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Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae
A review

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Abstract

The increasing interest of consumers in functional foods has brought about a rise in demand for functional ingredients obtained using ''natural'' processes. In this review, new environmentally clean technologies for producing natural food ingredients are discussed. This work provides an updated overview on the principal applications of two clean processes, supercritical fluid extraction and subcritical water extraction, used to isolate natural products from different raw materials, such as plants, food by-products, algae and microalgae. Although the extraction of some compounds with antibacterial, antiviral or antifungical activity is discussed, special attention is paid to the extraction of antioxidant compounds, due to their important role in food preservation and health promotion.

Keywords: Algae; Antioxidant; Food by-products; Functional foods; Microalgae; Nutraceuticals; Subcritical water extraction; Supercritical fluid extraction; Plants

1. Introduction

In recent years, there has been a growing interest in so-called functional foods because they can provide physiological benefits additional to nutritional and energetic, as, for instance, antihypertensive, antioxidant or anti-inflammatory (Goldberg, 1996). A functional food can be defined as a food that produces a beneficial effect in one or more physiological functions, increases the welfare and/or decreases the risk of suffering a particular disease. Furthermore, new types of products, derived from food, called nutraceuticals have recently been developed. These products, usually employed as food supplements, are marketed as tablets and pills, and can provide important health benefits.

Frequently, functional foods are obtained from traditional foods enriched with an ingredient able to provide or promote a beneficial action for human health. These are the so-called functional ingredients. These ingredients are preferred by consumers to have a natural origin (i.e. non-synthetic origin) being commonly extracted from natural sources, such as plants, food by-products or even algae and microalgae. These types of marine sources are receiving much attention, mainly because of their contents of functional ingredients, such as polyunsaturated fatty acids (Cohen & Vonshak, 1991; Mahajan & Kamat, 1995), β-carotene and other pigments (antioxidants) (Bhat & Madyastha, 2000;

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Madhava et al., 2000), sulphated polysaccharides (antiviral), and sterols (antimicrobials). (Borowitzka & Borowitzka, 1988; Ötles & Pire, 2001; Xue et al., 2002).

Among the different compounds with functional properties, antioxidants are the most widely studied (Bhat & Madyastha, 2000; Piñero-Estrada, Bermejo Bascós, & Villar del Fresno, 2001). These compounds can play an important role in food technology because of their usefulness against lipid peroxidation. Usually, food production, process and storage can generate important losses of endogenous antioxidants that limit their own protection against lipid oxidation. Moreover, the important role of antioxidants in human health has been demonstrated, thus increasing the interest in such products and their demand by consumers (Borowitzka & Borowitzka, 1988).

The traditional extraction methods used to obtain these type of products have several drawbacks; they are time consuming, laborious, have low selectivity and/or low extraction yields. Moreover, these traditional techniques employ large amounts of toxic solvents. At present, extraction methods able to overcome the above-mentioned drawbacks are being studied; among them, supercritical fluid extraction (SFE) and subcritical water extraction (SWE) are among the more promising processes (King, 2000). These extraction techniques provide higher selectivities, shorter extraction times and do not use toxic organic solvents.

The goal of this review is, therefore, to provide an updated overview of the principal applications of two environmentally safe technologies, SFE and SWE, for obtaining functional ingredients from natural sources, such as plants, by-products from the food industry, algae and microalgae.

2. Supercritical fluid extraction

2.1. Principles and instrumentation

When a fluid is forced to a pressure and temperature above its critical point (see Fig. 1), it becomes a supercritical fluid. Under these conditions, various properties of the fluid are placed between those of a gas and those of a liquid. Although the density of a supercritical fluid is similar to a liquid and its viscosity is similar to a gas, its diffusivity is intermediate between the two states, as can be seen in Table 1. Thus, the supercritical state of a fluid has been defined as a state in which liquid and gas are indistinguishable from each other, or as a state in which the fluid is compressible (i.e. similar behaviour to a gas) even though possessing a density similar of a liquid and, therefore, similar solvating power.

Because of its different physicochemical properties, SFE provides several operational advantages over traditional extraction methods (Anklam, Berg, Mathiasson, Sharman, & Ulberth, 1998). Due to their low viscosity and relatively high diffusivity, supercritical fluids have better transport properties than liquids, can diffuse easily through solid materials and can therefore give faster extraction yields. One of the main characteristics of a supercritical fluid is the possibility of modifying the density of the fluid by changing its pressure and/or its temperature. Since density is directly related to solubility (Del Valle & Aguilera, 1999; Raventós, Duarte, & Alarcón, 2002), by altering the extraction pressure, the solvent strength of the fluid can be modified. Other advantages, compared to other extraction techniques, are the use of solvents generally recognized as safe (GRAS), the higher efficiency of the extraction process (in terms of increasing yields and lower extraction times), and the possibility of direct coupling with analytical chromatographic techniques such as gas chromatography (GC) or supercritical fluid chromatography (SFC).

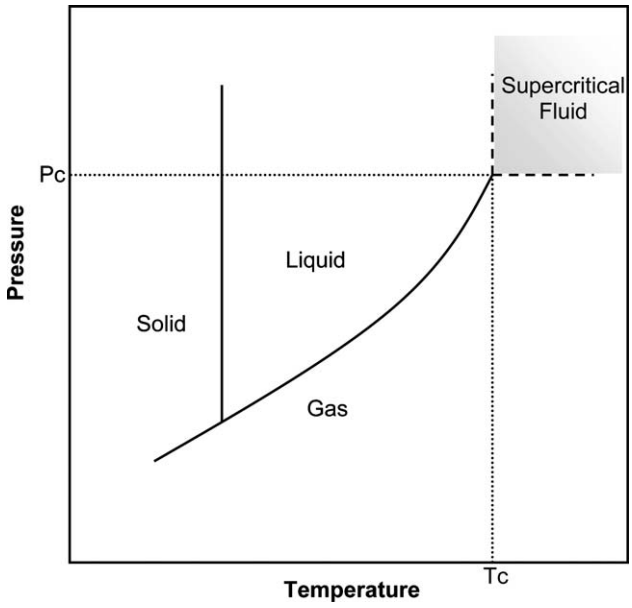

Fig. 1. Typical phase diagram for a pure compound.

Table 1
Range values of several physicochemical properties of gases, liquids and supercritical fluids

State of fluid	Density (ρ, g/cm^3)	Diffusivity (D_{AB}, cm^2/s)	Viscosity (μ, g s/cm)
Gas			
$p = 1$ atm; $T = 21$ °C	10^{-3}	10^{-1}	10^{-4}
Liquid			
$p = 1$ atm; $T = 15$–30 °C	1	$<10^{-5}$	10^{-2}
Supercritical			
$p = p_c$; $T = T_c$	0.3–0.8	10^{-3}–10^{-4}	10^{-4}–10^{-3}

Table 2
Critical properties of several solvents used in SFE

Solvent	Critical property			
	Temperature (°C)	Pressure (atm)	Density (g/ml)	Solubility parameter δ_{SFC} ($cal^{-1/2}cm^{-3/2}$)
Ethene	10.1	50.5	0.200	5.8
Water	101.1	217.6	0.322	13.5
Methanol	−34.4	79.9	0.272	8.9
Carbon dioxide	31.2	72.9	0.470	7.5
Ethane	32.4	48.2	0.200	5.8
Nitrous oxide	36.7	71.7	0.460	7.2
Sulfur hexafluoride	45.8	37.7	0.730	5.5
n-Butene	−139.9	36.0	0.221	5.2
n-Pentane	−76.5	33.3	0.237	5.1

As for the solvents, there is a wide range of compounds that can be used as supercritical fluids (see Table 2 where critical properties of several solvents used in SFE are given); carbon dioxide is the most commonly used because of its moderate critical temperature (31.3 °C) and pressure (72.9 atm). Carbon dioxide is a gas at room temperature, so once the extraction is completed, and the system decompressed, a substantial elimination of CO_2 is achieved without residues, yielding a solvent-free extract. On an industrial scale, when carbon dioxide consumption is high, the operation can be controlled to recycle it. However, supercritical CO_2, because of its low polarity (as can be seen in Table 2), where solubility parameter, δ, is shown that provides a measurement of the solvent polarity (Hildebrand & Scott, 1958), is less effective in extracting more polar compounds from natural matrices. To overcome this problem, modifiers (also called co-solvents) are commonly used. Modifiers are highly polar compounds that, added in small amounts, can produce substantial changes of the solvent properties of neat supercritical CO_2 (Valcárcel & Tena, 1997).

According to the specific requirements, the design of a supercritical fluid extraction system can be relatively simple or highly complex. Basically, it is possible to differentiate between analytical instruments and preparative systems (pilot or industrial scale). The analytical systems are utilized in sample preparation prior to, for example, a chromatographic analysis, to obtain milligrammes to grammes of extracts. There are several configurations, depending on their degree of automation. The preparative systems are used to extract grammes of compounds when working on a pilot scale or kilogrammes on an industrial scale. In these preparative systems, two different configurations can be found: for processing solid or liquid samples.

Basically, a preparative system on a pilot scale plant (see Fig. 2) consists of a solvent pump, that delivers the fluid throughout the system, a modifier pump if necessary, an extraction cell or extraction column, according to the system configuration (that is, for solids or liquids, respectively), and one or more separators (also called fractionation cells) in which the extract is collected and the solvent depressurized. Likewise, the extraction cell or column and the separators are commonly equipped with independent control of temperature and pressure, in such a way that fractionation of the extracted compounds can be carried out by a stepwise depressurization. Therefore, different compounds can be obtained within each separator, depending on their differential solubility in the supercritical fluid. Additionally, it is possible to install a refrigerated system especially designed to trap the most volatile compounds, as well as a recycling system to recycle the fluid employed.

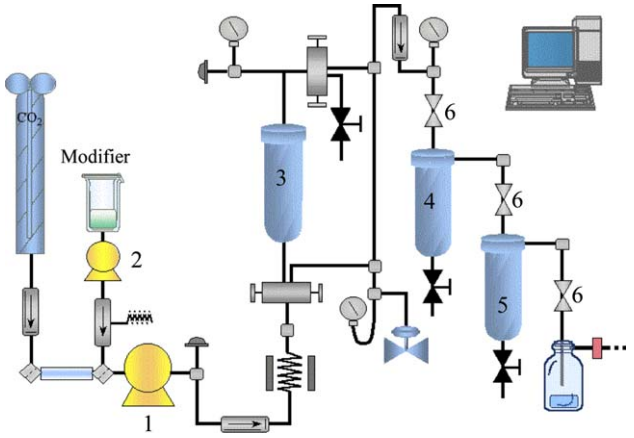

Fig. 2. Diagram of a supercritical fluid extraction pilot plant equipped with two fractionation cells. (1) CO_2 pump; (2) modifier pump; (3) solid samples extraction cell; (4) fractionation cell 1; (5) fractionation cell 2; (6) valve.

As has been mentioned, the most important difference between pilot plants to process solid or liquid samples is in the use of an extraction cell or an extraction column. Solid processing is always done in batch in a discontinuous or semi-continuous process, while liquid processing is usually carried out under countercurrent conditions in a continuous mode. In liquid-sample extractions, the supercritical fluid (usually CO_2) is moving upwards while the sample feed, introduced into the system from the top or centre of the column, is moving downwards by gravity.

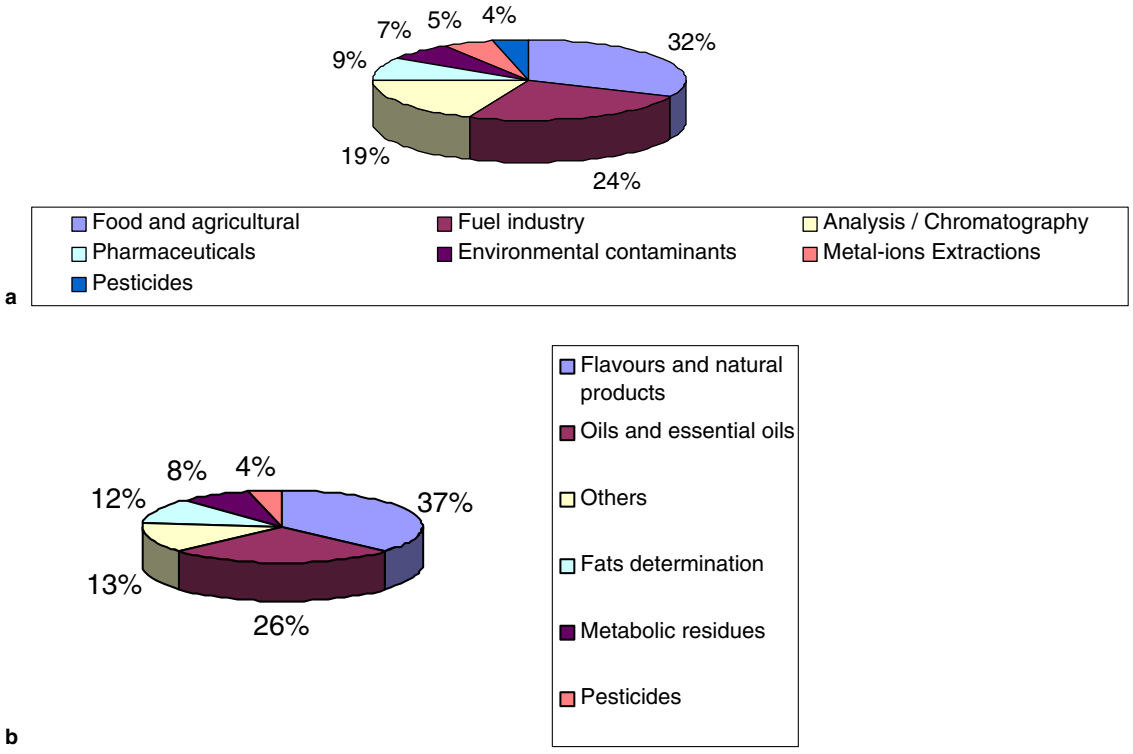

Fig. 3. (a) Graphical representation of the results obtained of a literature search in *Current Contents* database, using "supercritical fluid" and "carbon dioxide" as search parameters, between 1999 and 2000 Rozzi et al. (2002). (b) Graphical representation of SFE applications in food science and technology based on the literature search in *Food Science and Technology Abstracts (FSTA)* database, using "supercritical and fluid" as search parameters between 2001 and 2003.

Fig. 3a shows the general distribution, according to Rozzi and Singh (2002), of the development done with supercritical fluids in different fields from 1999 to 2000 according to the *Current Contents database* (http://go5.isiknowledge.com/portal.cgi). As can be seen in Fig. 3a, the main field of application for supercritical fluids is food and agriculture (32%), followed by the fuel industry (24%). On the other hand, Fig. 3b shows also the number of papers published in the "Food Science and Technology" field between 2001 and 2003 (using in this case the *Food Science and Technology Abstracts (FSTA) database*). As can be seen in Fig. 3b, flavours and natural products (37%) followed by oils and essential oils (26%) are the two main fields of application for supercritical fluids.

2.2. Plants as natural sources of functional ingredients

Numerous vegetable matrices have been used as natural sources for compressed fluid extraction. Legumes, spices, aromatic plants, and even fruit beverages, such as natural orange juice (Señorans et al., 2001) have been processed to obtain natural antioxidant compounds.

Several studies have compared the antioxidant activities of plant extracts obtained using supercritical fluid extraction with those using traditional extraction methods. For example, eucalyptus (Fadel, Marx, El-Sawy, & El-Gorab, 1999) and *Lippia alba* (Stashenko, Jaramillo, & Martínez, 2004) have been used to compare different extraction methods in terms of composition and activity. The research performed with eucalyptus demonstrated the differences that exist between the composition and functional properties of extracts obtained with SFE (using supercritical carbon dioxide at 200 bar and 50 °C) and hydrodistillation. Antioxidant activity was found to be greater in supercritical fluid extracts than in hydrodistillation extracts.

In spite of the fact that the main compounds were the same in the studied extracts, their quantitative composition changes. For example, supercritical fluid extracts have a higher content of sesquiterpenes and oxygenated compounds. As for the antioxidant activity, this seems to be related to the concentration of both, *p*-cymen-7-ol and thymol in the extract. The concentrations of both compounds, mainly the amount of *p*-cymen-7-ol, in SFE extracts, was higher than that found in hydrodistillation extracts. Those values were in agreement with the higher antioxidant activity found in supercritical extracts compared to extracts from hydrodistillation.

A more exhaustive comparison was carried out by Stashenko et al. (2004) with extracts obtained from *L. alba*. In this study, 40 compounds were identified

and quantified from extracts obtained using hydrodistillation, microwave-assisted hydrodistillation and supercritical fluid (carbon dioxide) extraction. The results showed both quantitative and qualitative differences among the extracts. The most complex extracts, in terms of amount of isolated compounds, were those obtained with SFE. Likewise, significantly more sesquiterpenes were quantified in the supercritical fluid (SF) extracts than in those obtained with the other extraction techniques tested. However, the opposite was found for the amount of monoterpenoids detected in the SF-extract.

Among the studies performed with leguminosae as natural sources of antioxidant compounds, those using Tamarind (*Tamarindus indica* L.) proved of greatest interest. Tsuda, Mizuno, Ohshima, Kawakishi, and Osawa (1995) studied the antioxidant activity of extracts obtained from tamarind seed coat using SFE (with CO_2) under different conditions. Results showed that the antioxidant activity of the extracts increased when the extraction pressure and temperature were raised. The addition of a co-solvent, suitable for the food industry (ethanol), on the extraction process was also studied in order to show the influence of polar compounds on the final antioxidant activity of the extracts. In either case, the addition of 10% modifier (v/v) increased the antioxidant activity of the extracts. Luegthanaphol et al. (2004) in their research on Tamarind, established a comparison between SF extracts and extracts obtained using solvent extraction (ethyl acetate and ethanol). The results of this study showed that the antioxidant activity of ethanol extracts was better than that of SF extracts.

For other matrices, supercritical fluid extraction is considered the most suitable method for producing fractions with high antioxidant activity. One example is the extraction of coriander (*Coriander sativum*). Yépez, Espinosa, López, and Bolaños (2002) demonstrated the possibility of obtaining odourless and flavourless extracts from coriander with high antioxidant activity, using supercritical fluid extraction with CO_2. Extraction under moderate conditions (45 °C and 177 bar), with CO_2 densities close to 0.74 g/ml, provided extracts with high antioxidant activity and yields. Moreover, Esquível, Ribeiro, and Bernardo-Gil (1999) showed that low temperatures and pressures were sufficient for concentrating antioxidant compounds from savory oil (*Satureja hortensis*). In this study, a constant temperature extraction (40 °C) was maintained while changing pressures. The most suitable pressure was 120 bar; higher pressures did not provide any improvement in the recovery of antioxidants. Depressurization was carried out using three collectors. Likewise, Esquível et al. (1999) suggested that it would be possible to isolate carvacol (the main component of savory oil) by optimizing the separation parameters.

Aromatic plants are among the most studied plants with antioxidant activity. Ribeiro, Bernardo-Gil, and Esquível (2001) studied the antioxidant activity of supercritical extracts from lemon balm (*Melissa officinalis L.*). The plant material was subjected to pressures from 100 to 180 bar and temperatures from 35 to 40 °C. However, in this case, the solid residues were analyzed to determine their antioxidant activity (instead of on the supercritical carbon dioxide extracts). The results obtained showed that the best antioxidant activity was achieved by optimizing the extraction conditions at 100 bar and 35 °C for 4 h.

Ginger (*Zingiber officinale* Roscoe) is another aromatic plant which has been widely studied due to its multiple functional activity. Zancan, Marques, Petenate, and Meireles (2002) carried out a study to prove the effect of temperature and pressure, as well as the addition of a co-solvent, on the kinetics of ginger extraction and extract antioxidant activity. By means of an experimental factorial design it was concluded that the addition of a co-solvent was not necessary in order to increase the mass transfer rate or yield. The factors selected to carry out the factorial design were: extraction temperature (25–35 °C), extraction pressure (200–250 bar) and solvents (namely CO_2, CO_2 + ethanol, CO_2 + isopropyl alcohol). The best results, in terms of antioxidant activity, were obtained when the extraction was carried out with a modifier at low temperatures, low pressures and relatively long extraction times; the antioxidant activity seemed to be related to the preferential extraction of gingerols and shogaols. A recent study has compared this aromatic plant with similar plants, such as rosemary (*Rosmarinus officinialis* L.) and turmeric (*Curcuma longa* L.) (Leal et al., 2003). Extracts of these three plants were obtained using supercritical carbon dioxide with/without modifier (ethanol and/or isopropyl alcohol). Experiments were carried out at pressures between 100 and 300 bar and temperatures between 30 and 40 °C. Once the antioxidant activity assays were performed, it was concluded that the extracts which lowered antioxidant activity were those from turmeric and ginger, whereas rosemary extracts provided higher values of antioxidant activity.

Rosemary is one of the plants with higher antioxidant activity and is therefore, frequently studied (Lopez-Sebastián et al., 1998; Mendes et al., 1995; Quirin, 2003). The antioxidant activity of supercritical extracts of rosemary is extremely high; even at low concentrations, the resultant extracts are heat-resistant and do not change the colour, taste, or flavour of the food in which they are used. Rosemary extracts dissolve easily in different types of foods (Gerard, Quirin, & Schwarz, 1995).

Tena and Varcárcel (1997) studied the SFE of rosemary using carbon dioxide. This method was previously compared with other traditional methods formerly developed using liquid solvent sonication.

SF extracts showed a higher carnosic acid concentration, one of the main compounds in rosemary responsible for its antioxidant activity (Hidalgo, Ubera, Tena, & Valcárcel, 1998). Therefore, the highest antioxidant activity was found in these extracts. As an additional advantage, it was found that these extracts did not show any colour. In studies by Hidalgo et al. (1998) the optimum extraction conditions for antioxidants from rosemary were: 383 bar, and 120 °C for 20 min. Bauman, Hadolin, Rizner-Hras, and Cnes (1999) studied the extraction of antioxidant components from rosemary using supercritical carbon dioxide at 100 °C and 475 bar. Their extraction yields ranged from 5 to 6%. The antioxidant activity of these extracts was compared with the activity of known synthetic antioxidants, such as BHA and BHT, providing a much higher activity than those found using the synthetic antioxidants. In work from our group (Ibáñez et al., 1999), a two step extraction method was suggested, with sequential recovery of two fractions with different antioxidant activities and different chemical compositions. The SFE experiments were carried out on an analytical scale and the conditions selected were: 100 bar and 40 °C for the first fraction and 400 bar and 60 °C for the second fraction. The antioxidant compounds were preferentially extracted in the second fraction. Both fractions were collected on a device specifically designed to improve the performance of the sample collection. This device consisted on a reservoir that, by using an extra cooling system, limited extract losses after CO_2 decompression.

These conditions were then scaled up to a pilot plant (Ibañez et al., 2000a; Señoráns, Ibáñez, Cavero, Tabera, & Reglero, 2000). In this study, instead of using a sequential two steps extraction process, two separation cells were used to carry out the fractionation. This system yielded two different fractions in terms of analytical and functional composition. Different extraction and fractionation conditions were tested, using carbon dioxide as supercritical fluid and ethanol as modifier. The extraction conditions ranged from 300 to 350 bar and from 40 to 60 °C. For all the experiments, the first separator was maintained at the given extraction temperature (from 40 to 60 °C) while the fractionation pressure was set in a range from 150 to 200 bar. The temperature in the second separator was kept equal to 25 °C in all experiments and the pressure varied between 20 and 55 bar.

Another study, performed in our research group to test the effect of the CO_2 quality on the antioxidant activity of rosemary extracts (Ibáñez et al., 2001), demonstrated that the CO_2 quality significantly affected the antioxidant activity of the extracts collected, probably due to the residual composition of O_2 and water of the carbon dioxide. Thus, the better the quality of the CO_2, the higher was the antioxidant activity of the extract.

Extraction of vitamin E from natural sources has received increasing interest due to the high antioxidant activity associated with this family of compounds. Besides its well known antioxidant activity, recent studies have demonstrated that synthetic vitamin E, is less effective than natural vitamin E (Hadolin, Skerget, Knez, & Bauman, 2001). Several natural sources have been used to isolate vitamin E using supercritical carbon dioxide extraction. Thus, Hadolin et al. (2001) studied the extraction of vitamin E-rich oil from a plant (*Silybum marianum*) that naturally grows in the Mediterranean area. It was pointed out that extractions at 60 °C and 200 bar produced the most concentrated extracts in terms of α-tocopherol (0.08%), while the extraction yield was relatively high (19%).

An other important source of vitamin E is wheat germ. The first studies conducted with this raw material were published by Saito and Yamauchi (1990), Saito, Yamauchi, Inomata, and Kottkamp (1989); these studies showed the capability of SFE, coupled to supercritical fluid chromatography (SFC), for tocopherol enrichment. Moreover, more recent studies have confirmed SFE (with CO_2) can achieve, extraction yields for tocopherols from wheat germ similar to traditional hexane extraction (Gomez & de la Ossa, 2000; Muñoz, Gomez, & del la Ossa, 1999). Also, the production of wheat germ oil by SFE has been studied (Gomez & de la Ossa, 2000; Panfili, Cinquanta, Fratianni, & Cubadda, 2003), providing oils with a very high contents of tocopherols. Ge et al. (2002b, 2002a) extracted vitamin E from wheat germ under the following extracting conditions: 275 bar, 40 °C and CO_2 flow rate equal to 2 ml/min for 90 min. The amount of total vitamin E extracted under these conditions was higher than those obtained using traditional extraction methods (with *n*-hexane or chloroform/methanol mixtures). Also, the quantities of α, γ and δ-tocopherol were much higher using SFE. However, the *n*-hexane extracts, and mainly, the chloroform/methanol extracts, showed higher selectivity towards β-tocopherol.

Additionally, other cereal sources have been studied for the production of tocopherols. By means of the combined use of SFE, together with preparative supercritical fluid chromatography (Prep-SFC), tocopherol enrichment in soybean flakes and rice bran was achieved (King, Favati, & Taylor, 1996). In this study, the optimum extraction conditions were found at 250 bar and 80 °C and one of the most important factors influencing the extraction process was the solvent-to-feed ratio (mass CO_2/mass seed charge). Nevertheless, considering only the SFE step, the enrichment factor obtained for tocopherols was 4.3, but additional enrichment was achieved in the Prep-SFC stage.

2.3. Functional ingredients from food industry by-products

Frequently, the processes that take place in the food industry generate products (the so-called by-products) that are discarded, causing subsequent environmental problems. In recent years, companies have devoted effort to find value-added application for these food by-products.

Supercritical fluid extraction has been used in many of these enrichment studies. Thus, several studies have been developed to extract β-carotene and lycopene from by-products of tomato industry (Baysal, Ersus, & Starmans, 2000; Rozzi, Singh, Vierling, & Watkins, 2002). These compounds are natural pigments, belonging to the carotenoid group, and their antioxidant properties are well known. Baysal et al. (2000) studied the optimisation of β-carotene and lycopene extraction from tomato paste waste by employing a factorial design. By previously determining the total amounts of these compounds in the tomato paste, it was possible to establish an optimum recovery of 54% of total lycopene using, as extraction conditions, CO_2 at 300 bar and 55 °C for 2 h. The CO_2 flow rate was kept constant at 4 kg/h and ethanol was used as modifier (5%). The optimum value for β-carotene recovery was around 50%. Extraction conditions were similar to those previous selected for lycopene extraction, except for an increase in the extraction temperature up to 65 °C. Likewise, these authors suggested the use of higher extraction temperatures and pressures to increase extraction yields, although a 100% recovery would never be expected due to the degradation that these compounds can suffer during the extraction process. Rozzi et al. (2002) studied the extraction of lycopene of tomato seeds and skins with supercritical CO_2. In this work, more extreme extraction conditions were tested, with extraction temperatures ranging from 32 to 86 °C and extraction pressures from 138 to 483 bar. The authors showed that the amount of lycopene extracted increased at higher pressures and temperatures up to a maximum recovery of 61% at 86 °C and 345 bar. The CO_2 flow rate was 2.5 ml/min and 3 g of sample were used in this analytical system. The results presented (Rozzi et al., 2002) demonstrated the possibility of lycopene extraction from tomato by-products using supercritical CO_2 without the addition of co-solvents. However, it is important to point out that high solvent/feed ratios (S/F = 166) were used in this process.

Other interesting by-products are those from the wine industry. Their interest is related to the type and amount of phenolic compounds that are found in grape seeds and skins. Isolation of phenolic compounds from grape seeds has been attempted using supercritical carbon dioxide (Murga, Ruíz, Beltrán, & Cabezas, 2000; Palma & Taylor, 1999). Palma and Taylor (1999) observed that the recovery of catechin and other phenolic compounds from grape seeds was higher when using supercritical CO_2, with methanol as modifier, rather than when using traditional solid–liquid extraction. Supercritical fluid extraction is faster and allows fractionation of the phenolic compounds of the grape seeds (Murga et al., 2000) by changing the pressure and adding co-solvents at different percentages. In a recent study, Louli, Ragoussis, and Magoulas (2004) employed supercritical fluid extraction to increase the added-value of extracts from by-products of the wine industry obtained by extraction with ethyl acetate. These extracts had antioxidant activities similar to the synthetic antioxidant BHT. The properties of the starting product were significantly improved by the subsequent supercritical CO_2 extraction that caused an increase of the antioxidant activity, allowing odourless and clearer extracts. These characteristics make the extracts more appropriate for use as natural antioxidants in the food industry. The selected parameters for performing the extraction were a pressure of 150 bar and a temperature of 45 °C. The addition of 0.5% of co-solvent did not significantly improve the results.

The use of by-products from the olive oil industry to extract tocopherols was suggested by Ibáñez et al. (2000b). In this study, the separation of tocopherols from the olive pomace (that is, the solid residue obtained using two-phase olive oil production systems) was achieved by means of a supercritical carbon dioxide extraction with two step fractionation. In the second fractionation step, where complete depressurization took place, the CO_2 density was very low and enrichment of the extract with tocopherols was observed. The extraction was carried out at a pressure of 350 bar and at a temperature of 50 °C, while the fractionation conditions that gave the best results were: 100 bar and 60 °C on the first separator and 10 bar and 25 °C on the second separator. Although the extract obtained using SFE was rich in tocopherols, these authors suggested a selective fractionation system of the different tocopherol isomers using supercritical fluid chromatography (SFC) with packed columns.

Other SFE work that suggested the utilization of food by-products to produce antioxidants, e.g. vitamin E, was performed by Mendes, Pessoa, and Uller (2002). It is based on soybean oil production and the by-product studied is obtained as a waste at the deodorization step during industrial production of soybean oil. Considering that soybean oil is the most widely consumed oil in the world, the use of this by-product (also called deodorizer distillate or soybean sludge) is of economic importance. Different extraction temperatures (from 40 to 80 °C) and pressures (from 90 to 170 bar) were studied. The highest extraction efficiency was obtained under mild extraction conditions, fatty acids being extracted by the supercritical CO_2. This fact allowed tocopherol enrichment inside the reactor cell.

The extraction of natural pigments from food by-products is of major importance, not only because of the increasing demand for natural ingredients by the food industry but also because some of these pigments may also have associated antioxidant activity that can even more, increase their added-value. The possibility of extracting natural pigments with antioxidant properties using SFE has been already mentioned. However, sometimes pigments themselves are the target compounds. This is the case for the extraction of carotenoids from carrots and tomatoes, i.e., the sources most frequently used to obtain natural carotenoids. Thus, Cadoni, De Giorgi, Medda, and Poma (2000) described the extraction and isolation of lycopene and β-carotene from tomato (both from skin and pulp). Among the different conditions studied in this work, the best results were obtained using an extraction pressure equal to 275 bar and an extraction temperature of 80 °C. The product obtained under these conditions had a composition of 65% lycopene and 35% β-carotene. However, taking into account that both compounds showed different solubility parameters on supercritical CO_2 (Cadoni et al., 2000), it was possible to select a two-step extraction to preferentially extract lycopene or β-carotene. For instance, on performing the extraction, in a first step, at 275 bar and 40 °C and, in a second step, at 275 bar and 80 °C, it was possible to obtain an end-product with 87% lycopene and 13% β-carotene, because β-carotene is preferably extracted under the initial extraction conditions. The isolation of lycopene was also studied by Ollanketo, Hartonen, Riekkola, Holm, and Hiltunen (2001) using tomato skin. After considering different extraction conditions (namely, different extraction temperatures, with/without co-solvents), they achieved an extremely high lycopene recovery when performing extractions with supercritical carbon dioxide at 1.5 ml/min flow rate, at 110 °C and 400 bar extraction pressure, for 50 min. Results were similar with and without acetone as modifier; leading to a lycopene recovery of 94% in just 15 min of extraction.

Using carrots as a natural source of carotenoids, Barth, Zhou, Kute, and Rosenthal (1995) carried out the optimization of the supercritical carbon dioxide extraction using a factorial design. The influence of different extraction conditions on carotenoid isolation was studied. Extraction temperatures of 30, 40 and 50 °C, pressures of 300, 400 and 500 bar and the addition of co-solvent (5% and 10% ethanol) were utilized. The authors concluded that optimum extraction conditions were achieved by working at 50 °C, 300 bar and 10% ethanol as modifier. In this study, it was confirmed that the amount of carotenoids extracted (including those with provitamin A activity) were higher in SFE extracts than in traditional solvent extracts. The traditional solvent extraction was completed after 6 h while the supercritical extraction was finished after 1 h.

2.4. Extraction of functional ingredients and other compounds of interest from algae and microalgae

In the search for feasible new sources of natural antioxidants that can be used in the food industry, algae and microalgae have been suggested as possible raw materials. Both organisms are widely known and consumed in certain countries, and numerous health benefits have been associated with their use. Therefore, algae and microalgae are potentially a great source of natural compounds that could be used as ingredients for preparing functional foods. Different compounds with antibacterial, antiviral and antifungical activity can be found in these type of organisms (Borowitzka & Borowitzka, 1988; Ötles & Pire, 2001; Xue et al., 2002), along with compounds with antioxidant activity.

Subra and Boissinot (1991) proved that, starting from a complex matrix, such as an alga (*Dilophus ligulatus*), it was possible to obtain extracts with different compositions and yields by changing the extraction pressure. Therefore, depending on the type of compounds of interest, optimum extraction conditions could be utilized for selective isolation of specific groups of compounds.

Many algae and microalgae are rich in polyunsaturated fatty acids. A consumption increase of these types of compounds has been associated with a decrease in the incidence of cardiovascular diseases (Cohen & Vonshak, 1991). Cheung (1999) studied the effect of the extraction conditions for obtaining fatty acids from *Hypnea charoides* algae, using supercritical CO_2, and suggested the usefulness of this extraction technique for converting this alga species as a new source of ω-3 fatty acids. Temperature ranges from 40 to 50 °C and pressure from 241 and 379 bar were studied. In general, the 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.

Several microalgae species have been used to obtain natural compounds of interest for the food industry, using supercritical fluid extraction. Mendes et al. (1995a, 1995b, 2003) applied this technique to several microalgae species to extract, for example, diolefines from *Botrycoccus braunii* cells. This organism can store high amounts of long-chain hydrocarbons (i.e. 25–31 carbon atoms), that can be utilized as substitutes of paraffinic and natural waxes. The authors proved that the solubility of these type of compounds in CO_2 increased with pressure and found that 300 bar provided the optimum with respect to yield and extraction speed.

SFE has also been used to extract carotenoids from microalgae *Chlorella vulgaris* (Mendes et al., 1995b). High pressures allowed high extraction yield. When microalgae cells were crushed, the carotenoid extraction improved slightly. A temperature increase produced an opposite effect at low pressures in terms of solute recovery. The optimum conditions were 55 °C and 350 bar.

Dunaliella salina is a microalga species capable of producing 14% of β-carotene relative to its dry weight. The optimum extraction yield for β-carotene extraction with supercritical CO_2 was found to be 300 bar and 40 °C (Mendes et al., 2003).

Another microalga species studied by Mendes et al. (2003) has been *Arthospira* (*Spirulina*) *maxima*. This microalga is capable of producing high amounts of γ-linolenic acid (GLA). The recoveries of GLA with pure CO_2, and with CO_2 plus ethanol as co-solvent, were compared with the results obtained using traditional organic solvents extraction. It was observed that, although both CO_2 and *n*-hexane provided similar extraction yields, the CO_2 allowed a higher recovery of GLA. The maximum extraction yield (0.44% GLA/dry biomass) was obtained using CO_2 with 10% of ethanol as modifier and performing the extraction at 350 bar and 60 °C.

Cyanobacterium *Spirulina platensis* was studied by Qiuhui (1999) to determine the amount of lipids and GLA present in the microalgae. The maximum extraction yield was obtained at 350 bar of pressure, in agreement with the results of Mendes et al. (2003). The temperature was set at 40 °C and the CO_2 flow rate was fixed at 24 kg/h for 4 h for this SFE.

3. Subcritical water extraction

3.1. Principles and instrumentation

Subcritical water extraction (SWE), i.e. extraction using hot water under pressure, has recently emerged as a useful tool to replace the traditional extraction methods. SWE is an environmentally friendly technique that can provide higher extraction yields from solid samples (Luque de Castro, Jiménez-Carmona, & Fernández-Pérez, 1999). SWE is carried out using hot water (from 100 to 374 °C, the latter being the water critical temperature) under high pressure (usually from 10 to 60 bar) to maintain water in the liquid state.

The most important factor to consider in this type of extraction procedure is the variability of the dielectric constant with temperature. Water at room temperature is a very polar solvent, with a dielectric constant close to 80. However, this value can be significantly decreased to values close to 27 when water is heated up to 250 °C (see Fig. 4) while maintaining its liquid state by applying the appropriate pressure. This dielectric constant value is similar to that of ethanol (Miller & Hawthorne, 2000).

The experimental device required for SWE is quite simple (see Fig. 5). Basically, the instrumentation consists on a water reservoir coupled to a high pressure pump to introduce the solvent into the system, an oven, where the extraction cell is placed and extraction takes place, and a restrictor or valve to maintain the pressure. 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.

Although this technique has been mainly used as a batch process, studies on the on-line coupling of a SWE system to a HPLC equipment via a solid phase trapping have been reported (Li et al., 2000).

3.2. Extraction from plants using SWE

Subcritical water extraction has been widely used to extract different compounds from several vegetable

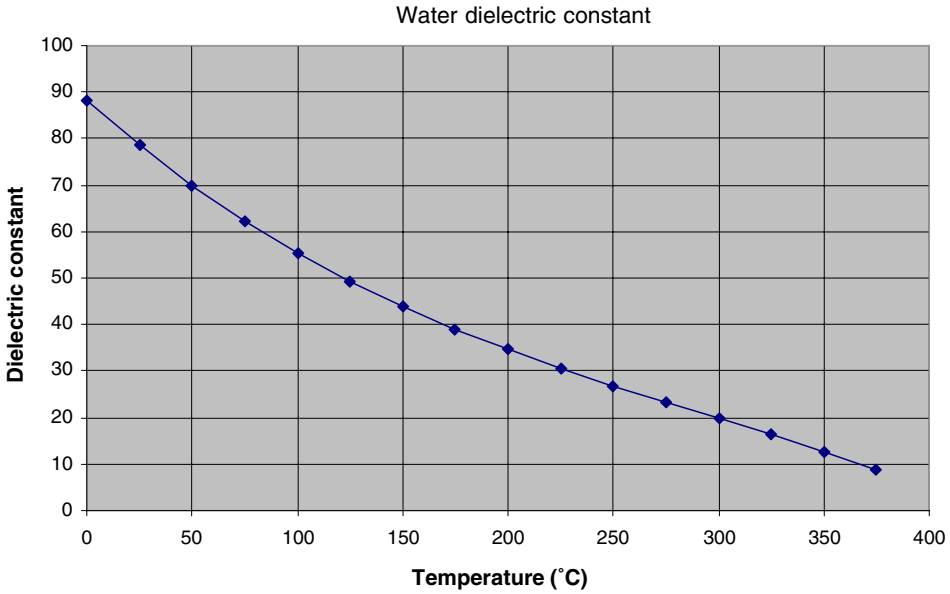

Fig. 4. Graphical representation of dielectric constant of water vs. temperature.

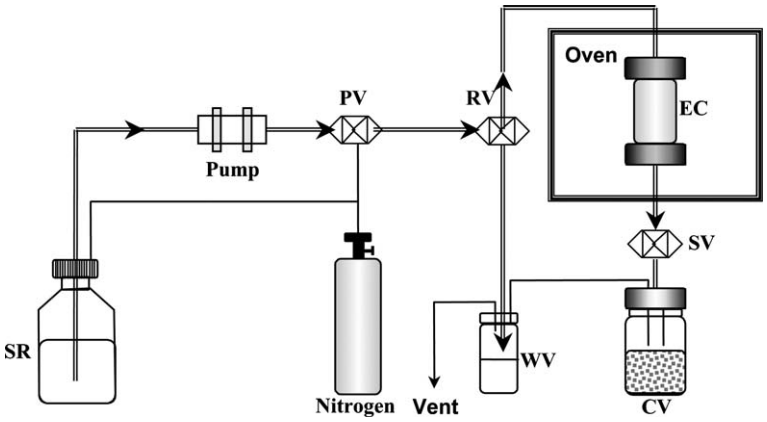

Fig. 5. Diagram of a subcritical water extraction system. SR, solvent reservoir; PV, purge valve; RV, pressure relief valve; EC, extraction cell; SV, static valve; CV, collector vial; WV, waste vial.

matrices. Likewise, one of the most deeply studied materials with SWE has been rosemary (*Rosmarinus officinalis* L.). Ibáñez et al. (2003) studied the extraction of antioxidant compounds of rosemary by SWE over a wide range of temperatures. Several temperatures, from 25 to 200 °C, were tested to study the extraction selectivity toward antioxidant compounds. There was a clear effect of water temperature on the extraction yield,which increased at higher extraction temperatures. The authors verified that the most polar compound (i.e. rosmanol) was the main compound extracted at low temperatures (25 °C). When the extraction was performed at 200 °C, a decrease in the capability of water to dissolve the most polar compounds was observed, while a high concentration of other compounds, such as carnosic acid, was obtained. Antioxidant extracts comparable to those achieved using supercritical carbon dioxide extraction could be obtained by SWE.

In addition to antioxidants from rosemary, the SWE extraction of aroma compounds from rosemary (Basile, Jiménez-Carmona, & Clifford, 1998). Savory (*Satureja hortensis*) and peppermint (*Mentha piperita*), has also been studied (Kuvátová, Lagadec, Miller, & Hawthorne, 2001a).

Some studies have been conducted to compare SWE to traditional extraction methods (such as Soxhlet extraction). Clove (*Syzygium aromaticum*) extractions, performed by Clifford, Basile, and Al-Saidi (1999) demonstrated that the amount of eugenol and eugenyl acetate recovered using subcritical water at 150 °C was similar to that achieved using Soxhlet extraction and hydrodistillation. These compounds are known to possess antioxidant properties similar to those of other natural compounds, such as α-tocopherol (Lee & Shibamoto, 2001).

In general, the use of subcritical water extraction, provides a number of advantages over traditional extraction techniques (i.e. hydrodistillation, organic solvents, solid–liquid extraction). These are, mainly, low extraction times, higher quality of the extracts (mostly for essential oils), lower costs of the extracting agent, and an environmentally compatible technique. These advantages have been verified for the SWE of several plants such as laurel (Fernández-Pérez, Jiménez-Carmona, & Luque de Castro, 2000), fennel (Gámiz-García & Luque de Castro, 2000), oregano (Soto Ayala & Luque de Castro, 2001) and kava (Kuvátová, Miller, & Hawthorne, 2001b), among others.

Ozel, Gogus, and Lewis (2003) studied the extraction of essential oil from *Thymbra spicata.* The influences of several factors, such as temperature (100, 125, 150 and 175 °C), pressure (20, 60 and 90 bar) and flow rate (1, 2 and 3 ml/min) were studied. It was shown that the best extraction yields (3.7%) were obtained at 150 °C and 60 bar, using a flow rate of 2 ml/min for 30 min. The essential oils of *Timbra spicata* were found to inhibit mycelial growth of several fungi species (Ozel et al., 2003).

3.3. Extraction from microalgae using SWE

SWE has already been used to extract antioxidant compounds from microalgae *S. platensis* (Herrero, Ibáñez, Señoráns, & Cifuentes, 2003). Similarly, a study using pressurized ethanol as extracting agent was conducted (Basile et al., 1998; Denery, Dragull, Tang, & Li, 2004). Denery et al. (2004) studied carotenoid extraction from the microalgae, *Haematococcus pluvialis* and *Dunaliella salina*, using ethanol. The extraction yield was shown to be similar to those obtained using traditional extraction techniques.

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