and short-chained fatty acids in the permeate (Sarrade et al. 1998). Antunes Correa et al. (2008) also studied the fractionation of fish oil and PUFA concentration with SC-CO₂. Phase equilibrium was measured at temperatures from 28°C to 50°C and pressures from 78 to 294 bar. They found that the best conditions to fractionate the fish oil were 78 bar and 28°C (Antunes Corrêa et al. 2008).

Finally, it is important to highlight that several methods based on supercritical fluid chromatography to obtain Omega-3 PUFA with a high purity have also been

reported in the literature. Most of these methods, as for supercritical fluid fractionation, deal with ethyl ester separation (Alkio et al. 2000; Petinello et al. 2000).

2.3.2 Pressurized Liquid Extraction (PLE)

PLE is also known as pressurized fluid extraction (PFE), enhanced solvent extraction (ESE), high-pressure solvent extraction (HPSE), or accelerated solvent extraction (ASE; Nieto et al. 2010). This technique was described for the first time in 1996 (Richter et al. 1996). In PLE, pressure is applied to allow the use of liquids at temperatures higher than their normal boiling point. Among them, ASE (which can be considered a new version of the Soxhlet apparatus but operating at high pressures and temperatures), pressurized hot water extraction (PHWE or SWE, subcritical water extraction), near-critical fluid extraction, and enhanced fluidity extraction are the most promising techniques in bioactive extraction from different raw materials (Mendiola et al. 2007; Herrero et al. 2006).

The combined use of high pressures and temperatures provides faster extraction processes that require small amounts of solvents (e.g., 20 min using 10–50 ml of solvent in PLE can be compared with a traditional extraction procedure in which 10–48 h and up to 300 ml are required). The increase on the extraction temperature can promote higher analyte solubility by increasing both solubility and mass transfer rate. In addition, high temperatures decrease the viscosity and the surface tension of the solvents, helping to reach areas of the matrices more easily, thus improving the extraction rate. On the other hand, PLE is broadly recognized as a green extraction technique, mainly due to its low organic solvent consumption, which meets perfectly the basics of Pollution Prevention addressed in Fig. 2.2 and with Green Chemistry and Engineering principles (Table 2.1).

2.3.2.1 Instrumentation

In general terms it could be said that instrumentation for PLE is quite simple, but nowadays there is little commercial equipment available. Basically, the instrumentation consists of a solvent reservoir coupled to a high-pressure pump to introduce the solvent into the system, an oven where the extraction cell is placed and extraction occurs, and a restrictor or valve to keep the pressure inside the system (Fig. 2.4). Extracts are collected in a vial placed at the end of the extraction system. In addition, the system can be equipped with a coolant device for rapid cooling of the resultant extract.

After preheating, the extraction cell is filled with solvent and kept in the oven at constant temperature and pressure for a user-set static time. The solvent, which contains the extracted analytes, is collected in a vial and the cell is then flushed and purged with nitrogen gas. Together these steps constitute a cycle that can be repeated

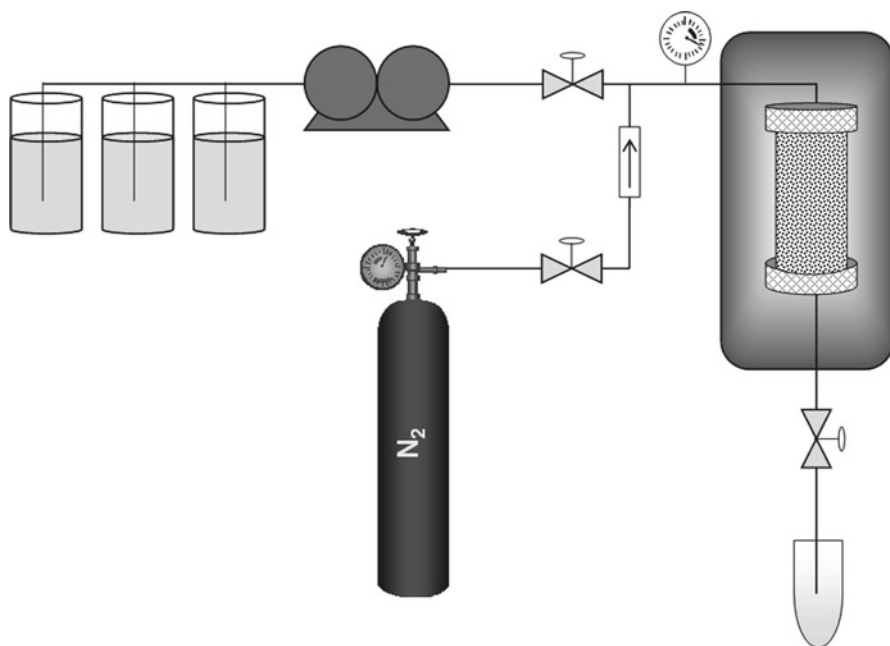


Fig 2.4 Basic scheme of a pressurized liquid extractor

several times, if necessary. The total extraction time is normally 15–45 min, although sometimes a longer extraction time is necessary (Nieto et al. 2010).

The PLE processes can be carried out in both dynamic and static mode, as SFE. The static mode has been the most utilized and is the more frequent when using commercial instruments. The dynamic mode, presumably, could improve the extraction rate by allowing a better contact between the matrix and fresh solvent pumped in a continuous way through the extraction cell and is used mainly with lab-made devices.

Different parameters can be optimized in order to obtain the highest recoveries; the most important are the extraction solvent, temperature, pressure, static time, and number of cycles. Other parameters (e.g., purge time and flush volume) have shown little influence on the final recoveries, so these are usually fixed. Each parameter can be optimized separately or using experimental designs (Nieto et al. 2010). Other aspects that have to be considered are the arrangement of the sample inside the extraction vessel and the collection of the analytes. In PLE the analyte recovery is not as critical as in SFE because, in most of the automatic systems, the solute is recovered in solution in a closed vial minimizing solute losses. Of course, for highly volatile compound recovery, a cooling step should be included.

Compared to SFE, PLE is more versatile in terms of the extraction solvents that can be used and therefore, is more flexible in terms of bioactive compounds to be extracted. In PLE the solvent will be selected depending on the polarity of the target compounds. However, this technique is considered by far less selective than SFE.

Therefore, it is important to keep in mind that, even if the extraction of the bioactives is attained, it would be possible to find other interfering compounds in the obtained extract.

2.3.2.2 Applications to Macroalgae, Microalgae, and Cyanobacteria

Since the early times of PLE, there has been a lot of effort focused in obtaining bioactive compounds from marine sources. Probably the main reason for the extremely important development of PLE-based techniques is the possibility of its automation along with the reduced extraction time and solvents required, as previously mentioned.

PLE has been used, for example, to concentrate carotenoids from *Dunaliella salina* microalgae (Breithaupt 2004; Herrero et al. 2006b). Results showed that the extraction temperature was the factor having the strongest influence (positive) on both the extraction yield and the antioxidant activity of the extracts (determined using the TEAC method). The best yields were obtained with ethanol at the highest extraction temperature and time tested (160°C and 17.5 min). Results of chemical characterization by high-performance liquid chromatography coupled with diode array detection (HPLC-DAD) pointed out that the extracts contained, besides all-trans- β -carotene and isomers, several different minor carotenoids that seemed to contribute strongly to the antioxidant activity of the extracts.

As seen for *Dunaliella*, ethanol was selected as the optimum solvent to extract antioxidants from *Synechocystis* sp. and *Himanthalia elongata* (Plaza et al. 2010a) and antimicrobial compounds from *Haematococcus pluvialis* (Santoyo et al. 2009). Generally, the best extraction conditions in these applications have been obtained at mild temperatures, around 100°C. The different species produced active extracts in terms of both antioxidant and antimicrobial activities. The obtained pressurized liquid extracts have been chemically characterized by GC–MS (gas chromatography–mass spectrometry) and HPLC-DAD in order to find the compounds responsible for their associated bioactivity. Several fatty acids and volatile compounds with antimicrobial activity have been identified in the different extracts, such as phytol, fucosterol, neophytadiene, or palmitic, palmitoleic, and oleic acids. On the other hand, antioxidant activity has been mainly attributed to the presence of carotenoids and phenolic compounds, among others.

Among cyanobacteria, several species have been screened for their content in bioactive compounds. For example, *Spirulina platensis* has been investigated as a natural source of different functional compounds together with PLE (Herrero et al. 2005a; Herrero et al. 2007). Different factors were optimized such as solvent polarity (hexane, petroleum ether, ethanol, and water), extraction temperature, and time and distribution of the sample inside the extraction cell. By combining PLE with a multistep chemical analysis including preparative thin layer chromatography (TLC), DPPH staining to test the antioxidant activity of the separated bands, and HPLC-DAD

to identify the separated compounds in each band, it was possible to determine different chlorophylls and carotenoids in the *S. platensis* PLE extracts, among them, β - and α -carotene, β -cryptoxanthin, asthaxanthin, zeaxanthin, lutein, echinenone, oscillaxanthin, and myxoxanthophyll (Jaime et al. 2005). Among the different solvents tested to maximize the attainment of antioxidant compounds, ethanol was found to be the most appropriate, considering both the high yields and the good antioxidant activities achieved.

Extraction of bioactive compounds from macroalgae has great potential and applications will continue to grow in the following years Plaza et al. 2008. PLE has been used, for example, to isolate carotenoids from brown macroalgae, such as *Eisenia bicyclis* (Shang et al. 2011), *Cystoseira abies-marina*, and *Himanthalia elongata* Plaza 2010. Results showed that ethanol at high temperatures provides high recoveries of fucoxanthin and other oxygenated carotenoids. Besides ethanol, Plaza et al. (2010) tested other solvents including water and hexane, and analyzed both the antioxidant and the antimicrobial activity of extracts, finding the highest bioactivities (antimicrobial and antioxidant) in ethanol extracts. In terms of antimicrobial activity, this seems to be related to the presence of several fatty acids such as linoleic or α -linoleic and certain volatile compounds such as phytol or furanones.

Depending on the selected solvent, PLE could be an ideal technique for the separation of bioactive phenolic compounds. In this sense, marine algae (*Stypocaulon scoparium*, *Porphyra tenera* -nori-, and *Undaria pinnatifida* -wakame-) and freshwater algae (*Spongiochloris spongiosa* and *Anabaena doliolum*) have been studied (López et al. 2011; Onofrejová et al. 2010). In general terms it could be stated that pressurized methanol/water mixtures provided the highest recoveries of phenolic compounds and, therefore, with the highest antioxidant activity. In the work carried out by Onofrejová et al. (2010), a combination of PLE and solid phase extraction (SPE) was used concluding that PLE–SPE extraction and HPLC-ESI-MS (HPLC-electrospray ionization mass spectrometry) analysis of phenolic compounds in selected algal species can detect bioactive phenolic compounds in subnanomolar concentrations (Onofrejová et al. 2010). Using optimized extraction conditions, the average recovery for studied phenols was 96%. In addition, the antioxidant activity analysis indicated that algae PLE–SPE extracts could be used as a source of antioxidants. This extraction procedure could be useful for the rapid extraction of bioactive phenols in various cyanobacteria or algae materials and their food products.

2.3.2.3 Applications to Invertebrates

PLE has also been used as an extraction technique for enrichment of high-value compounds from low-value raw materials. For instance, ethanol at elevated temperature and pressure has been used as a Green solvent for the extraction of astaxanthin from shrimp wastes (consisting of head, shell, and tail of shrimp). Parameters as such temperature and extraction time were shown to have a significant influence on the extraction yield whereas pressure only had a minor effect. Under optimal

conditions, astaxanthin yields of around 24 mg/kg shrimp waste were obtained (Quan and Turner 2009).

In addition, PLE has been used as a first step in the isolation of novel natural products from marine sponges. The PLE extraction has been carried out using three independent runs of three solvents, hexane, dichloromethane, and methanol, obtaining the highest percentage yield of the total organic extract in the first extraction. Meroditerpenes, such as isoaspic acid, cacospongin, and jaspquinol, with antimicrobial properties against *Staphylococcus epidermidis* have been isolated from *Cacospongia* marine sponge (Rubio et al. 2007) whereas sesquiterpenes, such as aignopsanoic acid A and methyl aignopsanoate A, which are moderately active against the parasite responsible for sleeping sickness, along with isoaignopsanoic acid have been obtained from *Cacospongia mudofijiensis* (Johnson et al. 2009). In a recent work, the utility of methanol partitioning and PLE to extract 12 bioactive natural products (including isoaspic acid and aignopsanoic acid A) from five marine sponges has been investigated. In this work, an aqueous extraction prior to extractions with hexane, dichloromethane, and methanol, was carried out to remove residual inorganic salts. In all cases, a pressure of 117.2 bar and temperatures equal to 22°C and 100°C were tested. Results obtained in this study showed that the extraction efficiency of PLE was three times higher than the solvent partitioning method (Johnson et al. 2010), demonstrating that it could be an effective high-throughput methodology to process marine sponges for the rapid discovery of novel and bioactive marine natural products.

2.3.2.4 Applications to Marine By-Products

PLE has been used to recover the total lipid content from fish tissue. In addition to the known advantages, less tissue than in conventional methods is required because high extraction efficiency is attainable by PLE. Different solvents have been tested for the extraction of total lipids from fish tissues (salmon and halibut muscle; Dodds et al. 2004). Each sample was extracted at 100°C and 138 bar with two static extraction cycles of 5 min each using chloroform/methanol, hexane/isopropanol, and methylene chloride. The performance of each solvent system was evaluated using gravimetry and GC analysis of FAME prepared from the extracts. Extraction using chloroform/methanol gave the highest recovery of FAs. On the other hand, Isaac et al. (2005) evaluated the reliability and efficiency of PLE for the extraction of lipid content from cod and the effect of sample treatment on the extraction efficiency. Under the best extraction conditions (using 2-propanol:hexane (65:35, v/v) as the first extracting solvent and hexane:diethyl ether (90:10, v/v) as second solvent, at 115°C, 100 bar, and two static cycles of 10 min each) the total lipid yields from homogenized cod (lean fish) and herring (fat fish) muscle exceeded those of conventional batch extractions (modified Jensen methods) by a factor of 10% (Isaac et al. 2005).

Lately, Spiric et al. (2010) carried out statistical studies to compare the fat content, the fatty acid profiles and cholesterol in carp fish muscles obtained by two extraction procedures, modified Soxhlet and PLE. PLE was performed using

hexane:isopropanol (60:40 v/v) at 100°C and 103 bar in two static cycles of 10 min each. Results obtained showed that modified Soxhlet gave higher Omega-6-fatty acid content than PLE, although there was no statistically significant difference in the Omega-3-fatty acid between both methods. They concluded that PLE could be considered as an alternative to the modified Soxhlet extraction (Spiric et al. 2010).

2.3.3 Pressurized Hot Water Extraction (PHWE)

Pressurized hot water extraction (PHWE), also known as subcritical water extraction, pressurized low polarity water (PLPW) extraction, or superheated water extraction (SHWE) is a particular use of PLE with water as extracting solvent. Water has many advantages in terms of versatility and environmental impact and can be used in extraction processes to isolate functional ingredients from different raw materials including plants and food wastes. PHWE is based in the use of water at temperatures above its atmospheric boiling point, while keeping it as liquid by applying pressure; under these conditions, physical and chemical properties of water change dramatically, for instance, the dielectric constant of water decreases from around 80 at room temperature (25°C) to around 33 at 200°C, that is, close to a polar organic solvent such as methanol. Moreover, the viscosity and surface tension are both reduced with increasing temperature, whereas diffusivity is increased, altogether enhancing the extraction process in terms of efficiency and speed. In addition, water's solubility parameter is also modified by temperature, thus favoring the solubility of a different type of compound and modifying its selectivity. Water is also the greenest solvent that can be used, perfectly complying with the rules of Green Chemistry and Green Engineering. For more information on the extraction process and the most important parameters to control (temperature, pressure, extraction time, flow rate, selectivity, etc.), readers are referred to a number of book chapters and review articles published covering general (Turner and Ibañez 2011; Teo et al. 2010), food and drug (Herrero et al. 2006), and medicinal plant (Glomohamad et al. 2008; ONg et al. 2006; Wang and Weller 2006) applications.

The conditions at which PHWE will be used will mainly depend on the target compound and the target application. In this sense, pressurized hot water is a very versatile medium in both sub- and supercritical conditions. For instance, supercritical or near-critical conditions can be used for supercritical water oxidation, hydrolysis, and molecular transformations, such as biomass conversion, whereas in the subcritical region, extraction of health-beneficial compounds can be performed.

2.3.3.1 Instrumentation

The instrumentation for PHWE is essentially the same as for PLE (Fig. 2.4), but basic instrumentation may differ depending on whether a static or dynamic process is used and whether a commercial or homemade setup is employed. Static PHWE consists in

a batch process with one or several extraction cycles with replacement of solvent in between. In dynamic PHWE, the extraction solvent is continuously pumped at a selected flow rate through the extraction vessel containing the sample; thus, it requires a slightly more sophisticated high-pressure or HPLC pump to control the water flow rate as well as a pressure restrictor or a micrometering valve rather than a static open/close valve. Among other advantages, dynamic operation may avoid, to some extent, thermal degradation of bioactive compounds inasmuch as water is continuously flowing through the matrix at a certain fluid velocity that improves the efficiency of the extraction avoiding the excessive heating of the sample.

The main advantages of homemade systems compared to commercial setups are the possibility to perform both dynamic and static extractions with fewer operating restrictions, the working temperature range, and the possibility to carry out different processes (reaction, drying, and extraction) by modifying the basic setup. For a complete description of how to build your own system, refer to Turner and Ibañez (2011).

2.3.3.2 Applications to Macroalgae, Microalgae, and Cyanobacteria

As seen for PLE, the use of solvents above their boiling points provides faster extractions; moreover the use of water instead of organic solvents makes the PHWE a greener extraction process. However, optimization of this type of extraction is often empirical and fails to take advantage of the experimental findings in associated fields or theoretical correlations (King 2006). Although PHWE is a promising technique, it needs more research in the marine field; only few applications can be found dealing with the extraction of bioactives from marine sources.

The extraction and characterization of compounds with antioxidant and antimicrobial activity from *Haematococcus pluvialis* microalga in red phase have been studied using PHWE (Rodríguez-Meizoso et al. 2010). Extraction yields achieved were as high as 30% (dry weight) using 200°C as the extraction temperature. Moreover, the extract obtained at 200°C presented the highest antioxidant activity, and temperature did not significantly affect the antimicrobial activity. Chemical composition was determined by HPLC–DAD, HPLC–QqQ–MS, and GC–MS. Short-chain fatty acids turned out to be responsible for the antimicrobial activity, whereas the antioxidant activity was correlated with vitamin E (present exclusively in the 200°C extract), together with simple phenols, caramelization products, and neoformed antioxidants produced by Maillard reaction during the extraction at high temperatures (Plaza et al. 2010b).

Recent evidence showed that PHWE at high temperatures may generate new bioactive (antioxidant) compounds, as seen, for instance in *Haematococcus pluvialis* microalga. Plaza et al. (2010b) studied both antioxidants naturally found in raw samples and those formed during PHWE via Maillard reaction and other chemical events. Samples of different natures such as microalgae (*Chlorella vulgaris*), macroalgae (*Sargassum vulgare*, *Porphyra* spp., *Cystoseira abies-marina*, *Sargassum*

muticum, *Undaria pinnatifida*, and *Halopitys incurvus*), and plants (rosemary, thyme, and verbenas) were studied (Plaza et al. 2010b). Results demonstrated that the extent of each reaction depends on the chemical composition and nature of the particular sample being extracted; for example, it has been shown how in samples richer in phenolic compounds (i.e., rosemary and thyme), the occurrence of these reactions is more limited. *Undaria pinnatifida* was the sample in which these reactions progressed to a higher extent. These observations were also in agreement with the tremendous difference between the antioxidant capacity shown by the two extracts obtained at low and high temperatures from this alga. Data supported the formation of new antioxidants during PHWE processing.

PLE has been tested to extract phycobiliproteins from the cyanobacteria *Spirulina platensis*. Capillary electrophoresis coupled with mass spectrometry (CE-MS) was used to monitor and optimize the pressurized liquid extraction of proteins from *Spirulina platensis* (Herrero et al. 2005b). The combined use of PLE and CE-MS allowed the attainment of extracts rich in phycobiliproteins in short extraction times (namely, yields of 20% can be obtained in less than 2 h under the optimum process in an automatic way). Different extraction conditions were tested, including time, extraction temperature and pressure, nature of pressurized solvent, distribution of microalga inside the extraction cell, type of packing, and so on.

2.3.3.3 Applications to Invertebrates

Water at high temperatures has been suggested to hydrolyze several marine sources, such as invertebrate viscera wastes (squid or scallop), to produce valuable compounds such as amino acids, fatty acids, organic acids, and so on.

A method for recovery of valuable compounds (amino acids, organic acids, fat and oil phases, soluble proteins, and peptides) from scallop viscera has been proposed by Tavakoli and Yoshida. In this work, reactions were conducted in the temperature range of 170°C (subcritical region)–400°C (supercritical region) and pressure range from 7.92 to 300 bar. Maximum yield of amino acids (0.15 kg/kg dry scallop waste) and organic acids (0.08 kg/kg dry scallop waste) were obtained at 240°C (reaction time of 50 min) and 280°C (reaction time of 40 min), respectively, with glycine the predominant amino acid and pyroglutamic acid the most abundant organic acid (Tavakoli et al. 2006a). These authors have also described the use of subcritical water hydrolysis for the treatment of squid wastes; they described a two-step process in which the first step was optimized to recover amino acids (maximum yield 0.1031 kg/kg dry entrails) and extracting fat and oil (using 200°C, 5 min) and the second step (performed at 240°C, 50 min) was used for processing the solid phase, achieving the highest amount of organic acids (maximum yield 0.055 kg/kg dry entrails; Yoshida and Tavakoli 2004). Later, they applied subcritical water hydrolysis to produce oil and fat phases and hydrolyzed the triglycerides to free fatty acids. Among fatty acids, DHA (0.103 kg/kg oil) and EPA (0.062 kg/kg oil) were obtained at 240°C (20 min) and 200°C (40 min), respectively. From the oil and

fat phases, it was possible to carry out the transesterification of fatty acid to the corresponding fatty acid methyl ester, which are important compounds in biodiesel fuel production (Tavakoli et al. 2006b).

In another interesting work, Uddin et al. (2010) compared the production of valuable compounds, mainly amino acids, by subcritical water hydrolysis from raw and deoiled squid viscera (Uddin et al. 2010). Deoiled viscera were obtained using SC-CO₂ at 45°C and 250 bar (Uddin et al. 2009). The highest yield of amino acids was found at low (180°C) and high (280°C) temperatures from raw and deoiled squid viscera, respectively, being about 1.5 times lower from raw viscera than from deoiled viscera. These results demonstrate the possibility to integrate green technologies based on supercritical and subcritical technologies to produce valuable compounds from low-cost raw materials.

2.3.3.4 Applications to Marine By-Products

Sub- and supercritical water technologies have been used to recover useful substances from fish wastes. For instance, Yoshida et al. (1999) studied the production of useful compounds by subcritical water hydrolysis from fish meats. Amino acids such as cysteine, alanine, glycine, and leucine (from 0.004 to 0.024 kg/kg dry meat) were produced at 270°C and 55.1 bar. The production of other useful organic acids (lactic acid, pyroglutamic acid) was also observed. In addition, the oil phase extracted with hexane contained EPA and DHA (Yoshida et al. 1999). Following this work, the authors proposed a simplified kinetic model to explain the hydrolysis reaction under subcritical and supercritical water conditions to generate major products such as oil, amino acids, or organic acids from fish meat (Yoshida et al. 2003). Kang et al. (2001) investigated the recovery of amino acids from white croaker fish entrails by sub- and supercritical water reaction using semibatch and batch reactors (Kang et al. 2001). They observed two consecutive reactions in the treatment: hydrolysis of proteins to amino acids, and decomposition of amino acids to other products such as organic acids. Therefore, a proper control of sub- and supercritical conditions was necessary to obtain the target compounds. The maximum yield of total amino acids (137 mg/g dry fish) was obtained under subcritical conditions (250°C, 40 bar) at 60 min reaction time by using a batch reactor. Under supercritical conditions (380°C, 450 bar) the yield decreased due to the rapid decomposition compared to the production rate of amino acids.

2.3.4 Ultrasound Assisted Extraction (UAE) and Microwave-Assisted Extraction (MAE)

The last two green techniques discussed in this chapter are ultrasound-assisted extraction and microwave-assisted extraction. UAE uses acoustic cavitation to cause disruption of cell walls, reduction of particle size, and enhancement of contact

between the solvent and the target compounds. MAE uses microwave radiation that causes motion of polar molecules and rotation of dipoles to heat solvents and to promote transfer of target compounds from the sample matrix into the solvent (Ying et al. 2011).

Both methods are very versatile due to the possibility of using several solvents of different polarities; in fact, both can couple extraction and reaction at the same time. Moreover, both techniques allow fast extractions, which is a key point to avoid degradation of labile compounds. Both techniques are fast, use low amounts of solvents, and are cost effective, therefore the development of both methods could represent a key point in Sustainable Development.

2.3.4.1 Instrumentation for Ultrasound-Assisted Extraction

In UAE only a small portion of the ultrasound spectrum is used, namely power ultrasound. Power ultrasound, having frequencies between 20 kHz and 100 MHz, are now well known to have significant effects on the rate of various physical and chemical processes such as cleaning, degassing, solubilization, homogenization, emulsification, sieving, filtration, and crystallization. Power ultrasound involves the mechanical and chemical effects of cavitation. When a liquid is irradiated by ultrasound, microbubbles form, grow, and oscillate extremely quickly, and eventually collapse powerfully if the acoustic pressure is high enough. These collapses, occurring near a solid surface, generate microjets and shock waves that result in cleaning, erosion, and fragmentation of the surface. Microdischarges due to high electrical fields generated by deformation and fragmentation of the bubbles and the formation of radicals could be responsible for the observed chemical effects (Ötles 2009).

The two ultrasound apparatus most commonly used for extraction are the ultrasonic cleaning bath and the more powerful probe system. For small extraction volumes, an ultrasound horn with the tip submerged in the fluid can be sufficient. Large volumes of fluids have to be sonicated in an ultrasound bath or in continuous or recycled-flow sonoreactors (Ötles 2009). Although most of the research effort in UAE has concentrated on ultrasound itself, some studies have also examined the coupling between ultrasound and other techniques. When combined with supercritical fluid extraction, UAE enhances mass transfer of the species of interest from the solid phase to the extraction solvent (Hu et al. 2007).

UAE of bioactive compounds is increasingly efficient at directly transferring knowledge into technology for commercial development. This novel process can extract analytes under a concentrated form (low volumes of solvent) and free from any contaminants or artifacts. The new systems developed so far clearly demonstrated the advantages of UAE in terms of yield, selectivity, operating time, energy input, and even preservation of thermolabile compounds (Ötles 2009).

2.3.4.1b Instrumentation for Microwave-Assisted Extraction

Microwave heating for sample digestion in elemental analysis (atomic absorbance spectroscopy, AAS; inductively coupled plasma-mass spectrometry, ICP-MS) has been a recognized technique for decades. The application of microwave irradiation to the extraction of compounds from biological samples has been a more recent development. MAE was first described in 1986 (Ganzler et al. 1986). In this description, a domestic microwave was used as a sample preparation technique prior to chromatography. A variety of compounds from seeds, foods, and feeds were extracted; the process was found to be more efficient than classical Soxhlet or LLE.

In general, samples for MAE are homogenized and mixed with a solvent and the suspension irradiated at greater than 2,000 MHz for short periods of time. Heating is usually repeated several times with periods of cooling in between to prevent boiling. Efficiencies seen with this technique approach those of classical Soxhlet extraction but can be performed much more rapidly. Further modifications of the technique mimic sweep codistillation or steam distillation with air, sweeping the surface of the heated sample and being collected by a condenser that protrudes through the oven housing (Worsfold et al. 2005). Techniques have also been developed using closed-system microwave heating, the most used nowadays, which lets the mixture of sample and solvent increase the pressure due to reaching the boiling point; therefore, the extraction takes place in a similar condition as PLE (see above).

There are two approaches of applying microwave energy, namely, the mere bulk heating of a mixture through the use of absorbing containers and solvents (oven-type apparatus with samples in closed-vessel conditions), and the more refined, albeit demanding, selective heating of the target materials. These two approaches led to the development of two main types of laboratory extraction instruments.

By using selective heating it is possible to operate safely and much more efficiently under open-vessel conditions (i.e., atmospheric pressure); under these conditions, operating temperatures remain low. Once fitted with an appropriate reflux column, the system is autocontrolled in terms of temperature Ötles 2009. A technology combining microwave and Soxhlet extraction was designed in 1998 (Garcia-Ayuso et al. 1998). This extraction technique, called microwave-assisted Soxhlet extraction, uses two sources of energy, namely microwaves, applied on the extraction chamber of a modified Soxhlet, and electrical heating applied on the distillation flask. The combination of both techniques, UAE and MAE, is also possible (see Fig. 2.5) and it was developed as a modification of microwave-assisted Soxhlet extraction.

2.3.4.2 Applications to Macroalgae, Microalgae, and Cyanobacteria

Despite the possibilities that MAE and UAE techniques offer, there are not many applications dealing with the isolation of bioactive compounds from marine resources to this date.



Fig. 2.5 Apparatus for simultaneous US/MW irradiation and detail of immersion horn (25 kHz) inserted in the cavitating tube (19 kHz); the inset shows the latter as seen from the top (Reprinted from Cravotto et al. (2008) with permission from Elsevier)

Different species of the microalgal genus *Dunaliella* have been treated with these techniques, mainly for carotenoid extraction. The performance of MAE in *Dunaliella tertiolecta* (chlorophyte) and *Cylindrotheca closterium* (diatom bacillariophyte) was studied in terms of pigment extraction (Pasquet et al. 2011). The process performed on *Dunaliella tertiolecta* led to rapid pigment extraction, mainly because of the absence of frustule in microalgae cells thus allowing immediate solvent penetration into the matrix. In contrast, presence of the frustule in the diatom *Cylindrotheca closterium* constituted a mechanical barrier to pigment extraction by MAE. On the other hand, MAE was identified as the best extraction process for *Cylindrotheca* pigments as it combined rapidity, reproducibility, homogeneous heating, and high extraction yields. Compared to conventional processes, MAE offers the advantage of an homogeneous thermoregulation of the medium, as no heat transfer is required to heat cells located in the center of the flask. Microalgal organites are also homogeneously heated, allowing synchronous pigment extraction, whatever their subcellular localization (cytoplasmic or chloroplasmic). It was also observed that the extraction process had no effect on cell integrity and shape.

In order to compare two green extraction methods, SFE and UAE, *Dunaliella salina* was subjected to both and the pigment extraction was compared (Macías-Sánchez et al. 2009). Results indicated that the supercritical CO₂ extraction process was comparable to the ultrasound-assisted extraction when methanol was used as solvent, but not when using N,N'-dimethylformamide (DMF). Extraction yields obtained for carotenoids and chlorophylls using DMF were higher than those

obtained with methanol. Results demonstrated that DMF was more selective than methanol in the recovery of carotenoids from chlorophylls. This behavior can be attributed to the fact that chlorophyll is heterogenically bound to other compounds in the chloroplast and at least two or even three fractions of chlorophyll exist in the chloroplast.

Oil recovery from different microalgae (*Chaetoceros gracilis*, *Chaetoceros mulleri*, *Chlorella vulgaris*, *Dunaliella* sp., *Isochrysis* sp., *Nannochloropsis oculata*, *Tetraselmis* sp., *Tetraselmis chui*, *Tetraselmis tetrathele*, and *Thalassiosira weissflogii*) has been recently studied using UAE (Araujo et al. 2011). Regarding oil production, the choice of salinity in the culture media was an important parameter and reflected directly on the suitability of the microalgae towards oil production. The diatoms *C. vulgaris* and *C. gracilis* were the most suitable microalgae for large-scale oil production. Comparing these results with the extraction of lipids of *Chaetoceros* by SFE (Mendiola et al. 2007b) revealed that the use of UAE is more suitable than SFE to obtain extracts from diatoms; this fact can be attributed to the better penetration of the solvent in the cells with frustule because of cavitation.

Cravotto et al. (2008) developed a method to work simultaneously with MAE and UAE in order to extract oil from soy and from the microalga *Cryptocodinium cohnii* (Cravotto et al. 2008). This microalga is very rich in DHA but presents a very tough cell wall. Extraction times were reduced up to tenfold and yields increased by 50–500% in comparison with conventional extraction methods. GC analyses showed only slight or negligible differences in methyl ester profiles of oils extracted under high-intensity MAE/UAE and Soxhlet. It should also be pointed out that techniques using US or combined US/MW irradiation should be well suited for other processes, such as two-step extraction and transesterification for the production of biofuels.

As already mentioned in Sect. 2.3.1.2, the combination of SFE and UAE has been studied to isolate phenolic compounds from algae (Klejdus et al. 2010).

2.3.4.3 Applications to Marine By-Products

Only one work in which MAE has been employed as an extraction technique from fish wastes has been reported in the literature. Batista et al. (2001) used this technique to extract lipids from mackerel fillet and cod liver for the determination of the fatty acid profile composition (Batista et al. 2001). MAE extraction was carried out using a solvent mixture of ethyl acetate:cyclohexane, 1:1 v/v. After lipid extraction, the esterification was performed using trimethylsulfonium hydroxide and the FAME were determined by GC-FID. The lipid content extracted from mackerel fillet and cod liver was $5.6\% \pm 0.4\%$ and $62.6\% \pm 3.1\%$, respectively. Results obtained in this work demonstrated that MAE can be a good alternative to conventional methods for lipid extraction.

2.4 From Analytical to Industrial Scale: Multiple Integrated Processes as Future Trends

As previously mentioned, there has been an enormous interest in the field of pressurized fluids, mainly when using safer, less harmful, and green solvents. The versatility of pressurized solvents is due to their very different physicochemical properties (density, diffusivity, viscosity, dielectric constant) and the possibility of controlling these parameters and, therefore, their solvating power and selectivity, by changing the pressure and temperature of the extraction system.

Most applications of the green extraction processes reported in this chapter have been carried out at analytical scale, although some of them have also been tested at pilot scale and, to a lesser extent, at industrial scale. Readers may have realized that the processes that have been studied at pilot or industrial scale refer only to the use of SFE; the reason is simple: SFE is a more mature process and industrial plants can be found worldwide for multiple applications. Nevertheless, it is important to realize that, when talking about pressurized fluid systems, the basic design of the extraction equipment is quite similar, although the solvents are basically different (and with different physicochemical properties). Systems used to carry out processes such as SFE, PLE, and PHWE consist of a solvent supply, a pump for transporting the solvent, a heater for heating the solvent, a pressure vessel where the extraction occurs, a pressure control in the system, and a collection device for the extract (see Figs. 2.3 and 2.4).

Lab scale studies can be used to determine factors required for scale-up. Moreover, knowledge of phase equilibria, mass transfer rate, and solubility data may also be necessary to scale up the extraction process and equipment. For a more in-depth discussion on the design and scale-up of pressurized fluid extractors, readers are referred to Pronyk and Mazza (2009); in this review, aspects such as modeling of the extraction process (del Valle and de la Fuente 2006), mass transfer effects (internal and external), and effects of processing parameters (temperature, pressure, flow rate, material properties, and length to diameter ratio) on the extraction process, that is, in mass transfer and solubility of the target compounds, and how this affects the design of the extraction system, are carefully considered. Readers are also referred to other publications related to the scale-up of SFE (Meireles 2003; Berna et al. 2002) and PHWE (Lagadec et al. 2000) for further information.

As for MAE and UAE, few reports can be found considering pilot scale units (Terigar et al. 2011; Boonkird et al. 2008) undoubtedly, more research is needed in this field to demonstrate the usefulness of these techniques at larger scale.

Therefore, considering the interesting applications discussed in the present chapter for bioactive compound extraction from marine resources, it is expected that once both viability and feasibility of the process at large scale are studied, not only SFE but also other pressurized fluids such as ethanol and water, could be used for both pilot and industrial process development. We should consider that, as for CO₂, water and ethanol have important advantages in terms of the low environmental impact of the solvent and, therefore, their use might be an alternative to more conventional extraction processes, using less green solvents. Undoubtedly, other aspects

of the process should also be considered such as the total amount of sample, other chemicals needed, energy usage, and the need for solvent recycling and storage. However, in terms of sustainability it is a good point to be able to switch to a more Greener solvent such as water or ethanol.

As for future trends, the idea of developing multiple integrated processes, able to face some of the challenges in our society such as environmental impact, sustainability, energy preservation, and health is suggested. Previously, King and Srinivas (2009) and Turner and Ibañez (2011) discussed this proposal. The idea of building a multiunit operations system with the possibility of using different fluids can provide unique characteristics and advantages to develop ad hoc platforms tuned to the different processes that want to be optimized. Main characteristics of this “green” processing platform are: it should work with environmentally benign solvents such as liquefied or supercritical CO₂, for nonpolar to moderately polar solutes, and with pressurized hot water (between its boiling and critical points) for a wider range of polarities, considering also the use of ethanol as cosolvent together with water or carbon dioxide. As mentioned previously, the use of pressurized hot water could be exploited to include extraction of bioactive compounds from natural raw materials such as marine resources, reaction of targeted substrates (oxidation, hydrolysis, etc.), and biomass conversion for renewable fuels.

Several examples can be found in the literature about integrated processes that may favor the extraction and purification of bioactives; some of them already deal with some green processes to extract bioactive compounds from marine resources (Liau et al. 2010) whereas others can be used as a base for converting the reported processes to more green, sustainable, and efficient ones (Siriwardhana et al. 2008; Athukorala et al. 2006). In the first approach (Liau et al. 2010), a process was studied considering SFE of lipids and carotenoids from the microalgal species of *Nannochloropsis oculata* and supercritical antisolvent precipitation of a carotenoid-rich solution. In this approach, both processes were considered independently but it can be easily inferred that the development of a multiple integrated process can allow purification of carotenoids in a cleaner and efficient way. Other processes that can be easily included in the green platform are those dealing with enzymatic hydrolysis and extraction; the pioneer work of Turner et al. (2006) demonstrated the viability of a process combining enzymatic hydrolysis in hot water, using a thermostable β -glucosidase to catalyze hydrolysis of quercetin glucosides in onion waste, plus extraction with water at high temperatures. The developed process was preferred over more conventional extraction/hydrolysis processes based on methanol extraction and hydrochloric acid hydrolysis at 80°C, regarding primary energy consumption and global warming potential (Lindahl et al. 2010). Following a similar approach, integrated treatments considering enzymatic hydrolysis plus extraction carried out on different algae, such as *Hizikia fusiformis* (164), *Ecklonia cava*, and other brown algae (Athukorala et al. 2006; Moreda-Piñeiro et al. 2007; Turner et al. 2006; Heo et al. 2005) could be optimized in a more efficient and Green way using the above-mentioned concepts and ideas. Moreover, studies have demonstrated that processes such as enzymatic hydrolysis are accelerated under pressure conditions, thus giving even stronger support to the possibility of improving the processes through the use of integrated pressurized fluid technologies (Moreda-Piñeiro et al. 2007).

2.5 Conclusions

Different aspects have been addressed in this chapter. First of all, we attempted to demonstrate the important possibilities offered by marine resources (such as micro- and macroalgae, cyanobacteria, invertebrates, and marine by-products) as a source of natural bioactive compounds with health benefits, with potential use in the food industry. The new integrated approach consisting of a screening of extraction conditions and an in vitro measuring of functional activities together with an exhaustive chemical characterization will provide us with a new tool to discover new bioactive compounds and to help the further design of processes to obtain such products in the most Green, sustainable, and efficient way, complying with the rules of green chemistry and green engineering. In this chapter we discuss and present some selected applications of the extraction of target compounds with, among others, antioxidant, antimicrobial, and antiproliferative activities from different sustainable marine sources. Either the use of microalgae as bioreactors to produce and enrich target compounds, the gathering and reuse of low-cost by-products from the fish industry, or the collection or growing of macroalgae in nonprofitable lands, is a good start for a process tending to recover new and valuable compounds from these sources in a sustainable, economical, Green, and efficient way. We have shown many possibilities at laboratory scale, which are the basis of the knowledge, and that can be used as the first step for a bigger production scale.

Undoubtedly, the replacement of environmentally burdensome solvents such as acetonitrile, methanol, dichloromethane, and toluene, long used in such extraction processes, for more green solvents such as CO₂, ethanol, and water is one of the goals and one of the proposals that we are offering to the reader in this chapter. Some technologies are mature enough to be used at large scale; others require more study and development but, in any case, it is advisable that new steps be taken to help build a more rational use of our natural resources. The possibility, mentioned in this chapter as a future trend, to build new platforms able, in a sustainable way, to run integrated processes including pretreatments, extractions, reactions, and transformations in a more integrated way is one of our main goals and might help all of us to build a better future.

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