# Compressed fluids for the extraction of bioactive compounds

Miguel Herrero, María Castro-Puyana, José A. Mendiola, Elena Ibañez

**The improvement of sample-preparation and extraction techniques for determinations of natural bioactive compounds is very important. New concepts relate to not only enhancement of extraction efficiencies but also environmental impact. This evolution towards Green Analytical Chemistry is to new extraction and sample-preparation processes that should be faster, more reproducible and more environmentally friendly.**

**Compressed fluid-based sample-preparation techniques (e.g., supercritical fluid extraction and pressurized liquid extraction) demonstrate good capabilities. In this review, we update knowledge on the techniques together with the main technical developments and the most notable recent applications for the extraction of bioactive compounds.**


*Keywords:* Agricultural by-product; Bioactive compound; Extraction; Green Analytical Chemistry (GAC); Natural sample; Plant; Pressurized liquid extraction (PLE); Sample preparation; Subcritical water extraction (SWE); Supercritical fluid extraction (SFE)

*Abbreviations:* ASE, Accelerated solvent extraction; DHA, Docosahexaenoic acid; EAE, Enzyme-assisted extraction; EPA, Eicosapentaenoic acid; IL, Ionic liquid; MIP, Molecularly-imprinted polymers; PFE, Pressurized fluid extraction; PHSE, Pressurized hot solvent extraction; PHWE, Pressurized hot water extraction; PLE, Pressurized liquid extraction; PLPW, Pressurized low polarity water extraction; n-3 PUFA, n-3 polyunsaturated fatty acid; RAM, Restricted access media; scCO2, Supercritical CO2; SFE, Supercritical fluid extraction; SHWE, Superheated water extraction; SPE, Solid-phase extraction; SWE, Subcritical water extraction; TG, Triglyceride; UAE, Ultrasound-assisted extraction; WEPO, Water extraction and particle formation on-line

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## 1. Introduction

Interest in the search for bioactive compounds of natural origin has arisen in the past two decades, driven by the increasing number of scientific papers demonstrating the efficacy of such compounds against several diseases. Applications in food science and technology have also undergone this evolution, mainly due to the increasing evidence correlating diet and some chronic diseases. Different sources of bioactive compounds have been studied, plants, agricultural by-products and marine products being among the most promising. Some plant antioxidants, derived from fruits and vegetables, have already been associated with lower risks of coronary heart diseases and cancer [1,2]. As for marine sources, they have a great potential, mainly considering their huge diversity, their sometimes unique chemical structures and their ability to work as natural bioreactors, favoring the synthesis of valuable compounds depending on the cultivation conditions or through biotechnology [3].

Extraction techniques are studied worldwide not only from the production point of view, but also as sample-preparation techniques able to contribute to meeting the requirements for the development of faster, more efficient, cheaper, high-throughput and ''greener'' analytical methodologies. Sample preparation is typically considered one of the ''bottlenecks'' of any analytical procedure, in not only throughput but also greening the analysis. Sample preparation has been the step in the analytical procedure evaluated most since it is considered the most difficult to implement.

The goals of Green Analytical Chemistry (GAC) cover sample-preparation tools [4], namely:

(1) reduction in the amount of sample to be treated and reduction/elimination of pollutant solvents or acids (miniaturization);
(2) simultaneous extraction of multiple compounds; and,

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(3) increasing automation and throughput determination, leading to understanding of the important role that techniques based on compressed fluids can play.

In this article, which covers the literature published during the period 2008–12, we review basic principles and main advances in these techniques {e.g., supercritical fluid extraction (SFE), pressurized liquid extraction (PLE) [also called accelerated solvent extraction (ASE), pressurized hot solvent extraction (PHSE) or pressurized fluid extraction (PFE)] and subcritical water extraction (SWE) [also called pressurized hot water extraction (PHWE), pressurized low polarity water extraction (PLPW) or superheated water extraction (SHWE)] [5,6]}. We briefly present the principles and the instrumentation of compressed fluid extraction, together with some technical developments and key applications in extraction of bioactive compounds.

As can be seen in Fig. 1, depending on the polarity of the compressed fluid selected, different ''green'' pressurized sample-preparation techniques can be used. All of them rely on the use of minimum amount of the food-grade solvents for a selective extraction of bioactives while preserving their bioactivity and chemical structure. They all show great versatility and efficiency, since the physicochemical properties of solvents (density, diffusivity, viscosity, and dielectric constant) can be modified by changing the pressure and/or the temperature of the extracting fluid, which also modify their selectivity and solvating power.

## 2. Principles and instrumentation of compressed fluids

Despite several differences in the basic principles of SFE, PLE and SWE, they all have in common that they must operate under medium-to-high pressures. In this section, we address their main characteristics.

SFE is based on the use of solvents at temperatures and pressures above their critical points, while PLE and SWE operate using liquids at temperatures above their normal boiling points and pressures enough to keep the extracting fluid in the liquid state.

Fig. 2 shows a basic scheme of the equipment used to perform compressed fluid extractions. It consist on a container of the extraction solvent (A) (usually $CO_2$ for SFE, water for SWE, or other organic solvents for PLE), a pump to pressurize the fluid (B), an oven containing the extraction vessel (C), a restrictor to maintain the high pressure inside the system (D), a trapping vessel (E) and optionally a modifier pump or $N_2$ container (F). Nevertheless, from this starting point, the instrumentation employed might be more or less sophisticated. For example, a dynamic extraction might require more accurate high-pressure pumps in order to maintain a precise flow rate during the whole extraction procedure. Besides, in this case, a heating coil should be included inside the oven to ensure that the solvent reaches the extraction cell at the set temperature. Although the extraction cell is a simple device, it should be capable of withstanding high pressures and temperatures [up to 200–250°C, when working with pressurized liquids (e.g., water or ethanol)]. For operations with SWE and PLE, a nitrogen circuit can be included to purge all the system after the extraction, as well as to ensure that all the extracting solvent has reached the collecting vial once the extraction is finished.

The general extraction procedure might be divided into three phases, comprising desorption of analytes from the matrix, their diffusion into the solvent that has penetrated the matrix itself, and their transfer to the rest of the extracting solvent [7].

Despite the great number of advantages found in these techniques, the use of experimental designs to optimize the extraction conditions is recommended, since undesirable effects might also take place at high temperatures (e.g., degradation of bioactive compounds).

The main valuable characteristic of SFE is the highly reduced (often to zero) employment of toxic organic solvents. Carbon dioxide is the solvent most commonly used to extract bioactive compounds from natural sources using SFE. Despite some other solvents having been proposed for SFE (namely, e.g., propane, butane, and dimethyl ether), none of them fulfill the principles of GAC as well as $CO_2$. $CO_2$ has interesting properties for bioactives extraction:

(1) its critical conditions are easily attainable (31.1°C and 7.39 MPa);
(2) it is a non-toxic, non-flammable solvent; and,
(3) it is considered Generally Recognized as Safe (GRAS) for use in the food industry.

At supercritical conditions, solvents present high diffusivity, whereas their solvent strength and density can be easily modified by tuning the temperature and the pressure applied. Another important characteristic of this technique, when using supercritical $CO_2$ ($scCO_2$), is the possibility of attaining solvent-free extracts. Once the extraction procedure is finished, depressurization of the system turns $CO_2$ to gas, while the compounds extracted from the matrix precipitate. However, an important drawback of $CO_2$ is its low polarity, which can be overcome by employing low amounts (1–10%) of polar modifiers to change the polarity of the supercritical fluid and to increase its solvating power towards the analyte of interest.

The basic principle of PLE relies on the use of solvents to carry out extractions at high pressures and temperatures, always below their critical points, so that the liquid state of the solvent is maintained during the whole

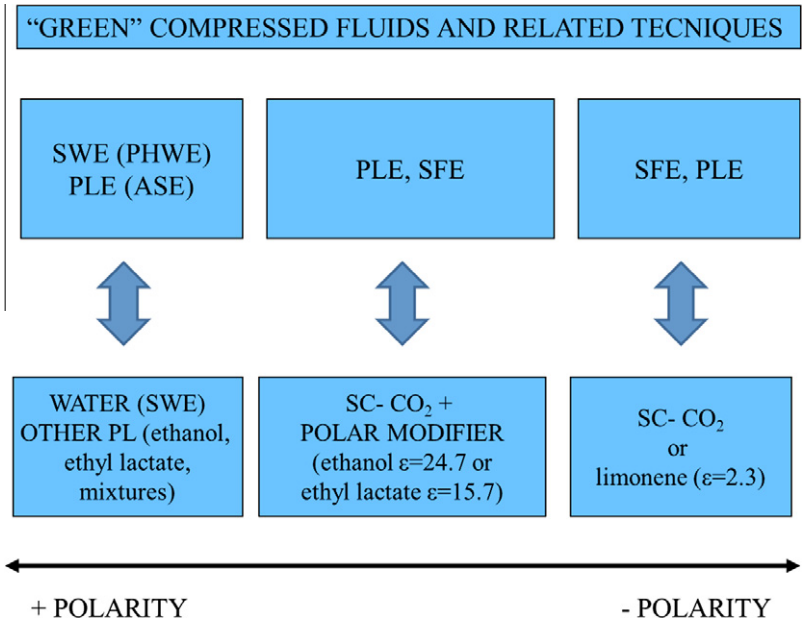


**Figure 1.** Green techniques based on the use of compressed fluids for extraction of bioactive compounds. ϵ, dielectric constant.

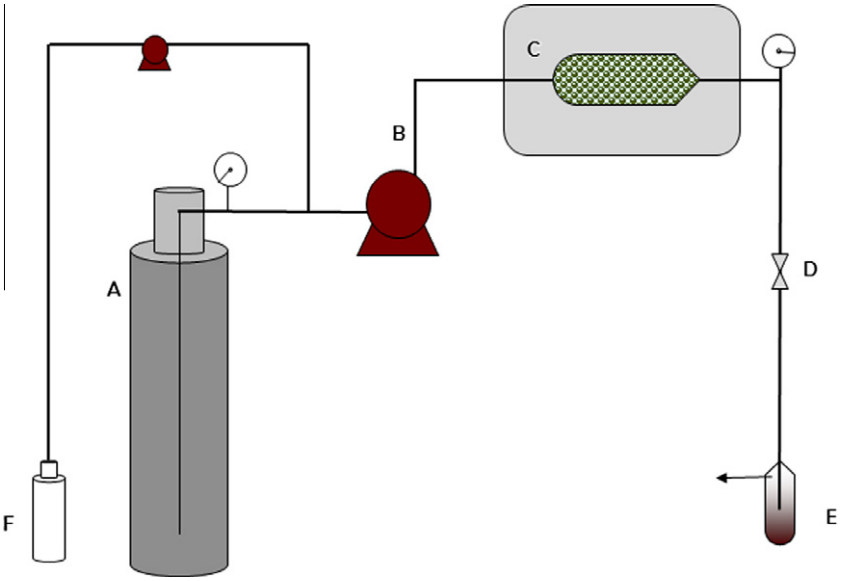


**Figure 2.** Typical compressed fluid extractor. The different parts of the system are: solvent reservoir (A), pump (B), heated extraction cell (C), static/dynamic valve (D), collection vessel (E) and modifier/N2 container (F).

extraction process [5]. By applying those conditions, faster extraction processes result, in which, typically, higher extraction yields are obtained with low volumes of organic solvents (e.g., 20 min using 10–50 mL of solvent in PLE can be compared with a traditional extraction step in which 10–48 h and up to 200 mL are required), decreasing in this way the dilution of the sample. These characteristics are mainly due to the improvement in mass-transfer kinetics obtained under high temperature and pressure [8,9]. The use of high temperatures increases the solubility of the analytes in the solvent and decreases solvent viscosity and surface tension, thus allowing a better penetration of the solvent into the matrix. Although a large number of commercial PLE instruments are available in the market, different applications have been also reported with home-made instruments [10–13]. In both cases, it has to be considered that, given the operating pressures and

temperatures usually employed, corrosive-resistant materials have to be used.

SWE is a particular type of PLE based on using hot water as the pressurized liquid. Usually, temperatures higher than the boiling point of water (100°C) and lower than its critical temperature (374°C) are used, while pressures of 3.5–20 MPa are employed to keep the water in the liquid state during the whole extraction process. Under these conditions of pressure and temperature, physical and chemical properties of water change dramatically. Among the main parameters that can influence SWE (extraction temperature, time, pressure, the addition of an organic solvent or surfactant, and water-flow rate), temperature is the main factor that affects extraction efficiency and selectivity. An increase in temperature:

(1) facilitates analyte diffusion (diffusivity of water at 25°C is about 10 times lower than that of water at 200°C);
(2) favors mass-transfer kinetics by disrupting intermolecular forces (i.e. van der Waals forces, hydrogen bonds and dipole attractions);
(3) decreases the viscosity of water (enabling better penetration of matrix particles); and,
(4) decreases the surface tension (allowing the water to better wet the sample matrix) [8].

In spite of the improvement in all these properties, the most important effect of the increase of liquid water temperature is undoubtedly the weakening of hydrogen bonds, resulting in a lower dielectric constant ($\epsilon$). The dielectric constant (measure of polarity) of water, at enough pressure to be maintained in its liquid phase, varies from ~80 at 25°C (being extremely polar) to 25–27 when temperatures of ~250°C are used [14], which falls between those of methanol ($\epsilon$ = 33) and ethanol ($\epsilon$ = 24) at 25°C [14,15] (see Fig. 3).

As can be observed, the dielectric constant values of water resemble those of other, less polar solvents at room temperature, so, under these conditions, water could be used as an alternative to dissolve medium-polar and even non-polar organic compounds. Basically, the experimental set-up for SWE is similar to that described above (see Fig. 2). The advantages of a home-made set-up, compared to commercial systems, are:

(1) the range of working temperature;
(2) the possibility of carrying out both dynamic and static extractions; and,
(3) different processes (extraction, reaction, drying) just modifying the basic set-up.

More information about how to build an SWE system can be obtained from Turner and Ibáñez's book chapter [8].

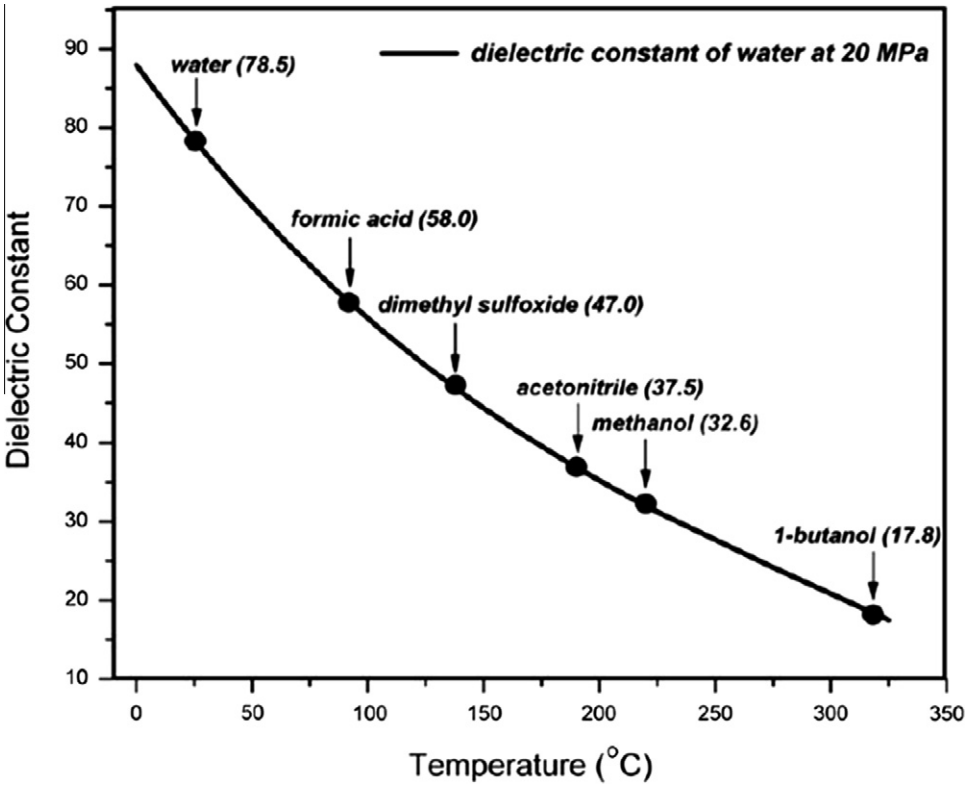


**Figure 3.** Dielectric constant of water as a function of temperature at 20 MPa. Solid circles superimposed on the plot are the values corresponding to different organic solvent at 25°C and 0.1 MPa (values given in parentheses) (Reproduced from [132], with permission from American Geophysical Union).

## 3. Supercritical fluid extraction (SFE)

### *3.1. Methodological and technological advances*

*3.1.1. Method optimization.* Method optimization plays an important role in SFE. Several parameters must be considered [e.g., extraction temperature and pressure, addition, amount and type of modifier, amount of sample (as well as particle size) and use of dispersing agents].

*3.1.2. Pressure and temperature.* Pressure and temperature have a strong influence on the solvent properties (e.g., density) so they are strongly related to the solubility of the target compounds in the supercritical fluid.

*3.1.3. Proportion and type of modifier.* Proportion and type of modifier are also key factors responsible for solubility of the target compounds in the supercritical fluid; in this sense, the organic modifier most commonly employed to extract bioactives is ethanol in a range 5–10% of $CO_2$ flow; other modifiers (e.g., methanol, acetone or even small amounts of water) have been also used to isolate polar bioactive compounds. However, to extract high molecular-weight non-polar compounds, vegetable oils (e.g., olive oil) have demonstrated ability as co-solvents at a proportion of 10% [16].

Recently, the use of greater amounts of modifier (up to 50%) was tested to obtain fractions enriched in γ-linolenic acid from *Arthrospira platensis* (*Spirulina platensis*); using $CO_2$-expanded ethanol at 30 MPa, 40°C and a ratio $CO_2$:ethanol 1:1 in the optimum, a recovery up to 35.3% was achieved [17]. Gas-expanded liquids (GXLs), which were used in this work for the first time in food products, demonstrated their performance as intermediate between PLE and supercritical fluids for the extraction of medium-polar compounds. Thus, in this case, GXL extraction takes place, and can offer several important advantages related to an improved extraction yield with lower consumption of organic solvents compared to classic solid–liquid extractions. GXLs are liquids whose volume has been increased when pressurized with a condensable gas (e.g., $CO_2$). Under these conditions, at least two fluid phases or a single phase above the bubble-point curve but below the critical composition exist. The properties of the liquid phase are substantially different from those at atmospheric pressure. GXLs have been shown to have improved mass transfer through reduced viscosity, increased solute diffusivity and decreased interfacial tension [18].

*3.1.4. Particle size and use of dispersing agents.* Particle size and use of dispersing agents have parameters more related to the efficiency of the extraction procedure and can be modified to avoid clogging and to increase extraction rate. The particle size (or crushing degree) is a very significant factor in the mass-transfer rate, so they will have maximum influence on the extraction yield.

Table 1 shows a selection of reviews published since 2002, which we strongly recommend for readers to gain a deeper understanding of the factors involved in SFE.

In recent years, several sample-preparation methods combining ionic liquids (ILs) and supercritical fluids emerged. Room-temperature ILs have been revealed as a new type of green solvent, generally comprising quaternary nitrogen cations. These solvents have interesting properties (e.g., negligible vapor pressure, thermal stability, tunable viscosity, and miscibility with water and organic solvents). They have been proposed as environmentally friendly solvents for ''green chemistry'' because they are good substitutes for traditional volatile and flammable organic solvents. However, there is some controversy about the greenness of the ILs, due to their incomplete physical, chemical and toxicological data. ILs have become partners of supercritical $CO_2$ in many applications that were reviewed recently by Keskin et al. [30]. Although more research is needed for a routine use of ILs in SFE, the development of new applications using ILs is increasing and the number of publications has risen exponentially since the mid-2000s.

The main field for combining ILs and SFE is in reactions. Their variable water and organic solvent miscibility allows the development of convenient extraction methods. Their characteristics can be easily modified (even changing pressure and temperature) and tuned to a targeted process by adjusting the cation/anion couple. Basically, the methods studied most involved enzymatic reactions in IL media followed by extraction of the desired compounds using $scCO_2$; this scheme has been proposed for a wide range of biocatalyzed reactions, mainly using lipases for different esterification purposes [31].

Enzymes in supercritical media are used in combination with not only ILs. Nowadays, the main trend in this field is so-called ''enzyme-assisted extraction'' (EAE) that is used with liquids and supercritical phases. Even if its main application has been in plant matrices to break cell walls at larger scale, the possibilities offered by this new approach to sample preparation are huge, mainly related to increases in extraction efficiency and/or selectivity of the extraction process. For a more in-depth knowledge in this field, we refer to the recent reviews of Sowbhagya and Chitra [32] and Subramaniam [33].

However, in the past few years, other technological developments have been studied in order to improve SFE efficiency for sample preparation; among them, the combination of SF with ultrasound-assisted extraction (UAE) has gained interest due to the higher extraction yields that can be obtained in shorter times. Its mechanism of action has been recently deciphered by Van Iersel et al. [34], who described how ultrasound

**Table 1.** Summary of some of the most relevant reviews published in SFE, SWE and PLE in the past 10 years

| Technique | Title | Year | Ref. |
|---|---|---|---|
| SFE | Steps of supercritical fluid extraction of natural products and their characteristic times | 2012 | [19] |
| SFE | Extraction of volatile oils by supercritical fluid extraction: Patent survey | 2011 | [20] |
| SFE | Supercritical fluid extraction: Recent advances and applications | 2010 | [21] |
| SFE, PLE, SWE | Use of compressed fluids for sample preparation: Food applications | 2007 | [5] |
| SFE | Supercritical fluid extraction in plant essential and volatile oil analysis | 2007 | [22] |
| SFE | Supercritical $CO_2$ extraction and purification of compounds with antioxidant activity | 2006 | [23] |
| SFE, PLE | Extraction methods and chemical standardization of botanicals and herbal preparations | 2004 | [24] |
| PLE | Natural dyes extraction from cochineal (*Dactylopius coccus*). New extraction methods | 2012 | [10] |
| PLE | Application of accelerated solvent extraction in the analysis of organic contaminants, bioactive and nutritional compounds in food and feed | 2012 | [25] |
| PLE | Techniques to extract bioactive compounds from food by-products of plant origin | 2012 | [26] |
| PLE | Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review | 2011 | [7] |
| SWE | Pressurized hot water extraction (PHWE) | 2010 | [15] |
| SWE | Pressurized hot water as a novel extractant of natural products: A review | 2010 | [27] |
| SWE | Extraction of functional substances from agricultural products or by-products by subcritical water treatment. | 2008 | [28] |
| SWE | Pressurized hot water extraction of bioactive or marker compounds in botanicals and medicinal plant materials | 2006 | [14] |
| SWE, SFE | Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae: A review | 2006 | [6] |
| SWE | Extractions with superheated water | 2002 | [29] |

irradiation of high-pressure fluids leads to improvements in mass and heat transfer in high-pressure fluids due to the creation of an extremely fast, local phase separation, which propagates through the mixture with a velocity equal to the speed of sound, in the vicinity of the critical point. The UAE + SFE combination has been used {e.g., in carotenoid extraction, showing an extraction yield up to 2.5 times higher than using only SFE [35]}. Pilot-scale devices have been also designed for a larger scale extraction of bioactive compounds [36].

In a recent paper by Klejdus et al. [37], a new methodology was proposed to isolate and to identify natural bioactive substances in biological matrices based on the use of a new SPE/SFE hybrid extraction. A new extractor device was designed, allowing the insertion of the SPE cartridge into the cell; after careful optimization of the extraction and elution conditions, it was possible to recover 13 phenolic compounds from different cyanobacteria and microalgae.

Sample collection has also improved through optimization of the factors involved in the effectiveness of the trapping method. Traditionally, the collection of extracts has been carried out using collection vessels where depressurization takes place, causing $CO_2$ gasification and extract precipitation. Since $CO_2$ gas is vented through an exhaust pipe, it is common to have losses of compounds (mainly volatiles) together with the $CO_2$ gas. Thus, trapping can result in significant loss of material or can increase the possibility of adding noise to the global analytical processes. Although different trapping methods have been developed (e.g., solid trapping, liquid trapping or a combination of both), for correct selection of the most appropriate approach, it is crucial to consider different factors [e.g., analyte volatility and polarity, volatility of the extracting agent, volatility of modifier (if used), and solvent flow rate]. Novel sample collectors use centrifugal forces to retain extracts while allowing their drying; one example of this use is the equipment developed in 2008 by KD Scientific that can be coupled to SFC and SFE systems [38]. The system consists of a flexible eluent tube that directs the flow from the SFC system into the fraction-collection tube while the rotor is spinning. Extract (liquids or solids, even volatiles) are trapped in the bottom of the collection containers due to the centrifugal force created by the rotor at 1500 rpm.

### *3.2. Applications*

Since the end of the 1970s, supercritical fluids have been used to prepare samples and to isolate compounds from natural products, but for a long time applications relied on only a few products. In recent years, many applications were developed to extract a wide variety of

**Table 2.** The most remarkable advancements in SFE published in the period 2008–12

| Product | Compounds of interest | Solvent | T (°C)/P (MPa) | Mode/cycles | Sample dispersion | Extraction time (min) | Ref. |
|---|---|---|---|---|---|---|---|
| *Arthrospira platensis* (*Spirulina platensis*) | Fatty acids γ-linolenic | $CO_2$:ethanol (1:1) | 40/300 | Dynamic | Sea sand | 60 | [17] |
| *Boletus edulis* | Fatty acids | $CO_2$ | 40/35 | Dynamic | – | 214 | [39] |
| *Borago officinalis* | Fatty acids | $CO_2$ + methanol | 65/35 | Static dynamic | Sea sand | 10 + 10 | [40] |
| *Camellia sinensis* | Fatty acids and antioxidants | $CO_2$ | 45/32 | Static | – | 90 | [41] |
| *Chamaecyparis obtusa* | Essential oil | $CO_2$ | 50/12 | Dynamic | Diatomaceous earth | 90 | [42] |
| Corn and Fish oils | Fatty acid ethyl esters | $CO_2$ | 40/160 | Dynamic | – | Continuous | [43] |
| *Evodia rutaecarpa* | Evodiamine, rutaecarpine | $CO_2$ + methanol | 62/28 | Dynamic | Sea sand | 78 | [44] |
| *Hemerocallis disticha* | Lutein, zeaxanthin | $CO_2$ | 80/60 | Static + dynamic | Speed Matrix | 30 + 30 | [45] |
| Kale, spinach | Polyphenols, flavonoids | $CO_2$ + 5% methanol | 50/25.8 | Static | – | 30 | [46] |
| *Lamiaceae* plants | Essential oils | $CO_2$ | 40/ 30 | Dynamic | – | 90 | [47] |
| *Lippia dulcis* | Hernandulcin and other sesquiterpenes | $CO_2$ | 35/120 | Static + dynamic | Sea sand | 60 | [48] |
| *Magnolia officinalis* | Honokiol and magnolol | $CO_2$ | 80/40 | Static + dynamic | – | 60 + 40 | [49] |
| *Mitragyna speciosa* | Alkaloids | $CO_2$ + ethanol | 65/30 | Dynamic | – | 45 | [50] |
| *Nelumbo nucifera* | Alkaloids | $CO_2$ + diethylamine + water | 70/30 | Dynamic | – | 60 | [51] |
| Nuclear waste | Radioactive Elements | $CO_2$ + modifier | 45/260 | Dynamic | Cellulose | 300 | [52] |
| Olive leaves | Phenolic compounds | $CO_2$ + ethanol | 40/15 | Static | – | 120 | [53] |
| Olive leaves | Oleuropein | $CO_2$ + methanol | 100/30 | Dynamic | Diatomaceous earth | 90 | [54] |
| Peach kernels | Fatty acids | $CO_2$ | 50/30 | Static | – | 150 | [55] |
| Pharmaceutical preparations | Piroxicam | $CO_2$ | 15/450 | Methyl-β-cyclodextrin + Static | – | 30 | [56] |
| *Psidium guajava* | Total phenols | $CO_2$ + ethanol | 50/30 | Static/4 cycles | Diatomaceous earth | 30 | [57] |
| Rosemary (*Rosmarinus officinalis*) | Phenolic compounds | $CO_2$ | 40/30 | Dynamic | – | 300 | [58] |
| Rosemary (*Rosmarinus officinalis*) | Phenolic compounds | $CO_2$ + ethanol | 40/15 | Static | – | 120 | [59] |
| *Salvia desoleana* | Sclareol | $CO_2$ | 40/25 | Dynamic | – | 240 | [60] |
| *Salvia officinalis* | Essential oil | $CO_2$ | 40/30 | Dynamic | – | 80 | [61] |
| *Schizochytrium limacinum* | Fatty acids DHA | $CO_2$ + ethanol | 40/350 | Urea complexation + static | – | 30 | [62] |
| Sea buckthorn (*Hippophae rhamnoids*) | Tocopherols, lycopene and β-carotene | $CO_2$ | 35/40 | Static | – | 60 | [63] |
| Spearmint (*Mentha spicata*) | Essential oil | $CO_2$ | 35/9 | Static | – | 30 | [64] |
| Spearmint (*Mentha spicata*) | essential oil | $CO_2$ | 50/30 | Dynamic | – | 180 | [65] |
| Spinach | Lutein | $CO_2$ + ethanol | 50/30 | Static | Cellulose | 90 | [66] |
| strawberry (*Arbutus unedo*) | Total phenolics | $CO_2$ + ethanol | 48/60 | Dynamic | – | 60 | [67] |
| Thyme (*Thymus vulgaris*) | Volatiles | $CO_2$ | 40/9 | Dynamic | Sea sand | 240 | [68] |

bioactive compounds from very different sources [5]. Table 2 shows some of the most relevant applications of SFE published in the period 2008–12.

SFE was mainly used to isolate bioactive non-polar compounds (e.g., lipids and carotenoids). Lipids were isolated from many natural sources (e.g., dairy products, oils, algae or microalgae). Normally, pressures ~10–30 MPa and temperatures ~40–50°C [5] were used. Another important application of SFE was the extraction of essential oils from plants. Essential oils have a complex composition, containing from a few dozen to several hundred constituents, especially hydrocarbons (terpenes and sesquiterpenes) and oxygenated compounds (alcohols, aldehydes, ketones, acids, phenols, oxides, lactones, acetals, ethers and esters). Besides their fragrance, the mixture of compounds confers several bioactivities (e.g., antimicrobial and antioxidant). Among the most well-known advantages of SFE towards the extraction of essential oils is the use of low temperatures that preserve the integrity of the sample. In 2007, Pourmortazavi and Hajimirsadeghi reviewed all the factors affecting the SFE of essential oils from several plants [22].

But not only non-polar bioactive compounds are extracted using supercritical fluids; as mentioned above, addition of small amounts of modifiers obtains fractions enriched in polar compound (e.g., phenolic compounds and metal-ligand complexes). The normal working pressures and temperatures are similar to those used to extract non-polar compounds, but the presence of modifiers (e.g., ethanol, methanol, water, acetone or mixtures) favors the extraction (e.g., flavonoids, lignan and simple phenolics) [5,21]. Even if it is true that SFE may provide lower yields than other conventional extraction methods (e.g., Soxhlet), SFE is faster, as reviewed by Stalikas [69].

New applications were recently developed dealing with the addition of derivatizing agents to promote extraction (e.g., bioactive, pharmaceutical compounds and metals). In these applications, the addition of the derivatizing agent helped improve the later detection of the target analytes while increasing their extraction rate by increasing the solubility of the analyte-ligand complex.

Urea complexation is a useful technique to enrich unsaturated fatty acids from mixtures. Upon crystallization, urea forms inclusion complexes with some long-chain aliphatic compounds. Saturated fatty acids form complexes readily, their formation being less efficient with increasing number of double bonds or in the presence of branched chains.

Lin et al. [70] used urea complexation to enrich the n-3 PUFA content of triglycerides (TG) in Menhaden oil under supercritical carbon dioxide. Through the saponification of Menhaden oil followed by urea inclusion, 80.1 wt% of n-3 PUFA could be concentrated, containing 29.4 wt% EPA and 41.8 wt% DHA, under the following conditions: pressure up to 10 MPa, temperature equal to 50°C, 10% of ethanol as co-solvent and 5 h conversion time. To carry out the conversion of n-3 PUFA into TG, an immobilized 1,3-regiospecific lipase was selected. Lin et al. [70] concluded that, under supercritical conditions, conversion was 40% higher than that under ambient conditions after 5 h.

Moreover, complexation in supercritical media was demonstrated to be a very useful tool for pharmaceuticals (e.g., Shinde et al. reviewed the use of cyclodextrins to prepare inclusion complexes of certain drugs [56] and showed how solubility can be enhanced about 70 times by complexation with methyl-β-cyclodextrin). Special attention must be paid to the drug, since extraction conditions greatly depend on drug structure (e.g., 15°C and 45 MPa were the conditions for piroxicam and 100°C and 10 MPa for borneol).

Complexation and extraction with supercritical fluids were also used to isolate radioactive metals; in this case, SFE with organic ligands was employed to recover actinides and other radionuclides from diverse matrices, including oxides of these elements or by-products [71]. This application requires not only significant optimization of complexation and extraction conditions, but also safe facilities {e.g., as designed by Kumar et al. [52]}.

A novel interesting trend in the application of SFE is the development of integrated processes, which can be defined as combining more than one specific unit process into a single piece of equipment [e.g., extraction can be followed by conversion (e.g., feeding SC-$CO_2$ and oil from the extractor to an enzymatic reactor for hydrolysis and/or esterification)]. The main challenge in this approach is the compatibility of the optimal operating conditions for extraction and reaction [16,43]. Different solvents other than $CO_2$ are under study in integrated processes of biocatalysis + extraction; in this sense, dimethyl ether seems to be a promising solvent, mainly for hydrophobic compounds. Some of its uses were recently reviewed by Catchpole et al. [72].

## 4. Pressurized liquid extraction (PLE)

### *4.1. Methodological and technological advances*

As already mentioned for SFE, method development in PLE also involves optimization of different factors influencing the outcome of the extraction process for each particular sample. As already stated, temperature is critically important to the extraction procedure. Theoretically, the highest temperature could provide the best results in terms of extraction yield. Nevertheless, when dealing with bioactive compounds, this parameter has to be closely examined and optimized, since it is widely known that high temperatures might have negative effects on the bioactivity of some thermo-labile compounds. Even if it has been repeatedly observed that

bioactive extracts might be obtained at high temperatures (maintaining the solvent's liquid state), this parameter should be studied and selected for each type of matrix or bioactive being extracted. However, possible formation of new components in the extracts, as a result of the occurrence of different reactions during the extraction process, has to be considered [73,74]. This point, which is clear shortcoming when the natural composition of a sample is sought, has been considered by some authors as an additional advantage in terms of the bioactivity of the achieved extracts [75]. It has been observed how, during PLE at high temperatures (135–160°C) of microalga *Chlorella vulgaris*, pyropheophytin formation increased. This chlorophyll derivative, not naturally present in the sample, might have potent antimutagenic activity, and consequently, according to Cha et al. [75], properties of PLE extracts might be enhanced by formation of this kind of component [75].

Some parameters (e.g., pressure and time) have been repeatedly reported to possess a less critical effect. Different works have pointed out the null influence of the extraction pressure beyond the point at which the solvent is maintained liquid [7,76]. Thus, in this sense, most applications devoted to the use of PLE to obtain bioactive natural products set an extraction pressure high enough to maintain the solvents in the liquid state, and the influence of extraction pressure is not further studied [26]. The influence of extraction time will directly depend on the PLE mode applied, static or dynamic. Most commercial instruments only allow static extractions, in which a certain volume of solvent, under the desired conditions of pressure and temperature, is maintained in contact with the sample for a given time. Thus, under static conditions, equilibria between those sample components still bound to the matrix and those already solubilized in the solvent might be reached. If this is the case, the efficiency of the extraction procedure will not increase beyond this point. A solution that has been widely employed to solve this shortcoming in part and to increase the total yield is sequential extractions of the same sample, using several consecutive static extraction cycles. For example, by using four consecutive extraction cycles, it was possible to increase the extraction yield of phenolic compounds from parsley, compared to an equivalent increase of the static extraction time using only one cycle [77]. However, this approach does not always provide the same results, as, in some applications, one extraction cycle is enough to extract the target compounds [78].

Under the dynamic mode, fresh solvent is continuously introduced into the extraction cell. Consequently, the equilibria might be displaced and the efficiency of the extraction procedure might be increased. This approach was shown to provide higher extraction yields than static extractions {e.g., isolation of diterpene taxanes from *Taxus canadensis* [79], and extraction of phenolic compounds from different *Pinus* species [80]}.

As for the solvent selection, although this is obviously a quite straightforward approach, care must be taken about the possible change in behavior under PLE conditions, compared to room temperature; this is especially important in the case of water, as previously mentioned.

As for the most commonly used solvents in PLE, those regarded as GRAS (e.g., ethanol, water or its mixtures) are preferred, although others more toxic and harmful (e.g., petroleum ether or dichloromethane) have also been used [81] for the extraction of very non-polar target compounds {e.g., extraction of volatiles and essential oils that are more efficiently extracted with solvents (e.g., n-hexane) [82–84]}.

However, as already mentioned, one of the most important trends in this field is the search for, and the application of, new environmentally green and food-grade solvents. In this regard, the possibility of using bioethanol [obtained as the fermentation product of glucose from renewable biomass (e.g., edible starch and non-edible cellulose)] to obtain bioactive gingerols from ginger (*Zingiber officinalis*) has been successfully explored [85]. These compounds could be efficiently recovered using 70% bioethanol at 100°C using a static extraction time of 5 min. More recently, the employment of ethyl lactate as a low polarity solvent for the extraction of γ-linolenic acid from a microalga has been also explored [17]. The ability of this food-grade solvent combined with ethanol was assessed and its capabilities for some applications were demonstrated.

Another important niche of research on this topic in the near future will be application of ILs as pressurized solvents. Already applied in other kinds of extraction {liquid-phase microextraction (LPME) [86], solid-phase microextraction (SPME) [87], liquid-liquid extraction [88], microwave-assisted extraction (MAE) [89] ultrasound-assisted extraction (UAE) [90] or, as already mentioned, SFE [30]}, ILs present some characteristics that make them potentially applicable in PLE (e.g., high solvent power, high chemical and thermal stability, non-flammability and non-volatility) [91].

Wu et al. [92] demonstrated the usefulness of pressurized ILs to extract flavonoids from *F. sophorae Inmaturus* herb. The main parameters influencing the outcome of the extraction were IL selection and concentration; optimum conditions for the recovery of rutin and quercetin from this plant included the use of a 1 mol/L concentration of [C4mim][Cl] at 120°C using a single static extraction cycle of 5 min [92].

However, much work remains to be done in this field, as there is huge variability of ILs and the information available from an extraction point of view is still scarce, as already pointed out. Besides, a potential problem that should be solved is the low volatility of these components, which would make difficult to separate the IL from

the interesting compounds after the extraction procedure.

In an effort to limit the use of organic solvents, Chang et al. [93] developed an extraction protocol based on the use of surfactants as extraction fluids under PLE conditions. This method was applied to the extraction of different flavonoids from *Costus speciosus* flowers before micellar electrokinetic chromatography (MEKC). This surfactant-assisted, pressurized extraction was carried out with SDS and Triton X-100 during 30 min under pressures of 2–3 MPa. Although Chang et al. [93] did not study the influence of the extraction temperature, this approach could be further developed by combining different surfactants and solvents in order to increase the efficiency of extraction of bioactives.

However, analyte recovery in PLE is not as critical as it is in SFE, since most automatic systems available in the market recover the solutes in solution in a closed vial, thus minimizing solute losses.

### *4.2. Applications*

Although the majority of PLE applications developed so far are aimed at the extraction of contaminants from different natural, food and environmental samples, this technique has also demonstrated its usefulness for the extraction of bioactive compounds from natural matrices.

Table 3 shows some of the most relevant PLE applications (published during the period 2008–12) devoted to the extraction of bioactives. As can be observed, plants are, by far, the samples most frequently studied, although different papers have been published dealing with the extraction of bioactive compounds from algae and microalgae, and other natural matrices. PLE has mostly been used to obtain antioxidants (e.g., phenolic compounds and carotenoids).

Generally, commercial instruments work in static extraction mode and employ extraction times up to 20 min. It is also common to find applications in which the sample is re-extracted several consecutive times, in order to extract the target compounds fully. This strategy was shown to be useful in performing three consecutive static extraction cycles {e.g., anthocyanins from strawberry [95], sweet potato [102] and grape skins [112], phenolic acids from food-industry byproducts [96], flavonoids from spinach [118] or alkaloids [128]}.

As can be observed in Table 3, temperatures of 100–160°C have been widely applied to extract natural bioactives. Nevertheless, the use of higher temperatures should not be ruled out. PLE with ethanol at 200°C was shown to be the most appropriate process to obtain extracts with the highest antioxidant activity from rosemary compared to other pressurized extraction methods including SWE and SFE [77].

PLE has been widely compared to other extraction techniques {e.g., maceration, UAE [75], solvent partitioning [133], or Soxhlet extraction [85]} for extraction of bioactive compounds, and provides better results than these more conventional techniques. Advantages associated with PLE are mainly attainment of higher extraction efficiencies, involving significantly less volumes of solvents, less total extraction time and helping automation of the process.

In terms of automation, one important trend is development of new systems and approaches to couple PLE to other processes on-line {e.g., UAE, PLE and SPE may be combined to increase the extraction efficiency and the purity of the compounds desired in the extracts obtained [134]}. Although there have been some off-line couplings, in-line use remains to be fully explored. Similarly, in-cell enzyme pre-treatments prior to extraction might be a good option to increase the extraction yield of particular compounds, thanks to the release of components from the natural matrix being extracted. Although this approach might be best suited to water extractions, the employment of PLE with hydroalcoholic solutions, e.g., cannot be rejected. This strategy has been followed in research fields, other than extraction of bioactive natural products, to speed up enzymatic hydrolysis [135]. The possibility of using in-cell SPE materials to retain the compound of interest or the interferences can facilitate the isolation of the target compounds, since it includes a new element of selectivity in the system. This approach can even be improved by using specifically-designed materials [e.g., molecularly-imprinted polymers (MIPs) or restricted access materials (RAMs)] that can fit only the molecule of interest; although at present no applications can be found in isolating bioactives, we expect it to be a growing field of research in the near future. Special devices have been designed for this purpose and are described elsewhere [136]; they are mainly based on the design of modular approaches and cell assemblies for simultaneous extraction and clean-up of different types of sample, including extraction of bioactive compounds. As mentioned above, different types of approach (trap and release, class separation, dual-mode clean-up or matrix retainer and trap) can bear in mind the idea of isolating and purifying bioactive compounds.

## 5. Subcritical water extraction (SWE)

### *5.1. Methodological and technological advances*

Although SWE might be seen just as a variation of PLE, the use of water as extraction solvent is highly relevant from an environmental point of view, since it is considered the greenest solvent that can be used in an extraction process. Water has essentially negligible environmental effect, since it is non-toxic to health and the environment and it is safe to work with. For this reason, it is common to refer to SWE independently.

**Table 3.** The most remarkable PLE applications devoted to extraction of natural bioactives published in the period 2008–12

| Product | Compounds of interest | Solvent | T (°C)/P (MPa) | Mode/Cycles | Sample dispersion | Extraction time (min) | Ref. |
|---|---|---|---|---|---|---|---|
| **Phenolic compounds** | | | | | | | |
| *Hylocereus undatus* flowers | Flavonoids | Methanol | 120/10 | Static/1 | Diatomaceous earth | 15 | [94] |
| Strawberry | Anthocyanins | Methanol/water/formic acid 80:19:1 | 40/n.i. | Static/3 | Diatomaceous earth | | [95] |
| Potato wastes | Phenolic acids | methanol/water 90:10 | 160/10 | Static/3 | Ottawa sand | 5 | [96] |
| Oak wood | Phenolic compounds | Methanol | 150/10 | Static/1 | Diatomaceous earth | 5 | [97] |
| Olive leaves | Oleuropein | Ethanol | 115/10 | Static/2 | | 10 | [98] |
| Olive leaves | Phenolics | Ethanol | 150/10 | Static/1 | Sea sand | 20 | [99] |
| Cinnamon and peppermint | Phenolic compounds | 75% acetone | 100/10 | Static/3 | Diatomaceous earth | 10 | [100] |
| Wheat products | Alkylresorcinols | 1-propanol/water 3:1 | 100/10 | Static/3 | Ottawa sand | 10 | [101] |
| Sweet potato | Anthocyanins | 75% acidified methanol | 80/15 | Static/3 | Sea sand | 5 | [102] |
| *Heracleum leskowii* | Coumarins | Methanol | 110/n.i. | Static/1 | – | 10 | [81] |
| *Fructus schisandrae* | Lignans | Ethanol | 160/15 | Static/1 | – | 10 | [103] |
| *Citrus reticulata* | Flavones | 70% methanol | 160/10 | Static/1 | – | 20 | [104] |
| *Myrciaria cauliflora* skins | Anthocyanins | Ethanol | 80/5 | Static/1 | – | 9 | [105] |
| Apricots | Polyphenols | Methanol/water 70:30 | 60/10 | Static/1 | – | 60 | [106] |
| Rosemary | Phenolic compounds | Ethanol | 150/10 | Static/1 | Sea sand | 20 | [107] |
| Olive leaves | Phenolic compounds | Ethanol | 150/10 | Static/1 | Sea sand | 20 | [108] |
| Oregano | Phenolic compounds | Ethanol | 200/10 | Static/1 | Sea sand | 20 | [109] |
| Honey | Phenolics | Ethanol/water/HCl 70:25:5 | 40/10 | Static/3 | – | 15 | [110] |
| Rosemary, marjoram | Phenolic components | 56% methanol | 129/10 | Static/1 | Diatomaceous earth | 5 | [111] |
| Grape skin | Anthocyanins | Ethanol | 100/15 | Static/3 | Glass beads | 5 | [112] |
| Different plants | | 40% Ethanol | 50/13 | Static/2 | Sea sand | 10 | [113] |
| Red grape pomace | Procyanidins | 50% Ethanol | 80–140/6.8 | Static/1 | Sea sand | 10 | [114] |
| Microalgae | Phenolic acids | 80% methanol | 130/13 | Static/2 | – | 10 | [115] |
| Apple | Phenolic antioxidants | 60% ethanol | 102/10 | Static/1 | Diatomaceous earth | 5 | [116] |
| Onion | Flavonoids | 60% methanol | 40/10 | Static/2 | Ottawa sand | 5 | [117] |
| Spinach | Flavonoids | 70% ethanol | 150/13 | Static/3 | Ottawa sand | 5 | [118] |
| Parsley | Phenolic compounds | 50% Ethanol | 160/10 | Static/4 | Ottawa sand | 5 | [77] |
| | | | | | – | | |
| **Carotenoids and tocopherols** | | | | | | | |
| *Chlorella vulgaris* | Carotenoids | Ethanol | 150/15 | Static/1 | Sea sand | 20 | [119] |
| Carrot wastes | Carotenoids | Ethanol | 60/5 | Static/2 | – | 5 | [120] |
| *Eisenia bicyclis* | Fucoxanthin | 90% Ethanol | 110/10 | Static/1 | Sea sand | 5 | [121] |
| *Chlorella vulgaris* | Lutein | 90% Ethanol | 160/10 | Static/1 | – | n.i. | [75] |
| *Haematococcus pluvialis* | Astaxanthin | Ethanol | 100/10 | Static/1 | Sea sand | 20 | [122] |
| Shrimp waste | Astaxanthin | Acidified ethanol | 87/4.9 | Static/1 | – | 14 | [123] |
| *Chlorella elipsoidea* | Zeaxanthin | Ethanol | 115/10 | Static/1 | – | 23.3 | [124] |
| *Phormidium* | Carotenoids | Ethanol | 150/10 | Static/1 | Sea sand | 20 | [125] |
| Sterols | | | | | | | |
| *Himathalia elongata* | Fucosterol | Ethanol | 100/10 | Static/1 | Sea sand | 20 | [126] |
| Seeds and nuts | β-sitosterol | Methanol | 50/11 | Static/2 | Hydromatrix celite | 5 | [127] |
| Alkaloids | | | | | | | |
| *Macleaya microcarpa* | Quaternary benzo[c] phenanthridine | Ethanol | 80/15 | Static/3 | Glass beads | 10 | [128] |
| *Bupleurum falcatum* roots | Saikosaponins | 70% methanol | 120/10 | Static/1 | Diatomaceous earth | 10 | [129] |

(*continued on next page*)

**Table 3.** (*Continued*)

| Product | Compounds of interest | Solvent | T (°C)/P (MPa) | Mode/Cycles | Sample dispersion | Extraction time (min) | Ref. |
|---|---|---|---|---|---|---|---|
| **Essential oils** | | | | | | | |
| Turmeric | Volatiles | n-hexane | 147/7 | Static/1 | Diatomaceous earth | 17 | [82] |
| Thyme | Essential oil | n-hexane | 100/6 | Static/1 | Neutral glass | 10 | [83] |
| Other food and natural bioactive components | | | | | | | |
| Grape seed oil | Vitamin E | Hexane | 100/10 | Static/3 | – | 30 | [84] |
| Fermented red rice | Monacolin K | Ethyl lactate | 120/10 | Static/3 | – | 7 | [130] |
| Radix Saposhnikoviae | Chromones | 50% ethanol | 140/10 | Static/1 | – | 8 | [131] |
| Cereals | Tocopherols and tocotrienols | Methanol, acetonitrile | 50/11 | Static/1 | Hydromatrix celite | 5 | [132] |

Generally, one of the major limitations of SWE is the low water solubility of certain compounds and the instability of some of them and/or matrices towards elevated temperatures. Some authors have investigated the use of modifiers or additives to improve the extraction yields {e.g., Mukhopadhyay and Panja carried out the extraction of high amounts of natural sweeteners from licorice by SWE with 0.01% w/v ammonia [137] and Arapitsas and Turner [138] developed a fast extraction method of anthocyanins from red cabbage using pressurized hot water containing 5% of ethanol [138]}. Other authors have studied how to extend the applicability of SWE to compounds exhibiting limited water solubility by pH control {e.g., Euterpio et al. employed SWE for extracting curcumin from turmeric rhizomes by adjusting the pH of the water [139]}.

Regarding the type of extraction, the most frequent method used in SWE is static mode, in which equilibrium is reached between the sample components and the water phase (in which the components are solubilized). This implies that a careful optimization of the static extraction time is of outmost importance. For the extraction of bioactive compounds from natural sources, short static times (5–20 min) are generally applied. When dynamic mode is used, heated, pressurized water flows into the extraction cell continuously. This is theoretically more favorable for complete extraction, but extracts may be diluted, and more costly procedures will have to be applied to remove the water. A new online process has therefore been developed involving SWE plus drying the extracts in a single step [140]. This methodology (called WEPO) combines a continuous flow of water through the sample with the continuous production of an aerosol from the extract assisted by the sc-$CO_2$ nebulization system, which is instantaneously dried by a hot current of $N_2$. The applicability of this process to obtain dried extract in one step was recently demonstrated in the extraction of bioactive compounds with high antioxidant activity from rosemary leaves [12] and fresh onion [141]. Moreover, its greenness was recently assessed, in comparison to SFE and PLE, by using life-cycle assessment (LCA) [142]. This work used such tools to assess the environmental performance of an extraction method to realize which are the main bottlenecks associated with a particular process.

From an environmental point of view, the combination of SWE and enzymatic hydrolysis using a thermostable beta-glucosidase to catalyze hydrolysis of quercetin glucosides in onion waste was demonstrated to be a viable process preferred (in terms of primary energy consumption and global warming) over more conventional extraction based on methanol extraction and hydrochloric acid hydrolysis [143,144].

**Table 4.** Remarkable SWE applications to obtain bioactive compounds from natural sources published in the period 2008–12

| Product | Compounds of interest | Solvent | T (°C)/P (MPa) | Mode | Sample dispersion | Extraction time (min) | Ref. |
|---|---|---|---|---|---|---|---|
| Liquorice roots | Mono-ammonium glycyrrhizate (MAG) | Water containing 0.01% ammonia | 110/0.5 | Static/1 cycle (stirring at 350 rpm) | n.i | 90 | [137] |
| Red cabbage | Anthocyanins | Water/ethanol/formic acid (94:5:1 v/v/v) | 99/5 | Static/1 cycle | n.i | 2 | [138] |
| Turmeric rhizomes | Curcumin | Phosphate-buffered water at pH 1.6 | 197/5 | Dynamic/0.5 mL/min | Sea sand | | [139] |
| Rosemary | Phenolic compounds | Water | 200/10 | Static/1 cycle | Sea sand | 20 | [12] |
| | | Water | 200/n.i | Dynamic/0.2 ml/min | Sea sand | 20 | |
| Onion | Flavonoids | Water | 120/8 | Dynamic/0.3 ml/min | n.i | 45 | [141] |
| Rosemary | Phenolic compounds | Water | 200/8 | Dynamic/0.2 ml/min | Sea sand | 40 | [142] |
| Grape pomace | Phenolic compounds/ flavonoids | Water | 140/11.6 | Static (1 cycle) + Dynamic (1-2 ml/min) | n.i | 30 + 100 | [145] |
| Grape Skin | Phenolic compounds | Water | 120/15 | Static/3 cycles | Glass beads | 5 | [146] |
| Seabuckthorn leaves | Phenolic compounds/ flavonoids | Water | 150/10 | Static/1 cycle | n.i | 15 | [147] |
| Pomegranate seed | Phenolic compounds | Water | 220/6 | Static/1 cycle (stirring at 120 rpm) | n.i | 30 | [148] |
| Rice brand | Phenolic compounds | Water | 175/2 | Static/1 cycle | n.i | 5 | [149] |
| Olive leaves | Phenolic compounds | Water | 200/10 | Static/1 | Sea sand | 20 | [108] |
| Citrus unshiu peel | Flavonoids | Water | 160/10 | Static/1 | Diatomaceous earth | 10 | [150] |
| Bitter melon | Phenolic compounds | Water | 200/10 | Dynamic/2 ml/min | n.i | 120 | [151] |
| Onion skin | Flavonoids | Water | 165/10 | Static/1 | Diatomaceous earth | 15 | [152] |
| Hop (Humulus lupulus) | Prenylflavonoids | Water | 150/10 | Static/6 | Sea sand | 5 | [153] |
| Chlorella vulgaris | Carotenoids/PUFAs | Water | 200/10 | Static/1 | Sea sand | 20 | [119] |
| Haematococcus pluvialis | Fatty acids/vitamin E/phenols | Water | 200/10 | Static/1 | Sea sand | 20 | [154] |
| Himanthalia elongata | Polysaccharides | Water | 100/10 | Static/1 | Sea sand | 20 | [126] |
| Haematococcus pluvialis | Polysaccharides | Water | 100/10 | Static/1 | Sea sand | 20 | [155] |
| Dunaliella salina. | | | 160/10 | | | 15 | |
| Microalgae, algae, plants | Neoantioxidants | Water | 100 or 20/10 | Static/1 | Sea sand | 20 | [156] |
| Glycation model system | Neoantioxidants | Water | 200/10 | Static/1 | | 20 | [73] |

*n.i: no indicated; PUFAs: Polyunsaturated fatty acids.

### 5.2. Applications

The potential of SWE to extract bioactive compounds from natural matrices (e.g., plants or algae) has already been demonstrated. Table 4 shows a selection of some of the most remarkable SWE applications to obtain bioactive compounds from natural sources published in the period 2008–12. As can be seen, quite a large number of recent works described in the literature were applied to the extraction of antioxidants {e.g., from rosemary [12], grape pomace [145], grape skin [146], seabuckthorn leaves [147], pomegranate seed [148], rice bran [149], olive leaves [108], *Citrus unshiu* peel [150], bitter melon [151] or onion skin [152]}. Not only antioxidant compounds have been extracted by SWE but also other bioactive compounds possessing different activities {e.g., SWE has obtained extracts with anti-inflammatory properties from hops (*Humulus lupulus*) [153], or with antimicrobial [119,154] or antiviral activity [126,155] from algae}. In addition, SWE has been employed as a final step to separate dietary fibers from the residue obtained by supercritical $CO_2$ of citrus, demonstrating the importance of sequential methodologies for the recovery of valuable compounds from citrus-fruit waste [157].

Recently, the possibility to obtain antioxidant compounds in SWE extracts not naturally present in the original matrix as consequence of using high temperature was demonstrated [73,156] in both glycation-model systems and real samples. In both cases, neoformed antioxidants derived from Maillard or caramelization reactions were produced during SWE, which could increase interest in the bioactive extract obtained, although it undoubtedly modified the natural profile of bioactive compounds in the sample.

## 6. Conclusions

At present, with the significant introduction of mass spectrometry (MS) into all laboratories, the requirements for sample preparation have changed considerably. Since modern MS, combined with LC and/or GC, is able to provide higher sensitivities and selectivities, sample-preparation techniques have become simpler (e.g., there is no great need for enrichment or exhaustive clean-up, since both sensitivity and selectivity of the new systems have increased enormously).

However, when dealing with research into bioactive compounds linked to natural products, biomedicine, food science, and –omics technologies, several clues have to be considered to optimize the sample preparation step:

(1) we are dealing with natural matrixes, all of them very complex samples;
(2) most of the analytes are non-volatile or semi-volatile and the matrixes include solid and liquid samples;
(3) most of the bioactive compounds are present in low concentrations in the samples; and,
(4) in most cases, information is needed for a further processing of the natural matrix in order to extract the compound/s of interest.

With all this information, it is possible to understand the (still) important role of sample preparation in the extraction of bioactives. We have a long way to go to be able to achieve the degree of simplicity that is undoubtedly observed in other areas of analytical chemistry in terms of sample preparation.

For bioactive compound extraction, the first step is therefore usually a solvent extraction to enrich the target bioactive compounds from the complex matrix (e.g., plant, by-product, or marine source). In the present manuscript, we have discussed the advantages of some environmentally-friendly techniques [e.g., SFE, PLE, and SWE as alternatives to more conventional extraction techniques (e.g., Soxhlet)]. All of these techniques have in common the dramatic reduction in the amounts of solvents used, since other physical processes (e.g., pressure and temperature) are applied to improve the efficiency and to speed up the extraction process. These techniques allow the reduction of energy consumption and of the amounts of waste generated, and they can be easily miniaturized and automated, thus increasing throughput. They can also constitute the first step towards a larger scale production process, often so necessary in this type of research. All of these techniques can be seen as ''green pressurized sample-preparation'' techniques working with green (or food-grade) solvents.

Technological and methodological advances presented, even separated by techniques, can be understood from the need to develop more selective sample-preparation procedures that achieve better clean-up (from very complex samples) and improve analysis at lower concentrations (typical of bioactive components). In this manuscript, we presented several general approaches that can be applied to all techniques using compressed fluids:

(1) different designs (in-cell) for improving clean-up or selectivity of the extraction process; more selective solvents (ILs);
(2) cleaner and greener solvents (ethyl lactate);
(3) integrated processes (use of EAE plus pressurized fluids for more selective extraction of the compounds of interest and susceptible to being enlarged to a pilot-scale process); and,
(4) *in situ* derivatization plus extraction for greater requirements in terms of sensitivity (or lower detection limits).

We therefore think there is a huge area of research in front of us that can contribute to the development of new methodologies for higher throughput, while complying with the rules of GAC, and also provide with new tools

for the future development of more sustainable, clean industrial processes towards extraction of bioactive compounds from natural sources.

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