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SUPERCritical FLUID EXTRACTION: RECENT ADVANCES AND APPLICATIONS

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Abstract:

Among the different extraction techniques used at analytical and preparative scale, Supercritical Fluid Extraction (SFE) is one of the most used. This review covers the most recent developments of SFE in different fields, such as food science, natural products, by-product recovery, pharmaceutical and environmental sciences, during the period 2007-2009. The revision is focused on the most recent advances and applications in the different areas; among them, it is remarkable the strong impact of SFE to extract high value compounds from food and natural products but also its increasing importance in areas such as heavy metals recovery, enantiomeric resolution or drug delivery systems.

1.- INTRODUCTION

Solvent extraction is one of the oldest methods of separation known and certainly dates back to Palaeolithic age. The science of solvent extraction has evolved over a long period of time and much progress has been made in the understanding of solvation and the properties of liquid mixtures used in extraction processes. Hannay and Hogarth's (1879) early observations of the dissolution of solutes in supercritical fluid (SCF) media introduced the possibility of a new solvent medium. However, it is only quite recently (around 1960) that commercial process applications of supercritical fluid extraction have been extensively examined [1].

The massive wide scale use of organic solvents by a diverse range of global industries represents a serious threat to the environment. In part response, the Montreal Protocol was introduced in 1987 with an initial objective to restrict or eliminate the manufacture and use of particularly damaging ozone depleting solvents such as chlorofluorocarbons (CFCs). The Montreal Protocol is dynamic and evolving with the manufacture and supply of solvents other than CFCs gradually being restricted. Years of negotiation fostered by the United Nations Environment Programme has now resulted in more than 170 signatory nations to the Montreal Protocol with its London (1990), Copenhagen (1992) and Beijing (1999) amendments. Consequently, world-wide there is pressure for industry to adopt new sustainable processes that do not require the use of environmentally damaging organic solvents [2].

Since the end of the 1970s, supercritical fluids have been used to isolate natural products, but for a long time applications relayed only on few products. Now the development of processes and equipment is beginning to pay off and industries are getting more and more interested in supercritical techniques [3]. This interest is also reflected in the high amount of scientific papers dealing with Supercritical Fluid Extraction (SFE) published in recent years. Moreover, industrial applications of SFE have experienced a strong development since the early 1990's in terms of patents [4].

In 2007 we published a review paper dealing with the use of compressed fluids (SCFs among them) for sample preparation [5]; since then, several applications have appeared in the literature. Therefore the main purpose of the present review paper is to cover the recent advances and developments of SFE mainly in Food, Toxicological, Pharmaceutical and Environmental fields during the period 2007-09, including all the papers dealing not only with the use of SFE as sample preparation technique but also with the analytical scale SFE process development.

As will be seen throughout this paper, the main supercritical solvent used is carbon dioxide. Carbon dioxide (critical conditions = 30.9 °C and 73.8 bar) is cheap, environmental friendly and generally recognized as safe by FDA and EFSA. Supercritical CO₂ (SC-CO₂) is also attractive because of its high diffusivity combined with its easily tunable solvent strength. Another advantage is that CO₂ is gaseous at room temperature and pressure, which makes analyte recovery very simple and provides solvent-free analytes. Also, important for food and natural products sample preparation, is the ability of SFE using CO₂ to be operated at low temperatures using a non-oxidant medium, which allows the extraction of thermally labile or easily oxidized compounds [5]. The main drawback of SC-CO₂ is its low polarity, problem that can be overcome employing polar modifiers (co-solvents) to change the polarity of the supercritical fluid and to increase its solvating power towards the analyte of interest. For example, the addition of relatively small percentages (1–10%) of methanol to carbon dioxide expands its extraction range to include more polar analytes. The modifiers can also reduce the analyte–matrix interactions improving their quantitative extraction [6]. Due to the high amount of literature dealing with the basics of SFE, the reader is referred to some interesting books and papers [1-9].

The design of processes using supercritical solvents is strongly dependent on the phase equilibrium scenario, which is highly sensitive to changes in operating conditions. Therefore, phase equilibrium engineering plays a key role in the synthesis and design of these processes. Phase equilibrium engineering is the systematic application of phase equilibrium knowledge to process development

[10]. This knowledge comprises data banks, experimental data, phenomenological phase behavior, thermodynamic analysis, and mathematical modeling procedures for phase equilibrium process calculations. Each SC application has a set of specifications and physical restrictions. Rigorous simulations of equilibrium stage separations at near-critical conditions are needed for the design and optimization of supercritical processes. However, equilibrium calculations in the near-critical region can present serious convergence difficulties. In that respect, Michelsen's phase stability criterion, multiple-phase flash algorithms, and global phase computations are of particular interest for supercritical extraction applications. The bases of phases equilibrium are out of the scope of the present review, but reader can be referred to the works performed by E. Brignole's group [11-16] among others.

2.- SFE APPLIED TO FOOD AND NATURAL PRODUCTS EXTRACTION.

2.1. General aspects.

From a simple literature search, it can be easily deduced the impact of SFE as sample preparation technique for the analysis of target compounds from natural products and foods. In this section, the most remarkable applications included in this group are commented and summarized. It is worth to mention that SFE has been also widely used in this field for process development, that is, to extract target (bioactive or valuable) compounds from different matrices. Even though these processes usually offer clear advantages over traditional ones, the main drawback for industrial scale use is the lack of realistic economical studies. In this sense, some papers have been lately published dealing with the assessment of the industrial economical feasibility of some developed processes, such as essential oil extraction from rosemary, fennel and anise [17] and brewer's spent grain management [18]. Therefore, SFE can be regarded as a possible tool not only from a laboratory point of view but also for the natural products and food industries.

One of the main aspects that should be considered in SFE is the extraction optimization. The use of the optimum values for the different variables influencing the SFE extractions could significantly enhance the recovery or extraction yield of a target compound. With the aim to, effectively, optimize these variables (mainly extraction temperature, pressure, time, type and percentage of modifiers, sample size, etc.), different approaches have been applied. Those strategies can be grouped in phase equilibrium strategies and experimental design statistical optimization. Phase equilibrium engineering is the systematic application of phase equilibrium knowledge to process development. This knowledge comprises data banks, experimental data, phenomenological phase behavior, thermodynamic analysis, and mathematical modeling procedures for phase equilibrium process calculations [10]. The phase equilibrium and mass transfer are not modelling problems; they are limit stages of the process that can justify the results. It is very important to organize the experiments based in these fundamentals, to contribute to the understanding of the extraction problem.

Despite of the valuable information obtained by phase equilibrium engineering, it's a common practice to optimize the processes using experimental designs and statistical modelling. As reviewer said the information obtained with these methods is very specific and not easily applicable to other matrices, but it represents a very easy practical way for the optimization and scale up. The use of experimental designs is one of the most common strategies when it comes to set up robust extraction methods [19]. For instance, a simplex centroid design was used to determine optimum temperature, pressure, dynamic extraction time and modifier volume that maximize the yield of the essential oil of valerian (*Valeriana officinalis* L) attained by SFE using supercritical carbon dioxide [20]. Based on the graphical and statistical analysis of results, temperature and pressure were to be the influential variables on the extraction process, the optimum was found at 37 °C and 243-250 bar. With this strategy four independent variables were tested at five levels by using only 18 experiments. Response surface methodology (RSM) is another popular possibility; RSM was

recently been applied to optimize the process parameters for supercritical carbon dioxide extraction of passion fruit oil [21]. The use of RSM allowed the simultaneous graphical optimization of the extraction temperature, pressure and extraction time. The extraction yield was selected as response variable, although the particular fatty acid composition of the extracts was subsequently studied by GC-FID [21]. RSM provide more information about how the extraction is working, but requires more experimental points. In the case of the extraction of passion fruit oil [21], fourteen experiments plus six replicates in the centerpoint were used to test 3 variables at 5 levels. This methodology has been also employed to optimize the extraction of other matrixes such as *Pueraria lobata* to obtain flavonoids [22] as well as wheat germ [23], pomegranate [24] and brazilian cherry extraction [25]. Besides, RSM using a full factorial composite design involving 3 factors (temperature, pressure and extraction time) and 3 levels, was employed to fit a second order polynomial model for the maximization of lycopene SFE extraction from tomato pomace [26,27]. A full statistical analysis of the results allowed the determination of 57 °C, 530 bar and 1.8 h as optimum extraction temperature, pressure and time, respectively. Five levels were instead employed, together with the same factors, to the optimization of antioxidants extraction from roasted wheat germ. Yield, total phenolic and tocopherol contents and extracts antioxidant activity were used as response variables [27]. Full factorial designs require more experimental points, but provides more information in terms of the effect of variable combinations.

Orthogonal array designs can be effectively used to study the influence of 4 different factors simultaneously. This type of experimental design was employed to study the type of modifier, the dynamic extraction time, temperature and pressure of the supercritical CO₂ in terms of extraction yield produced and *dl*-tetrahydropalmitine extracted from *Corydalis yanhusuo*, an herbal medicine [28]. Three levels of each variable were considered, and their optimal values determined through an ANOVA analysis. These designs can be used to estimate main effects using only a few

experimental runs. These designs are also available to investigate main effects for certain mixed level experiments where the factors included do not have the same number of levels.

Thermodynamic simulations and theoretical models are also an alternative to process optimization [29,30]. For instance, a GC-EoS (Group Contribution Equation of State) model was applied to simulate the counter-current supercritical extraction of phytosterol esters [31]. Some extraction parameters have been studied particularly in detail, such as the effect of pressure on plant seeds extraction [32], the influence of milled seeds particle size distribution in the extraction process [33], or even deeper calculations of solubility changes in supercritical CO₂ according to the temperature and pressure applied [34]. In fact, it was shown how the particle size significantly influenced the yield of paprika extract. Likewise, sample moisture above 18% negatively affected the extraction [35]. Moreover, different mass transfer models have been also employed to modeling vegetable oils extraction [36].

In food and natural products analysis, the performance of SFE compared to other advanced and /or conventional extraction techniques has been frequently evaluated. SFE has been directly compared to classical solvent extraction [37,38], soxhlet extraction [39], hydrodistillation [40-43], solid-liquid extraction [44], as well as ultrasound assisted extraction [37]. Besides the very well known advantages over other extraction techniques, such as the low consumption of organic solvents and selectivity [42], SFE has demonstrated to provide faster extractions and higher yields than hydrodistillation for the extraction of the essential oil of *Valeriana officinalis* [40]. In contrast, hydrodistillation provided better results in terms of number of volatiles extracted, although the loss of these compounds in the depressurization step after SFE was a possibility to consider. SFE was selected over solid-liquid extraction for the enrichment of phenolic compounds from grape pomace [44]. SFE produced higher phenolic concentrations than the traditional extraction technique, although the composition of the extracts was not exactly the same; while solid-liquid extraction contained more proanthocyanidins, SFE was richer on simple phenolics. Besides, the extracts

obtained using this technique showed higher antiradical activity [44]. Moreover, SFE has demonstrated to be more effective than other conventional techniques for the extraction of antimicrobial compounds [38,39].

A clear trend in food and natural products analysis, as well as in other fields of analytical science, is the hyphenation of sample treatment procedures with analytical tools in order to produce effective on-line couplings. Some of these couplings have involved the use of SFE. For instance, SFE has been directly coupled to a CE instrument with fluorimetric detection to effectively carry out the clean-up of the sample and the direct CE analysis of riboflavin from chicken liver and powdered milk [45]. A scheme of the instrumentation employed can be seen in Figure 1. As it can be observed, a continuous flow device allowed the coupling between the SFE and CE instruments. In a first step, the sample clean-up was performed using supercritical CO₂; then, by actuating several switching valves, a carrier solution composed of methanol/water (95:5) was let to pass across the sample extracting the available vitamins and filling an injection loop. Later on, the valve was again actuated releasing the extract to the capillary inlet. There, a certain amount was injected in the CE system and analyzed [45]. Nevertheless, this is not the only direct coupling developed in the last years. *Tithonia diversifolia* was extracted using CO₂ with the aim to obtain tagitinin C (a sesquiterpene lactone, with anti-plasmodial activity), which was directly determined by Fourier transform-Infrared spectroscopy (FT-IR) [46]. A high pressure fibre optic flow cell was installed in order to allow the real-time determination of the tagitinin C being extracted. This novel set-up allowed the monitoring of the SFE process as well as the determination of the kinetic curve of this compound [46]. Once its usefulness was demonstrated, the developed instrument was successfully employed to optimize the extraction of this compound from *T. diversifolia*, studying by real-time monitoring, the effect of varying the extraction pressure and temperature [47].

Thanks to the production of a clean extract when using supercritical CO₂ as extraction solvent in SFE, the on-line coupling of this extraction technique with analytical tools, specially GC, is relatively straight-forward using different devices interfacing extraction and analysis [48,49]. Nevertheless, no application in the food and natural products field has been referenced in the last three years including this type of on-line coupling.

SFE has been also used in combination with other sample treatment processes; for example, polyphenols from cocoa seeds were effectively extracted and separated by combining SFE together with nanofiltration processes [50]. In this case, the extraction conditions were previously determined and then, the performance of different commercial nanofiltration membranes was studied. These membranes were placed in a special filtration cell before the collector. Besides, the permeability of the membranes to the supercritical CO₂ was also studied [50].

2.2. Food applications

Probably, the most extended use of SFE is in the food field. A high variety of samples, type of materials, target compounds and procedures have been published in the last years.

A relatively new group of applications that have been recently developed includes the extraction and fractionation of carbohydrates by SFE. This technique, using supercritical CO₂, as it is massively employed, is not at first sight the best option for this group of compounds, given the low polarity of carbon dioxide; however, using CO₂ with a relatively low amount of polar entrainer, authors demonstrated its usefulness. A full factorial design was used to evaluate the influence of extraction pressure, temperature and modifier flow rate (and consequently proportion of modifier) to effectively fractionate carbohydrate mixtures formed by lactose and lactulose. Results obtained were analyzed by multiple linear regression determining as optimum values 100 bar, 100 °C and 0.2 ml/min of co-solvent (4% modifier) for the selective recovery of lactulose [51]. This strategy was also applied to other carbohydrates [52,53]. All these works demonstrated the great importance of the modifier nature to obtain selective fractionations, being ethanol/water the most successfully

used co-solvent. In fact, the solubilities of different carbohydrates in supercritical carbon dioxide with ethanol/water as co-solvent were experimentally obtained and thermodynamically modelled [54]. The optimization of mannitol extraction from olive leaves has been also performed, although the extraction yield in terms of mannitol extracted was lower than that attained using Soxhlet [55].

Crude vegetable oils are widely used in the food industry for a number of applications. Usually oils have to be refined to remove undesirable compounds before consumption; during this refining process, valuable compounds contained in the oils can be also lost. SFE has been suggested as an alternative to refining procedures, to obtain extracts enriched with the particular compounds of interest; this is the case for instance of wheat germ oil [56], green coffee oil obtained by pressing [57], rice bran oil [58] or crude palm oil [59].

SFE has been also used to remove unwanted compounds from other matrices; for example, supercritical CO₂ with water as co-solvent has been employed to selectively extract caffeine from green tea while avoiding the extraction of antioxidants from the matrix [60]. The selectivity of the different extraction conditions studied was confirmed by HPLC.

Essential oils [61-65], fatty acids [61,62,65] and/or bioactive compounds [62,64,66,67] have been extracted from fruits and vegetables using supercritical CO₂. Soy isoflavones have been also widely studied; aqueous methanol seems to be the most appropriate modifier to carry out isoflavones supercritical CO₂ extractions [68], although the use of acetonitrile has been also tested [69]. Daidzein and genistein were successfully extracted at high pressures, from 350 to 500 bar [68,70]. In all of these studies, HPLC was the analytical tool selected to determine these flavonoids. These compounds as well as other bioactives have been extracted by SFE prior analysis from a number of matrices: stilbenes such as cajaninstilbene from pigeonpie [71], cinnamic derivatives from propolis [72] or carotenoids and flavonoids from black rice [73].

SFE has demonstrated to be a useful tool to investigate fatty acids profile in fish oils [59]. Fractionation of fish oils with supercritical CO₂ to obtain omega-3 enriched fractions was possible by operating at different temperatures and pressures [74,75]. These authors found that the tuning of the extraction parameters made supercritical extraction a helpful procedure to effectively change the lipids composition, so that a high value functional product is obtained. One of the few works dealing with SFE using a solvent different from CO₂ is related to fatty acid extraction from a fish oil sample [76]. In this case, the ability of ethane as an alternative to the more common use of CO₂ to extract fatty acids was evaluated. Besides, a thermodynamic modeling was used to obtain the most appropriate conditions to extract the highest possible amounts of eicosapentanoic (EPA) and docohexaenoic (DHA) acids. Ethane provided better selectivity and higher solubility for EPA and DHA esters as compared to other esters containing the same number of carbons. Applying 60 °C and 84 bar, a concentration of 60% of both fatty acids ethyl esters could be obtained using ethane as supercritical solvent [76].

Phospholipids are widely employed in the food industry, not only for their technological properties but also for their biological functions. Two SFE processes (without and with ethanol as modifier) together with a supercritical antisolvent procedure in a semi-continuous process have been suggested as an effective way to produce a very pure phospholipid extract from egg yolk powder at industrial scale [77]. SFE was also useful to concentrate more than 5-fold the phospholipids present in whey cream buttermilk powders [78]. These phospholipids were later on separated on a HPLC diol column and detected using an evaporative light-scattering detector (ELSD). This kind of products have been also investigated for their fatty acids content, extracted by SFE [79].

Supercritical CO₂ is the most widely used near-critical fluid for the extraction of lipids. However, CO₂ cannot extract complex lipids unless an organic co-solvent is also used. Among the other near-

and super-critical fluids that could be considered, we have found that dimethyl ether (DME) is a strong solvent for both neutral and polar lipids and has a wide range of potential use in food, pharmaceutical, and cosmetic applications. DME is non-toxic, non-reactive, does not cause a pH change in aqueous solution, and has a sufficiently high vapour pressure at room temperature that virtually complete solvent removal can be carried out easily and at moderate, or ambient, temperatures. Catchpole et al [80] combined the use of CO₂ and DME for the extraction of phospholipids from egg yolk and a special dairy stream. A two step extraction process is described in which neutral lipids are extracted with supercritical CO₂, and then polar lipids using near-critical DME. The polar lipid extract was enriched in phospholipids (~70% by mass), which included phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and sphingomyelin. Other complex lipid components extracted included gangliosides and cerebroside. Unlike the antisolvent process, proteins were not denatured during either CO₂ or DME processing of the spray dried powders, and the de-fatted powders are therefore suitable for a range of functional foods. A polar lipid extract could also be produced from spray dried powder by extracting first with DME to obtain a mixed neutral/complex lipid extract, then re-extracting the lipid extract with CO₂ to remove neutral lipids.

One strategy to increase the extraction efficiency of food materials is an enzyme pre-treatment [81]. It has been repeatedly shown that some enzyme treatments on the sample just prior extraction can effectively help to the extraction of the target compounds. This strategy was employed with soybean oil deodorizer distillate to increase the recovery of phytosterols esters present on this by-product [82]. After a two-step enzymatic treatment, phytosterol esters were concentrated in the raffinate up to 82% with a yield reaching 72%, using CO₂ at 250 bar and 55 °C and a solvent-to-feed ratio of 35 [82].

As it can be appreciated, these treatments are mainly focused to increase the extraction yield. To this aim, besides enzymes, other pretreatments can be performed. Recently, Zhang et al. [83],

compared the SFE extraction performance (yield and chemical composition of extracts) of almonds with and without autoclaving pretreatment. Authors found that the fatty acid profile suffered just minor modifications whereas the extraction yield was significantly increased due to the rupture of the almond cell walls during the treatment in the autoclave [83].

SFE has been also widely employed as a sample treatment technique prior to volatiles analysis in different beverages. Aroma compounds from sugar cane spirits were isolated by supercritical CO₂ [84]. The application of mild pressures and temperatures (100 bar and 40 °C, respectively) allowed the highest concentration of aroma compounds that were subsequently analyzed by GC-MS and using sensory evaluation. Recently, an application was proposed for the removal of ethanol from alcoholic beverages using supercritical carbon dioxide [85]. Vacuum distillation, membrane techniques and CO₂ supercritical fluid extraction (CO₂-SFE) can be used in the dealcoholization process. All these techniques have the disadvantage of eliminating the beverage aromas together with ethanol, but still, among them, SC-CO₂ is particularly attractive because water, salts, proteins and carbohydrates are not substantially removed or denatured. The removal of ethanol from alcoholic beverages implies a problem of ethanol separation from aqueous solutions, since most alcoholic beverages contain large amounts of water. In this paper [85] a different approach was followed. The GC-EoS thermodynamic model was used to correlate the CO₂+ethanol+water phase behavior and to simulate a countercurrent multistage separation process. As expected, extraction (T, P) with the same CO₂ density produced almost the same elimination of ethanol; at a given CO₂ density the ethanol content in the dealcoholized product can be controlled by tuning the solvent to beverage flow ratio.

One of the most interesting recent applications of SFE in food analysis sample treatment dealt with the determination of amino acids profiles in different genetically modified varieties of maize and

soybean [86]. Supercritical CO₂ modified with 35% methanol at 80 °C and 120 bar was employed to extract the amino acids, that were afterwards analyzed by GC-MS. These optimal extraction conditions were provided by a central composite design and the statistical analysis of the obtained data. The sum of the areas of the aromatic amino acids was selected as the experimental design response variable. This way, several genetically modified organisms (GMO) could be directly compared to their respective isogenic non-transgenic varieties. Some statistical differences among the amino acids contents in the samples studied were observed [86]. The extraction with modified CO₂ allowed a faster and more efficient recovery of amino acids from maize and soybean grains compared with previous techniques.

2.3.- By-products processing using SFE

Industrial activities generate a large variety of by-products and wastes ranging from manure to packing residuals. Strong research is focused in the development of new technologies and new uses for these materials in order to reduce their environmental impact. New processes are being developed to recover components producing high added value products [87]. Mainly SFE has been tested for the extraction of lipids and lipophilic components, but not only, as can be seen in Table 1. SFE has been widely used to value food industry by-products; these products are generated during food manufacturing and normally do not have any commercial value. By-products extraction allows the removal of valuable/interesting compounds that otherwise cannot be utilized. Rice wine lees were investigated for the extraction of polyphenols with antioxidant activity [88]. The amount of modifier (ethanol) used, combined with supercritical carbon dioxide, was shown to be the most critical factor to maximize polyphenols extraction. In this paper Soxhlet ethanol extraction was compared with SC-CO₂+ethanol, polyphenols content was only 43% of that obtained by Soxhlet ethanol extraction, on contrast, SFE needs much shorter extraction times.

The tomato-related industry produces a number of by-products, such as peels or pomace, in which the presence of carotenoids has been confirmed. These compounds act as pigments and have been described to possess several interesting functional activities, for instance antioxidant activity [109], ability to prevent cardiovascular diseases [110], macular degeneration [111] and cancer prevention [112]. These activities obviously make carotenoids very interesting for the food industry. Tomato pomace, containing skin and seeds of ripen tomatoes has been extracted using SFE in order to obtain not only carotenoids [113,114] but also tocopherols and phytosterols [89]. Later on, the extracts were analyzed by HPLC and GC in order to determine their contents on the interesting compounds. It was observed that the use of CO₂ at 460 bar and 80 °C produced extract with the highest carotenoid content, being more than 90% lycopene. On the other hand, extracts rich in tocopherols and phytosterols were obtained at 300 bar and 40 °C [89]. In a subsequent work, a central composite rotatable design model was used to optimize the SFE conditions to extract lycopene from tomato skin waste [90]. The complete statistical analysis of the data generated predicted 62 °C and 450 bar as optimum extraction conditions, using CO₂ with 14% ethanol concentration. Under these conditions the recovery of all-trans lycopene was 33%. In order to obtain lycopene from tomato, recently, a novel procedure has been proposed. Ciurlia et al. [91], studied the simultaneous extraction of tomato and hazelnuts in order to extract lycopene from tomatoes and essential hazelnut oil at a time. The application of CO₂ at 60 °C and 400 bar resulted on the extraction of the 72.5% of the lycopene present on the tomatoes, and 80 % of the hazelnut oil. The product was an over-saturated solution of lycopene in hazelnut oil. Besides, after centrifugation, a product five-folds more concentrated on lycopene was attained. HPLC-UV using a specially suited column for carotenoids analysis (C30 stationary phase) was employed to characterize the extracts and products obtained. Authors concluded that this novel approach was an effective way to increase the lycopene extraction from tomato powder [91].

Carotenoids and tocopherols recovery from Sea buckthorn pomace (a by-product of Sea buckthorn juice manufacture process) [92] and from fresh palm-pressed mesocarp fiber (by-product from palm oil production) [93] have been also studied. As could have been seen most of the papers dealing with carotenoids extraction requires very high pressures, this fact suppose a di

By-products of vegetable oil production are one of the most studied matrices [115,116]. Other matrices that have been also evaluated were hake by-products, to obtain omega-3 rich extracts [94], sugar cane crude wax, coming from sugar cane production, to obtain long chain *n*-alcohols [95], pomegranate seeds from juice production to obtain polyphenols [96], loquat seeds, in the search of phytosterols [97] and tea stalk and other tea plant wastes to extract caffeine [98].

Many recent works deals with oil recovery from different residues, namely, wood [105], corn [101] or even silkworm pupae [107]. Desilked silkworm pupae is the main by-product of the silk industry, and it constitutes 60% of dry cocoon weight; only China generates more than 700.000 tons of silkworm pupae per year; from this, only a small amount is as fertilizer and animal feed. Wey et al. [107] optimized, by response surface methodology, a method to obtain a high value oil from desilked silkworm pupae with SC-CO₂. In this case, the use of SFE contribute keep unaltered the composition of the oil, while other techniques for oil recovery can modify the fatty acid profile. Through an experimental design, considering four variables (pressure, temperature, extraction time and CO₂ flow), authors concluded that oil yield was linearly dependent on pressure and extraction time while the quadratic terms of pressure, extraction time, and CO₂ flow rate and the interactions between pressure and temperature and temperature and flow rate were also significant. A polynomial regression model was used to describe the experimental results, the optimal condition for silkworm pupal oil yield was found to be 324.5 bar, 39.6 °C, 131.2 min, and 19.3 L/h. Furthermore, unsaturated fatty acids were the predominant fatty acids in the silkworm pupal oil, as the GC-MS analysis concluded.

Biocrude is a replacement for geologically sourced crude oil, made from biomass. Biocrude is a next-generation non-fossil form of energy which can be transported and refined using existing petroleum facilities, this is why biocrude is different from biodiesel. The primary biofuel in use today is corn- or cane-based ethanol, which exhibits several negative characteristics; these include the very large amount of energy required to create ethanol from corn, and the corrosive effect of ethanol on piping and engine components. The rising price of grain leads to increase in the final cost of grain-based ethanol. The use of supercritical fluids in bio-fuel production has been recently reviewed by Wen et al. [117]. Woodwaste and forestry residues could be a promising source of biocrude whose possibilities have been studied by Yang & Gilbert [105]. In this work, the use of supercritical methanol was suggested since it seems to enhance the dissolution rate of lignocellulosic materials, to increase the linkage rate of α and β -ether linkages while keeping intact the condensed linkages. The birch wood sawdust was treated, without catalyst, in methanol at 20-100 bar, 200-400 °C. Once the reactor was cooled to room temperature, the gas inside was collected in a gas cylinder, and was analyzed by GC-TCD, while the residual products were analyzed by GC-MS. Besides, several catalysts (NaOH, K₂CO₃, or Rb₂CO₃) were assayed in order to tune the biocrude composition. In the optimized conditions (20 bar, 200 °C and 2 h) the obtained heavy oil products consisted of a high concentration of phenol derivatives, esters, and benzene derivatives, and also contained a higher concentration of carbon, a much lower concentration of oxygen, and a significantly increased heating value (>30 MJ/kg) when compared with the raw woody biomass. Hydro-liquefaction in supercritical methanol can thus be a promising technique for upgrading of woodwastes of a low-heating value to bio-crude with a significantly increased heating value.

2.4. Natural products applications.

SFE has for long been used to extract bioactive compounds from plant materials in order to characterize compounds responsible for a specific functional activity. In Table 2, a summary of the recent works published on this topic is shown. As it can be seen, antioxidant compounds have been the most frequently studied. It is interesting to note that in every application CO₂ with or without organic modifiers was the solvent employed. Concerning the analytical techniques used to characterize the extracts, GC and HPLC were utilized. High speed counter-current chromatography was also employed to characterize isocoumarins and other compounds from coriander [72] and *Cynanchum paniculatum* [118], respectively.

As it can be inferred from Table 2, most of these works were aimed on the characterization of bioactives from medicinal herbs, or on the extraction of these compounds to be used as ingredients intentionally added to other products, mainly food products [119-137]. The extraction of carotenoids from different samples has been studied. While 450 bar and 80 °C were used to extract these compounds from rosehip [32], milder conditions were applied to test *Eugenia uniflora* as a source of these compounds. However, in both cases CO₂ without the addition of any modifier and HPLC-UV were employed to obtain, characterize and quantify these compounds at 450 nm [138]. Other interesting compounds studied included coumarins [121,133], tocopherols, fatty acids [122], phytosterols [126] and terpenes [131,135], among others. Blood-thinning, anti-fungicidal and anti-tumor activities have been associated to coumarins [121]. An artificial neural network model was employed to determine the optimum extraction conditions; using supercritical CO₂ with a 2% of methanol as co-solvent at 55 °C and 250 bar for 150 min, a coumarin yield of 90.1 µg/g was obtained from *Cuscuta reflexa*, a medicinal herb [121]. Another example is the extraction and analysis of β-sitosterol and stigmasterol [126]. These compounds are classified as phytosterols and they are regarded as possessing several bioactivities including the reduction of cholesterol levels, anti-inflammatory, anti-mutagenicity and prevention of cancer. The SFE extracts of *A. roxburghii* were analyzed by HPLC-APCI-MS in order to determine the amount of these compounds. The SFE

optimized conditions produced higher yields in shorter times than traditional Soxhlet methods, reaching 2.9% and 3.6% for b-sitosterol and stigmasterol, respectively [126].

In general, as it can be observed in Table 1, a wide variety of samples and compounds of interest have been studied. The optimum extraction conditions obviously varied between samples, as did the most influential parameters; among them, sample pretreatment prior extraction, entrainer addition, particle size and temperature and pressure have been studied in detail. For instance, the entrainer was the key parameter when extracting xanthenes from *Garcinia mangostana* concerning the extraction yield [139]. On the other hand, the extraction pressure was the most important in other samples, such as *Hibiscus cannabinus* [75] while high pressures were used to extract bioactive compounds from sunflower leaves [140]. Particle size was also demonstrated to influence in great extent the outcome of the extraction procedure [136]. As for sample pretreatment, no significant differences were observed when processing dried samples compared to frozen ones [140]. In summary, a careful optimization procedure was needed for each particular sample, paying special attention to the specific target compounds. Moreover, one of the fundamental advantages of SFE over other extraction techniques was repeatedly demonstrated, that is, the tuneable selectivity according to the extraction conditions, mainly extraction pressure and temperature [138,142].

A quite similar overview can be inferred from the works dealing with algae and microalgae. These organisms have been highlighted in the search for feasible new sources of natural antioxidants and functional compounds that could be employed in the food industry [157]. Both types of organisms include a huge variety of species, increasing the interest of finding, among them, new sources of bioactives. Moreover, in the case of microalgae, the production of a particular interesting compound can be further improved by changing the growing conditions at which these organisms are cultured. It is well known that the production in these organisms of certain secondary metabolites is directly related to this growing conditions, such as temperature, salinity, light or nutrients available [158]. In

Table 3, a summary of the published works in the last years concerning the use of algae and microalgae to obtain bioactive compounds by SFE is shown.

Dunaliella salina is one of the most studied microalgae; for long, this organism has been cultured at industrial scale to produce β -carotene. In fact, the amount of β -carotene this microalgae can accumulate can reach 14% of its dry weight [171]. As it has been already mentioned, carotenoids are very appreciated for their antioxidant properties, among others. Macías-Sánchez et al. [161], recently studied the SFE of carotenoids from several microalgae, including *D. salina*. Several pressures and temperatures were tested in order to maximize carotenoid extraction using supercritical CO₂ with 5% ethanol as co-solvent. The total carotenoids in the different extracts was assessed spectrophotometrically. Authors found that optimum conditions varied among the different studied microalgae, being 400 bar and 60°C the best conditions for carotenoid extraction from *Dunaliella*. This organism was, besides, the one which produced higher amount of these components [161]. In a subsequent work, the same research group compared the efficiency of SFE compared to ultrasound assisted extraction (UAE) to extract these carotenoids, as well as chlorophylls, from *D. salina* [160]. They concluded that although both techniques were comparable in terms of carotenoids extracted (using methanol and CO₂ for UAE and SFE, respectively), SFE was by far more selective since it produced higher values of the ratio carotenoid/chlorophyll extracted. The kinetics of the carotenoids SFE extraction have been also studied in detail [172]. The β -carotene isomer composition as well as the antioxidant activity of *D. salina* SFE extracts were determined [159]. HPLC-DAD using a C30 column was employed in order to effectively resolve the different β -carotene isomers present on the extracts. Moreover, a certain relationship was found between the isomeric β -carotenoid composition and the antioxidant activity of the extracts, especially according to the concentration of *9-cis*- β -carotene present. *13-cis*, *all-trans*, *15-cis* and *9-cis*- β -carotene were separated and quantified together with α -carotene [159].

Other microalgae were investigated in order to determine their composition on particular carotenoids. For instance, 87 % of total lutein in *Chlorella pyrenoidosa* was achieved at optimized SFE conditions, which included the extraction at 250 bar and 50 °C using supercritical CO₂ with 50% ethanol as modifier [165]. This carotenoid is interesting not only for its antioxidant activity but also for being useful for prevention of age-related macular degeneration. Authors found that the selectivity of SFE allowed the attainment of high purity products, therefore suggesting that the process could be used for lutein commercial production [165]. Another interesting work studied the possibility of obtaining a functional food oil rich in antioxidants from *Chlorella vulgaris* [166]. Different parameters were studied in detail, and it was concluded that the degree of crushing of the microalgae mixed with soybean oil strongly influenced the extraction recovery. The resulting enriched soybean oil presented a double effect: firstly, the pigments present contributed to oil stability due to their antioxidant effect, and in the other hand, the oil worked as a protector of the carotenoids degradation.

Carotenoids have not been the only target compounds. In fact, the extraction of γ -linolenic acid from *Spirulina platensis* by SFE has been also studied [169,170], as well as the tocopherols content in the same microalgae [167,168]. Likewise, GC-MS was employed in order to characterize the volatiles extracted by SFE from the brown alga *Dictyopteria membranacea* [163].

2.5.- Applications of SFE to drugs extraction from natural sources: analysis and isolation

SFE results in a high valuable tool when it comes to find novel pharmaceutical activities of extracts from natural matrices. In this field, plants and marine living beings are the main subjects of research. For example, mistletoe (*Viscum album L.*) has been studied for its possible cytotoxic effect on Ehrlich carcinoma cells [151]. Most pharmacological studies on mistletoe have focused on the therapeutic properties of its polar extracts; but Čebović et al. [151] treated the mistletoe leaves with CO₂ at 350 bar and 34-35 °C to obtain new extracts, whose composition was analyzed using a

common GC-MS method with a HP5 column and a temperature gradient from 50 to 350 °C. Several new terpene compounds were identified by this method including trans- α -bergamotene, trans- β -farnesene, loliolide and vomifoliol. The extracts were injected in mice before and after carcinoma induction. The largest decrease was observed in mice pretreated with the *Viscum album* extract, although significantly reduced numbers of Ehrlich carcinoma cells were also observed in animals with developed carcinoma. The activities of antioxidative enzymes in the Ehrlich carcinoma cells suggested the absence of oxidative stress. However, changes in the antioxidative enzymes activities observed after administration of the *Viscum album* extract might be due to the induction of oxidative stress in the Ehrlich carcinoma cells.

Some other pharmacological activities related to the antioxidant activity of supercritical extracts have been studied. For example, antioxidant therapy has been proposed to improve the oxidative stress status of diabetic patients. Rupérez et al. [173] performed the metabolic fingerprinting and target metabolite analysis of *Dunaliella salina* microalga extracts on diabetic rats. As seen above, *D. salina* carotenoids have been previously characterized and associated to its antioxidant activity by using SFE [159]. In this case, Rupérez et al. used extracts with proved antioxidant and antimicrobial activities obtained by using PLE [174,175] and SFE [159,176]. Urine fingerprints of control and diabetic rats, both with and without treatment, were obtained by capillary electrophoresis with two different modes (normal polarity and MEKC and reverse polarity and CZE). CE was also used for target metabolite analysis, in this case glutathione as well as four short chain organic acids (acetoacetate, 3-hydroxybutyrate, lactate, and pyruvate) and urate. Chemometric data treatment was done using multivariate data analysis (principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA) to achieve more in-depth knowledge of the results.

SF extracts have also been tested on Leishmaniasis; Soares et al. [152] studied the leishmanicidal activity of a supercritical fluid fraction obtained from *Tabernaemontana catharinensis* (jasmine).

The extract was obtained at 250 bar, 45 °C using supercritical CO₂ plus ethanol (4.6%v/v) as co-solvent, alkaloidal fraction of the extract was analyzed with TLC, GC-MS, and other spectrometric methods such as IR, UV and H¹-NMR, concluding that besides coronaridine and voacangine (two previously characterized indolic alkaloids [177]), the alkaloidal fraction contain voacangine hydroxylindolenine, voacristine, voacristine hydroxylindolenine, and 3-hydroxylcoronaridine. Authors conclude that SFE is an efficient method for the extraction of bioactive indole alkaloids from plant extracts, while retaining the alkaloids' properties associated with inhibiting *Leishmania amazonensis* amastigote replication in macrophages without incurring host cell toxicity.

Nowadays the search of new antiviral compounds is more and more important in order to fight new types of virus. SFE has also been applied to the search of those new antiviral extracts from natural sources. For example, Šmidling et al. [153] described the examined cytotoxicity and extracellular and intracellular antiviral activity of fractionated extracts of wild and cultivated sage (*Salvia officinalis*) against vesicular stomatitis virus (VSV). In this work, dried sage ethanolic tinctures were treated with SC-CO₂ at 200-500 bar and 60-100 °C during long extraction times, 6h. The main compounds found in extracts by HPLC and GC were terpenoids and phenolic terpenes. The fraction obtained with CO₂ at 500 bar and 100°C was the least toxic for human amnion epithelial cells; moreover, it had an antiviral effect at the intracellular level: when added 5 hours before VSV infection, it caused 100% reduction of CPE (cytopathic effect) at concentrations of 99.5 and 199.0 µg/ml. The obtained results indicate that antiviral activity of this extract involved inhibition of the early steps of the virus infective cycle without a direct virucidal effect.

Hippophae rhamnoides L. (Sea buckthorn) is a branched and thorny nitrogen-fixing deciduous shrub, widely spread along Europe and Asia. It has been described that Sea buckthorn has significant antioxidative activity related to its content in carotenoids and tocopherols [92], but Basu, Upadhyay et al. examined new uses of Sea buckthorn: anti-atherogenic and cardioprotective activities [155] and wound healing properties [154]. Sea buckthorn seed oil extraction was carried

out at 60 °C and at 450 bar with a gas flow of 60 g/min for 3 h. GC-FID was used to analyze the fatty acid profile of supercritical extracted seed oil, while different HPLC-DAD methods were used to analyze the content in tocopherols and sterols. In the first work [155] it was observed that the HDL-cholesterol levels, HDL-C/Total cholesterol ratio and vasorelaxant activity of the aorta were significantly increased. In cholesterol-fed animals the Total cholesterol, Triglycerides, LDL-cholesterol and Atherogenic index were significantly increased and showed a decline following seed oil administration. The presence of Omega-3 and Omega-6 fatty acids, tocopherols, phytosterols and β -carotene were pointed as responsible of cardioprotective effects of SBT seed oil to which in combination may have synergistic effects on cardiovascular health. In the second work [154] *Hippophae rhamnoides* seed oil was co-administered by two routes on experimental burn wounds in rats. The *Hippophae rhamnoides* seed oil improved the wound healing process as evidenced by a significant increase in wound contraction, hydroxyproline, hexosamine, DNA and total protein contents in comparison to control. Gelatin zymography analysis of the granulation tissue after seed oil treatment showed increased expression of metalloproteinases, while SDS-PAGE and Western blot analysis showed an up-regulated expression of VEGF and collagen type-III in SBT seed oil treated wounds as compared to untreated control burn wounds.

3.- SFE IN PHARMACEUTICS

Proprietary pharmaceutical product development is driven by continuous innovations in drug discovery, drug polymorph preparation, dosage form design, and process engineering, while meeting rigorous regulatory standards. Innovations in all these areas are feasible with the application of technologies utilizing supercritical fluids (SCFs) [178]. Pharmaceutical companies are more and more urged to develop production processes with very low environmental impact, in particular to reduce the use of volatile organic compounds in medicine manufacturing as well as to

avoid residues in the finished product. Other benefits of supercritical fluid technologies, apart from their mass transfer related properties, are linked to the reduced complexity of the process which stems from a diminution of the number of steps as well as to the improved process understanding and control [2,179].

The main uses of supercritical fluids in pharmaceutical industry include processes such as particle and crystal engineering [180], formation of complexes with cyclodextrins (CDs), coating, foaming and tissue engineering, enzymatic reactions in supercritical media, extrusion, production of liposomes and biotechnological compounds [181], purification of pharmaceutical excipients [182], sterilization, solvent removal [179], enantioselective separations [183] and, of course, extraction and purification of active principles from raw materials and from synthetic reaction media.

In general terms, the main use of supercritical fluids in pharmaceuticals deals with the extraction of bioactive compounds from a mixture (purification from reactions, quantification of active enantiomer, extraction from natural matrices...) or with the extraction of the matrix. In this case, crystallization and particle formation have undergone an enormous development in recent years.

3.1.- Application of SFE to enantiomeric separations

Chiral separation is a very important issue for the pharmaceutical industry. The applicability of SFE as an effective and green technique for enantioseparations is known since the late 90s [8,184-187]. In these processes, diastereomeric salts or complexes of the racemic compounds and resolving agents are formed before the extraction step. The selected resolving agent is added in less than stoichiometric ratio to the racemic compound. The unreacted enantiomers are extracted with the supercritical solvent, and are collected as a powder after depressurization of the solution [188]. For example Molnár et al. [188] reported a new and simple resolution for the enantiomers of *trans*-1,2-cyclohexanediol by diastereomeric complex formation with tartaric acid and subsequent

supercritical fluid extraction, and showed the influence of the extraction parameters on the resolution efficiency. Tartaric acid and its derivatives are widely used and are considered low cost chiral agents in resolutions through diastereomeric complex or salt formation [189]. Racemic trans-1,2-cyclohexanediol and (2R,3R)-(+)-tartaric acid were dissolved in ethanol; the reaction product was extracted under different conditions ($P = 100\text{--}200$ bar, $T = 33\text{--}63$ °C) with CO₂/g *rac* using a laboratory scale supercritical unit. The (1*S*,2*S*)-(+)-rich mixture was collected in the separator at 40 bar and 40 °C. The enantiomeric excesses were determined by GC-FID analysis with a chiral column with permethylated β-cyclodextrin. Optimal conditions in terms of resolution efficiency, according to a 3² factorial design, were found within the studied range of the extraction pressure and temperature. In the best cases, the (1*S*,2*S*)- and (1*R*,2*R*)-diol enantiomers were obtained with $ee_{(1S,2S)} = 62\%$ and $ee_{(1R,2R)} = 93\%$ enantiomeric excess in one equilibrium stage, respectively.

Other example of the use of SFE for the resolution of racemic mixtures by using tartaric acid was developed by Kmezc et al. [189]. In this work, tartaric acid is used in combination with derivatives (O,O'-dibenzoyl-(2R,3R)-tartaric acid monohydrate (DBTA) and O,O'-di-p-toluoyl-(2R,3R)-tartaric acid (DPTTA)), for the separation of racemic N-methylamphetamine (*rac*-MA). After partial diastereomeric salt formation, the free enantiomers were extracted by SFE using CO₂ as solvent at 160 bar and 39 °C (flow rate: 0.9–1.1 g/s). Enantiomeric excess (ee_x) values of the samples were determined by optical rotatory measurement. DBTA and DPTTA are efficient resolving agents for *rac*-MA, the best chiral separation being obtained at a molar ratio of 0.25 resolving agent to racemic compound for both resolving agents ($ee_E = 82.5\%$ and $ee_E = 57.9\%$, respectively). Compared with the two other acids, TA is practically unsuitable for enantiomer separation ($ee_E < 5\%$). As could have been seen, SFE represents a faster and easier alternative to conventional techniques for the racemic resolution. On contrast, its generally needed the use of compounds that helps the separation which may produce contaminations in the final product.

Supercritical fluid chromatography (SFC) also shows enormous advantages for chiral separations [190-195]: packed-column supercritical fluid, subcritical fluid, and enhanced fluidity liquid chromatography have gained popularity in the analytical determination of drug substances and food compounds. The advantage of SFC on chiral separation lies in the low viscosity and high diffusivity of the mobile phase with CO₂; very high flow rates of 5–10 ml/min can be used to decrease elution times while maintaining the separation efficiency [191]. SFC method development can be done in a comprehensive way, as seen in the work developed by Gham et al. [194]. Three major considerations for a successful scale-up in SFC are the impurity profile, the cycle time, and the solubility in the supercritical mobile phase. These authors use these three factors in order to assist the scale-up of the enantiomeric separation of a proprietary pharmaceutical intermediate. In this work, chiral packed columns were used for the analytical and preparative scale in combination with a UV fixed wavelength detector. But SFC can be applied for chiral separation coupled to other detectors, such as MS/MS [195]. Coe et al. [195] used SFC-MS/MS for fast bioanalysis of R/S-warfarin in human plasma, and developed a pSFC-MS/MS (preparative-SFC-MS/MS) method two-fold faster than previously described. But probably the main development in the supercritical fluid field for the enantiomeric separation is the Simulated Moving Bed (SMB) technology, which can be defined as a continuous multi-column chromatographic process. This technology has been thoroughly reviewed recently by Rajendran et al. [190].

3.2.- Solvent removal and new drug delivery formulations

In order to enhance the bioavailability of poorly water-soluble drugs, an increasing number of pharmaceutical formulation technologies are being developed; these include micronization, complex formation and solid dispersions [196]. In the case of polar compounds which are not soluble in supercritical fluids (particularly CO₂), SCFs could be used as antisolvent; in this process, a solution consisting of an organic solvent, completely miscible with the SCF, and a solid material

dissolved in this solvent, is sprayed into a high-pressure vessel filled with SCF [197]. In these processes the supercritical fluid is used to extract the solvent instead of the analyte. In recent years, several reviews [7,179,180,198,199] and original researches [200-212] have dealt with particle formation processes. Some examples

Crystallization is one of the most widely purification techniques used after a chemical synthesis. The purification and fractional crystallization using a gas anti-solvent (GAS) type SCF process exploits the sharp change in solvation power that occurs as a solvent expands. Unlike conventional crystallization processes, supersaturation in gas anti-solvent crystallization processes depends on pressure; therefore, solutes with differing solubility will precipitate at different stages of expansion. A typical scheme of a supercritical crystallization apparatus can be seen in Figure 2. Thus, SCF-CO₂ processing allows the isolation of certain process impurities from active pharmaceutical ingredients (APIs) which may include reaction intermediates, enantiomers and additives. Nucleation occurs at the point where the solute concentration exceeds the solubility in the solution [7]. Generally, the success of supercritical antisolvent precipitation process depends on the solubility of the liquid solvent in the supercritical antisolvent and based on the fact that the solute is not soluble in the antisolvent. It also depends on the fast solubilization of the liquid due to the gas-like diffusion characteristic of SCF; this characteristic is fundamental to obtain small particles.

Argemí et al. [212] described in very thorough way the spectroscopic and chromatographic characterization of triflusal (2-acetoxy-4-(trifluoromethyl) benzoic acid) delivery systems prepared by using supercritical impregnation technologies. Triflusal is an antithrombogenic drug structurally related to acetylsalicylic acid. Characterization assays comprised the determination of the percentage of triflusal and its degradation product impregnated in polymeric supports and further monitoring of the releases of the two drug components over time in physiological conditions. The analytical methods employed consisted in a continuous-flow spectroscopic system to measure the

evolution of the drug concentration in the solution as a function of time, and an HPLC-UV method to determine the percentage of impregnation and to monitor the drug release. Also, the chromatographic approach provided additional information regarding the presence of triflusal hydrolysis product and its evolution over long periods of time. From the set of samples prepared at different experimental conditions, the best behavior from a pharmaceutical point of view corresponded to the case of poly(methyl methacrylate) beads impregnated according to the supercritical procedure for 48 h at 190 bar and 40 °C. In these samples, the amount of triflusal impregnated corresponded to an 11.4% and a constant release in aqueous media was expected to be longer than 2 months.

Wu et al. [200] studied the formation of solid dispersions of a poorly water-soluble drug piroxicam (a non-steroidal anti-inflammatory drug) in polyvinylpyrrolidone (PVP) comparing a supercritical fluid crystallization process and classical spray drying. Physicochemical properties of the products and drug-polymer interactions were characterized by powder X-ray diffraction, Fourier transform infrared spectroscopy, differential scanning calorimetry and HPLC-UV-Vis. Both processes resulted in improved dissolution of piroxicam; by comparison, supercritical processed solid dispersions showed distinctly superior performance dissolving piroxicam completely. Solutions were injected into the precipitation chamber through the inner capillary and meanwhile steady flow of CO₂ was introduced through the outer nozzle. After injection, compressed CO₂ flushed through the precipitation chamber to extract the residual organic solvent from the product. Authors demonstrated that supercritical processing is more powerful in controlling particle size. The physical transformation nature of this technique seems not to interfere with the interactions between drug and carrier, making possible the use of this method to control the in vitro performance without compromising the stability issue.

Salmaso et al. [201] prepared homogeneous dispersions of insulin and recombinant human growth hormone (rh-GH) in tristearin/phosphatidylcholine/PEG mixtures supercritical carbon dioxide gas

micro-atomisation to produce protein-loaded lipid particles. In this work [201], a home-made equipment “supercritical gas-assisted melting atomization process” [202] was used, this apparatus was pressurized with CO₂ at 150 bar and heated to 40 °C; several analytical techniques were applied to the homogeneous dispersions obtained, namely: differential scanning calorimetry analysis (DSC), transmission electron microscopy (TEM) and UV-Vis spectrophotometry. The insulin and rh-GH content in the lipid particles was assessed by DMSO extraction and HPLC analysis. With this procedure insulin could be administrated orally producing a significant hypoglycaemic effect comparable with the classical injected drug, leading relative pharmacological bioavailability of 7%. The importance of finding alternative ways for the administration of insulin was also studied by Kim et al. [203], who examined the effect of stabilizers on the physicochemical characteristics of the aerosol of inhaled insulin particles produced using a supercritical fluid technology. Growth hormone prepared using supercritical fluids [201] and administrated orally produced a relative pharmacological bioavailability of 3.4%.

Chu et al. [204] prepared fine particles of cefpodoxime proxetil (CPD) using a slightly different particle formation process called Aerosol Solvent Extraction System (ASES) with supercritical CO₂. CPD is an oral third generation cephalosporin, active against most Gram positive and Gram negative organisms. In this work, the degree of agglomeration was reduced using a high CO₂-to-solution weight ratio, and a low solution concentration. In particular, spherical particles, approximately 0.1-0.4 µm in size, were obtained when a 10.0 wt% CPD solution was used. As a result of the dissolution study, almost 90% of the processed CPD had dissolved within 10 min. The recovery yield of the CPD powder reached approximately 80% using a membrane filter.

Vatanara, Rouholamini et al. have studied the use of supercritical crystallization of several antiasthma drugs such as salbutamol sulphate [205] or fluticasone [206] using solution enhanced dispersion by SC-CO₂. The use of supercritical fluid extraction of the matrix, coupled to a controlled crystallization facilitates the inhalatory administration of those drugs. In these studies the

morphology and size distribution of precipitated particles were characterized using scanning electron microscope (SEM) and laser diffraction particle size analyzer respectively. FTIR spectra was used before and after processing to assess crystal modifications, and the amount of active principles determined using the HPLC methods of the European Pharmacopoeia.

Among the various approaches that have been used to improve the solubility and dissolution rate of drugs, complexation with cyclodextrins is one of the most promising. Cyclodextrins are cyclic oligomers of glucose with cone-like structures, whose exterior surface has hydrophilic properties, while the interior is hydrophobic in nature. This particular characteristic of cyclodextrins allows them to form non-covalent inclusion complexes with various drugs of proper size and polarity leading to changes in their physicochemical and biopharmaceutical properties, which enhance their solubility, dissolution rate, chemical stability and bioavailability and reduce their side effects and toxicity [213]. The use of SC CO₂ has been recently proposed for the preparation of various drug–cyclodextrin inclusion complexes for enhanced solubility and dissolution rate. The SC CO₂-inclusion method of cyclodextrins of several antifungal drugs such as itraconazol [214], econazole and fluconazole [215] have been studied. In both works higher inclusion yields were obtained in the SC CO₂ method as compared to physical mixing and co-precipitation methods. In vivo drug pharmacokinetic studies showed that the drug-β-cyclodextrin product prepared using SC CO₂ display higher bioavailability (in blood, liver and kidney of rats) as compared to the products obtained by conventional procedures. In the SC CO₂ inclusion method, the reaction cell was filled with a physical mixture of drug and β-CD. The system was then pressurized and heated up to the desired pressure and temperature. After keeping the system in a static mode for 3 h, at 450 bar and at temperatures ranging 50-130 °C, CO₂ with solvent residue was extracted from the cell. The solubility of fluconazole in SC-CO₂ was significantly larger than that of itraconazole and econazole. The higher solubility of fluconazole and the possibility of fluconazole to be melted at the SC CO₂ conditions lead to higher extent of interaction between the drug and β-CD as compared to

itraconazole and econazole, which was verified by DSC, FTIR and PXRD analysis. *In vivo* drug pharmacokinetic studies showed that ingestion of drug complexed with β -cyclodextrin using the solvent-free SC CO₂ results in a higher bioavailability of the drug as compared to the products obtained by physical mixing or co-precipitation method, indicating the superiority of the SC CO₂ method. Moreover, antifungal- β -cyclodextrin complexes prepared using SC CO₂ made the drug available to liver and kidney tissues for at least 6 h after ingestion, therefore, the dose could be lowered.

But not only cyclodextrins have been under research to form more soluble drugs complexes; a novel group of lipid based drug delivery systems, called pharmacosomes, is also growing [216]; their uses have been recently reviewed by Semalty et al. [216]. Pharmacosomes provide better biopharmaceutical properties to the drug, resulting in improved bioavailability. Li et al. [217] compared the physicochemical characteristics of the phospholipids complex of puerarin (an isoflavone used to treat fever, pain, diabetes, measles, diarrhea, and cardiovascular diseases including coronary artery disease, arrhythmia and hypertension) prepared by traditional methods (solvent evaporation, freeze-drying and micronization) and a supercritical fluid (SCF) technology. The phospholipid-puerarin complexes were prepared as follows: the reaction vessel was filled with puerarin and phospholipids solutions and CO₂ was added at 100 bar and 38°C, and left for 3 h. A pure constant carbon dioxide flow rate of 25 ml/min was then maintained in order to completely remove the residual solvent. Drug-phospholipids interactions were characterized using FTIR. SEM was used to determine any morphological changes. Pharmaceutical performance was assessed in dissolution rate and solubility tests using UV spectroscopy. The complexes prepared by using the described protocol exhibited more rapid dissolution and showed some advantages in terms of particle size and morphology.

4.- ENVIRONMENTAL APPLICATIONS OF SFE

There is no doubt that the industrial activity of mankind has generated too much carbon dioxide giving rise to an imbalance in the biosphere; perhaps ironically, the use of SCF carbon dioxide can significantly help to reduce further CO₂ emissions if the need to transport and incinerate waste organic solvents from manufacturing processes is decreased [2]. In fact, several recently developed applications of supercritical fluids not only tend to eliminate organic solvents, but also to reduce the environmental impact of human activities. In this sense, applications like removal of heavy metals from soils, sludges and wastes, reduction of secondary wastes generation, regeneration of inactive catalysts or methods for treating soils contaminated with non polar compounds (PAH, PCB...) are being studied [218,219].

4.1.- SFE in food Toxicology and Ecotoxicology

There are several compounds with serious health implications which determination can be done using SFE, the main areas of application include food toxicology and ecotoxicology. In addition, SFE has been adopted by the EPA as a reference method for extracting Petroleum Hydrocarbons (Method 3560, in 1996) PAHs (Method 3561, in 1996) and PCBs (Method 3562, in 2007) from solid environmental matrices. SFE has been recently used for extracting POPs from different plant materials [220], and several analytical applications dealing with POPs extraction from different animal tissues have also been reported [5].

As seen in Table 4, SFE is used to measure contaminants in sediments. It is important to quantify the impact of human activities in the environment, and soils and sediments are good markers of this impact. For example, Miyawaki et al. [221] developed a very fast strategy to quantify polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs),

polychlorinated biphenyls (DL-PCBs) and dioxins in soils and sediments. Farming and industrial soils samples were extracted using SC-CO₂ with water as modifier, at 300 bar, 130 °C and 50 min; contaminants were trapped using a SPE cartridge filled with alumina (maintained at 150°C) and eluted with hexane; their concentration was quantified by GC-MS. In order to evaluate the usefulness of this protocol, authors compared to the results obtained using the classical clean-up procedure followed by Soxhlet extraction. The average concentration of PCDD/DFs and DL-PCBs were comparable between techniques, and the reproducibility of the SFE method was below 21% of the relative standard deviations for all samples. Some advantages of the new protocol were the total analysis time, only 2 h, and the possibility of analyzing the extracts eluted from the solid phase trap without a further clean-up procedure. On contrast the classical procedure needs near 3 days for the total analysis.

In a similar way as seen for dioxins and PCB, other residues of human activities can be extracted and quantified by SFE. Mmualefe [232] evaluated the impact of agriculture in the Okavango river delta, by assessing the levels of 15 pesticides. In this work, three different extraction conditions were compared: pure CO₂ (one step: 400 bar, 60 °C), CO₂+H₂O (two steps: 60 °C, 200 bar and 60 °C, 400 bar) and CO₂+acetone (three steps: 60 °C, 200 bar; 60 °C, 350 bar and 60 °C, 400 bar). GC-TOF was used to identify the pesticides extracted, while GC-ECD was used for quantification purposes with external standard calibration. CO₂ + acetone as modifier and fractional extraction by pressure ramping gave the highest recoveries ranging from 55 to 86% for the 15 pesticides studied. The study showed an increase of pesticide concentration in the direction of the water flow from the Panhandle (point of entry) to the lower delta; these results demonstrated a clear need for a close monitoring of our natural resources for long-term preservation purposes.

Along with soils and sediments, algae and microalgae are the best indicators of contamination of water environments. SFE was used by Lage-Yusty et al. [235] to measure the residual levels of PAHs in edible seaweed, five years after an oil spill. Dehydrated seaweeds were treated at 100 °C and 256 bar during 50 min in continuous-flow mode at a rate of 1.5 mL/min of supercritical CO₂. In order to maximize the recovery of PAHs, a C18 trap was used to collect the extracts. After extraction, PAHs were eluted using acetonitrile and analyzed by HLPC-FLD. The levels found were below maximum limits established by the Spanish Food Safety authority (<200 mg/kg dry weight); meaning that no relevant effects were detected in terms of PAHs contamination in seaweed.

But not only inorganic or vegetable material can be used to measure contamination. Fish, for instance, is also a good indicator of environmental contamination, mainly in its fat fraction [223,237]. A single-step extraction and clean-up procedure was used by Rodil et al. [223] for the determination of 15 organohalogenated pollutants in aquaculture products (turbot, clam, mussel and cockle). The extraction procedure was screened by a fractional factorial design for the preliminary statistically significant parameters. To reduce the number of experiments, a fractional factorial 2⁵⁻¹ design, leading to 16 experiments, was used. The extraction temperature (60–120 °C), pressure (140–320 bar), static extraction time (5–20 min), dynamic extraction time (10–60 min) and carbon dioxide flow rate (1–2.5 ml min⁻¹) were studied. A Doehlert design, followed by a multicriteria decision-making strategy, was then performed in order to determine the optimum conditions for pressure and dynamic extraction time. As previously, a C18 trap was used to maximize the recovery of organohalogenated compounds. Under optimal conditions, the procedure developed along with GC-ECD and GC-MS/MS provides an excellent linearity, detection (0.01–0.2 ng.g⁻¹) and quantification limits (0.05–0.8 ng.g⁻¹) for most of the analytes investigated.

The last example of SFE application to toxicology consists on the measurement of veterinary drug residues developed by Abd El-Aty et al. [236]. The method consisted on the quantification of

orbifloxacin (third-generation fluoroquinolone antibacterial drug used in ruminant farming) in plasma and milk. Samples were adsorbed in filter paper and then extracted using SFE with SC-CO₂ containing methanol as modifier. The extraction conditions studied were 250-320 bar and 60-150 °C. The extracts were collected in methanol previous to the HPLC-FLD analysis. The analytical method was developed and validated in this work, and was applied to a pharmacokinetic study for evaluating the main pharmacokinetic parameters of orbifloxacin in plasma and milk of seven lactating does; the method showed an appropriate sensitivity, demonstrating the usefulness of SFE for future application in pharmacokinetic and clinical studies, both in human and animal medicine.

4.2.- Metals recovery using supercritical fluids

Removal of heavy metals from solid matrices and liquids remain a big challenge and, although various methods have been described for this purpose, SFE seems to be one of the most promising [219]. Complexing agents used in conventional solvent extraction can also be used in SFE complexation of metal ions, provided that they are soluble in supercritical CO₂. Solubilities of metal complexes in supercritical CO₂ vary significantly depending on the chemical nature of the complexes. Numerous chelating or complexing agents have been employed in the SFE of heavy metals, such as diisooctyl-thiophosphinic acid (Cyanex 302), sodium diethylthiocarbamate (Aliquat 336), bis(2-ethylhexyl)phosphoric acid) and bis(2-ethylhexyl)monothiophosphoric acid [219]. In fact, most of recent works deal with the optimization of the chelating agent to extract metals from different matrices, Table 5.

Wang & Chiu [239] developed a green method to remove chromated copper arsenate (CCA) from wood before its dumping, incineration, reuse or disposal, using supercritical carbon dioxide (ScCO₂) containing an organophosphorus reagent, Cyanex 302. Chromated copper arsenate has been used extensively as a wood preservative since 1940. After shelf life, treated wood caused

environmental hazard, therefore, metal removal is highly important. In this work, the extraction efficiency was improved by adding a chelating agent to the wood sample. The extraction was performed at 200 bar and 60 °, using CO₂ + 5% methanol as extracting solvent and considering 20 min static extraction followed by 40 min dynamic extraction. The eluent released from SFE system was trapped in a 30 ml vial with 10 ml of methanol inside. Extraction residues were analyzed by neutron activation analysis (NAA) and ICP-AES. The extraction efficiencies for the extraction of CCA were in the order of Cu >> As > Cr. The extraction profiles descend rapidly, followed by a plateau of metal extractions, suggesting that the process is limited to the leachable toxic heavy metals. By increasing pressure and static extraction time, the process efficiency can increase to 95% (Cu), 66% (As) and 50% (Cr), respectively, after eight successive batchwise extractions. The *in situ* chelation/SFE showed advantages over conventional solvent extraction, in which it greatly minimizes the generation of secondary wastes in decontamination processes and helps alleviate the land filling and potential chromated copper arsenate contamination problems.

Organophosphorus chelating agents are highly useful when it comes to extract metals by using SFE; they have even demonstrated its usefulness by extracting radioactive metals such as thorium [240]. Kumar et al. investigated the feasibility of supercritical CO₂ extraction of thorium from tissue paper using various organophosphorus reagents (TBP, TOPO, TPP, TPPO, TBPO). Optimum extraction conditions were: 200 bar, 60 °C, 2 mL min⁻¹ (CO₂ flow rate), 20 min static time and 20 min dynamic extraction time. The maximum extraction efficiency was obtained with 0.2 M TOPO in methanol where thorium was extractable with 68 ± 4% efficiency. Thorium is not the only radioactive metal that have been extracted using SFE, Gavrilesco et al. have reviewed recently the possibilities of SFE in the remediation of soils contaminated with uranium [246]. In fact, the surface decontamination of radioactive metal wastes using acid-in-supercritical CO₂ emulsions have been proposed by Koh et al. [241] as suitable candidate to decontaminate radioactive metal wastes using

an environmentally friendly process. Microemulsions were prepared at a molar ratio ($W = 20$) between the surfactant (NP-4) and water into the cell at 40 °C and 200 bar. After 1 h, microemulsions were transferred to a separator by depressurization, water droplets in unstable microemulsions started to agglomerate to each other and were finally separated from the surfactant. Then, with the pressure and temperature being maintained, the surfactant, still dissolved in CO₂, was separated from the water droplets and collected through dynamic flushing of CO₂. Finally, the collected surfactants were analyzed by HPLC. Organic acid-in-CO₂ microemulsions achieved over 95% efficiency, while using inorganic acid decontamination efficiencies were about 90%. In this process, 73% of the total surfactants was recovered and could be reused, which means that secondary waste can be minimized considerably. Therefore, the supercritical fluid surface decontamination may be an effective candidate for surface decontamination of radioactive metals in the near future.

Other applications of supercritical fluids in the environmental field deal with their use as oxidizing agents. Supercritical water oxidation has been widely studied [247-250]; these processes always use very high temperatures (>400 °C) and mild pressures (200-300 bar). At these conditions, water becomes a strong oxidizer, therefore, by using adequate chelating agents, the heavy metal extraction can be controlled. In fact, some authors even used H₂O₂ to favour the oxidation of metals, for example Bo et al. [248] investigated the application of H₂O and H₂O₂ to the elimination of heavy metals in medical waste incinerator fly ash. By using 320–420 bar and 450 °C during 1-4 h, heavy metals in exchangeable and carbonate forms in the ashes could be transferred into other relatively stable forms (Ba and Cr into residual fraction; Cu and Pb into organic matter fraction). H₂O₂ treatment could stabilize heavy metals in Fe–Mn oxides and residual fractions; however, the behaviour of As was quite different from heavy metals, and was extracted by H₂O and H₂O₂ after the treatment. Therefore, in this process, supercritical oxidation and extraction coexist. Authors

concluded that SC H₂O and H₂O₂ treatments could not only decompose hazardous organic matters but also effectively detoxify heavy metals in fly ashes.

5.- CONCLUSIONS

In this review, the interest of SFE not only as an analytical tool but also for process development is discussed. It seems clear from the literature that SFE has an enormous interests nowadays, with more than 200 references dealing with SFE in the last two years (2007-2009). In this review we have tried to widen the range of applications of SFE, including not only its use as sample preparation technique but also new and recent advances in different areas such as food science, pharmaceutical and environmental science. Readers are encouraged to treat the information provided as a tool to develop new processes at lab and pilot scale, to discover new ways for sample preparation, to learn how to deal with SFE optimization and, certainly, to be able to develop in the future emerging technologies able to fulfil the requirements of environmentally clean processes.

Thus, in this review, aspects such as factors that influence the SFE processes (temperature, pressure, enhancement effects, mode of operation, etc.) are discussed using examples. Also, new emerging technologies, for instance the use of supercritical fluids in particle design, sterilization, and separation of enantiomers, among others, are presented.

It seems clear that SFE is now a real option for product development, mainly those that will be used for human consumption, such as new foods, food ingredients/additives or pharmaceutical products. Moreover, SFE has also demonstrated some advantages in the environmental field; for example, to reduce solvent waste, to get new useful compounds from industrial byproducts, and to allow quantification and/or removal of toxic compounds from the environment.

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FIGURE CAPTIONS

Figure 1.- Scheme of a SFE-CE online coupling apparatus: M. Zougagh, A. Ríos “Supercritical fluid extraction as an on-line clean-up technique for determination of riboflavin vitamins in food samples by capillary electrophoresis with fluorimetric detection” *Electrophoresis* 29 (2008) 3213. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

Figure 2.- Scheme of the typical supercritical antisolvent precipitation equipment

Table 1.-. Summary of the works published on the recovery of valuable compounds from industrial by-products using SFE in the period 2007-2009

Matrix	Compounds	Extraction conditions	Other features	Analytical technique	Ref.
Rice wine lees	Polyphenols	CO ₂ , 80 bar, 30 °C	Antioxidant activity measurement: DPPH. Comparison with Soxhlet	UV Spectrometry	[88]
Tomato	Tocopherols and phytosterols	CO ₂ , 300bar, 40 °C	Full factorial response surface experimental design	HPLC-DAD (290, 320 and 360 nm), GC-FID	[89]
	Lycopene	CO ₂ + Cosolvents, 250-450 bar, 40-70 °C	Central composite rotatable design	HPLC-DAD (460 nm)	[90]
Tomato & hazelnuts	Lycopene and essential oils	CO ₂ , 400 bar, 60 °C	--	HPLC-UV (C30 stationary phase)	[91]
Sea buckthorn	Carotenoids and tocopherols	CO ₂ , 300-460 bar, 40-90 °C	Full factorial response surface experimental design	TLC, HPLC-FLD (Ex. 295nm, EM. 330 nm)	[92]
Palm-pressed mesocarp fiber	Sterols, carotenoids and tocopherols	CO ₂ , 100-300 bar, 40 °C	Continuous extraction 1-6 h	GC-FID, HPLC-DAD	[93]
Hake by-products	PUFA's	CO ₂ , 250bar, 40 °C	Freeze dried, Antioxidant activity measurement: TBARS	GC-FID	[94]
Sugar cane crude wax	Long chain <i>n</i> -alcohols	CO ₂ , 300-350bar, 50-100 °C	Full factorial response surface experimental design	GC-FID	[95]
Pomegranate seeds	Polyphenols	CO ₂ , 130-470 bar, 40-70 °C	Central composite rotatable design	GC-FID, HPLC-UC (292 nm)	[24,96]
Loquat seeds	Phytosterols	CO ₂ , 200-450bar, 40-80 °C	Comparison with Soxhlet	HPLC-DAD (210 nm)	[97]
Tea stalk	Caffeine	CO ₂ , 200 bar, 55 °C	--	HPLC-UV (272 nm)	[98]
Wax derivatives	Alcohol ethoxylates	Water and Toluene, 130 bar, 120 °C	--	GC, GPC, TOC	[99]
Fresh palm-pressed fiber	Water-soluble compounds	CO ₂ , 300 bar, 60 °C	Antioxidant activity measurement: FRAP and TEAC	GC-MS	[100]
Corn oil	Transesterification products	CO ₂ , 440 bar, 100 °C	Lipozyme treatment	HPLC-UV-ELSD (205 nm)	[101]
Brewer's spent grain	Tocopherol	CO ₂ , 350 bar, 40 °C	Economic analysis	HPLC-UV (290 nm)	[18]
Spent activated carbon	2,2,3,3-tetrafluoro-1-propanol	CO ₂ , 89-190 bar, 30-60 °C	Cold trap	GC-FID	[102]
Lignin residues	Glycerol, guaiacol	Water, n.a. press., 400 °C	--	GC-MS, GC-FID	[103]
Canola flakes	PUFA's	CO ₂ , 207 bar, 35 °C	Fungal fermentation	GC-MS	[104]
Wood biomass	Bio-crude	Methanol, 20-100 bar, 200-400 °C	SC-methanol	GC-TCD, GC-MS	[105]
Aqueous waste streams	Organic solvents	CO ₂ , 100 bar, 30-50 °C	--	GC-FID	[106]
Silkworm pupae	Oil	CO ₂ , 200-300 bar, 35-45 °C	Chemometric optimization	GC-MS, GC-FID	[107]
Pine (<i>P. Pinaster</i>) bark	Antioxidants	CO ₂ +Ethanol 100-300 bar, 30-50 °C	Comparison with Soxhlet	TLC, GC-MS, HPLC-UV (280 nm)	[108]

Table 2.-. Summary of the works published on the extraction of bioactive and interesting compounds from plants by SFE in the period 2007-2009

Plant material	Compounds of interest	Related functional activities	Extraction conditions	Analytical technique	Ref.
<i>Cynanchum paniculatum</i>	Paeonol	Anti-inflammatory, antidiabetic, cardiovascular protective	CO ₂ + methanol, 150 bar, 55 °C, 20 min (static) + 90 min (dynamic)	HSCCC, HPLC-DAD	[118]
<i>Ramulus cinnamomi</i>		Antioxidant, antimicrobial	CO ₂ , 230-410 bar, 40-50 °C	-	[119]
<i>Cassia tora</i> L. seeds	Volatile oil	Antioxidant	CO ₂ + ethyl acetate (10%), 250 bar, 45 °C	GC-MS	[120]
<i>Cucscuta reflexa</i>	Coumarin	Anti-fungicidal, anti-tumor	CO ₂ , 248 bar, 55 °C (optimized through artificial neural network model)	HPLC-UV (280 nm)	[121]
Cardamom (<i>Elettaria cardamomum</i>)	Volatiles, fatty acids, tocopherols	Antioxidant	CO ₂ , 300 bar, 35 °C	GC-MS; HPLC-FD (ex: 295 nm, em: 330 nm); HPLC-DAD	[122]
Black cumin (<i>Nigella sativa</i>)	Essential oil	Antimicrobial	CO ₂ , 400 bar, 40 °C, 35 min	-	[123]
<i>Rhodiola rosea</i> roots	Rosavin	Antioxidant, anti-stress, among others	CO ₂ + water (10%), 200 bar, 80 °C, 3 h	HPLC-UV (254 nm)	[124]
Ginger (<i>Zingiber corallinum</i> Hance)	Essential oil	Antipyretic	CO ₂ + methanol, 100 bar, 30 °C, 40 min	GC-MS	[125]
<i>Anoectochilus roxburghii</i>	Phytosterols	Anti-inflammatory, anti-cancer	CO ₂ + ethanol, 250 bar, 45 °C, 1 h (static) + 1 h (dynamic)	HPLC-APCI-MS	[126]
Valerian (<i>Valeriana officinalis</i> L.)	Valerenic acid	Tranquilizing	CO ₂ + ethanol, 360 bar, 37 °C, 20 min	GC-MS	[127]
	Sesquiterpenes		CO ₂ , 150 bar, 50 °C,	GC-FID; GC-MS	[131]
<i>Stevia rebaudiana</i>	Glycosides	Anti-inflammatory, diuretic, among others	CO ₂ , 211 bar, 80 °C, 60 min	HPLC-UV (210 nm)	[128]
<i>Braccharis dracunculifolia</i>	Phenolics	Antioxidant	CO ₂ , 400 bar, 60 °C, 20 min	HPLC-UV (280nm)	[130]
<i>Borago officinalis</i>	Seed oil		CO ₂ , 200 bar, 50 °C, 2.5 h (dynamic)	HPLC-DAD	[132]
Coriander (<i>Coriandrum sativum</i> L)	Volatile oil		CO ₂ , 90 bar, 40 °C, 100 min	GC-MS	[129]
	Isocoumarins		CO ₂ , 80 bar, 35 °C, 2 h (dynamic)	High-speed counter-current chromatography (HSCCC)	[133]
Chamomile (<i>Matricaria chamomilla</i>)	Essential oil	Anti-inflammatory, anti-spasmodic	CO ₂ , 250 bar, 40 °C, 90 min (dynamic)	HPLC-UV (220 nm)	[134]
<i>Vitex agnus castus</i>	Diterpenes, triterpenes, casticin		CO ₂ , 450 bar, 45 °C, 4 h (dynamic)	TLC; GC; HPLC	[135]
Lotus (<i>Nelumbo nucifera</i>)	Germ oil	Antioxidant	CO ₂ , 320 bar, 50 °C, 2 h (dynamic)	GC-MS	[137]
<i>Eugenia uniflora</i> fruits	Carotenoids	Antioxidant	CO ₂ , 250 bar, 60 °C, 120 min (dynamic)	HPLC-DAD (450 nm)	[138]
<i>Garcinia mangostana</i>	Xanthones	Antioxidant	CO ₂ + ethanol (4%), 200 bar, 40 °C	HPLC-ESI-MS	[139]
Sunflower		Natural herbicide	CO ₂ , 500 bar, 50 °C, 15 min	-	[140]

<i>(Helianthus annuus)</i> leaves			CO ₂ + water (2 %), 380 bar, 50 °C, 120 min	-	[141]
<i>Hyssop (Hyssopus officinalis L.)</i>	Sabinene	Anti-fungal, anti-bacterial	CO ₂ + methanol (1.5% v/v), 100 bar, 55 °C, 30 min (dynamic)	GC-MS	[142]
	Essential oil		CO ₂ , 90 bar, 40 °C (dynamic)	GC-MS	[136]
<i>Pinus sp.</i>	Flavonoids	Antioxidant act.	CO ₂ + ethanol (3% v/v), 200 bar, 40 °C	HPLC-UV (280 nm)	[143]
Rosehip (<i>Rosa canina</i>)	Carotenoids	Antioxidant	CO ₂ , 450 bar, 80 °C, 150 min	HPLC-UV (450 nm)	[144]
	Fatty acids		CO ₂ , 450 bar, 80 °C, 3 h (dynamic)	GC-FID	[145]
<i>Hibiscus cannabinus</i>	Oil	Antioxidant	CO ₂ , 200 bar, 80 °C, 150 min	-	[146]
<i>Salvia officinalis L.</i>	Essential oil		CO ₂ , 128 bar, 50 °C, 3 h (dynamic)	GC-FID	[147]
<i>Satureja fruticosa</i>	Volatiles		CO ₂ , 90 bar, 40 °C – two separators	GC-FID; GC-MS	[148]
<i>Eremanthus erythropappus</i>	α-bisabolol	Anti-inflammatory	CO ₂ , 150 bar, 40 °C (dynamic)	-	[149]
<i>Vativeria zizanioides</i>	Volatile oils		CO ₂ + ethanol (5%), 200 bar, 40 °C, 5 h	TLC; GC-FID	[150]
<i>Viscum album L.</i>	Cytotoxic compounds	anticancer drugs	CO ₂ , 350 bar, 35 °C	GC-MS	[151]
<i>Tabernaemontana catharinensis</i>	Antileishmaniasis drugs		CO ₂ +Ethanol, 250 bar, 45 °C	TLC,GC-MS, IR, UV and H1-NMR	[152]
<i>Salvia officinalis</i> ethanolic tinctures	Antiviral compounds	Anti tomatitis virus	CO ₂ , 200-500 bar, 60-100 °C	HPLC, GC	[153]
<i>Hippophae rhamnoides</i>	Coagulation related compounds	Antithrombotic and antiaterogenic	CO ₂ , 450 bar, 60 °C	GC-FID, HPLC-DAD	[154,155]
Bamboo shavings	Triterpenoids	antifatigue	CO ₂ +Co-solvents, 250-350 bar, 50-65 °C	Biochemical kits	[156]

Table 3.- Summary of the works published on the extraction of bioactive and interesting compounds from algae and microalgae by SFE.

Specie	Compounds of interest	Related functional activities	Extraction conditions	Analytical technique	Ref.
<i>Dunaliella salina</i>	β -carotene isomers	Antioxidant	CO ₂ , 437 bar, 40 °C, 10 min (static) + 90 min (dynamic)	HPLC-DAD (450 nm)	[159]
	Carotenoids, chlorophyll	Antioxidant	CO ₂ , 400 bar, 60 °C, 180 min (dynamic)	UV spectrometry (665, 480 nm)	[160]
	Carotenoids, chlorophyll	Antioxidant	CO ₂ + ethanol (5%), 400 bar, 60 °C, 3h (dynamic)	UV spectrometry (665, 480 nm)	[161]
<i>Nanochloropsis gaditana</i>	Carotenoids, chlorophyll	Antioxidant	CO ₂ + ethanol (5%), 500 bar, 60 °C, 3h (dynamic)	UV spectrometry (665, 480 nm)	[161]
<i>Synechococcus</i> sp.	Carotenoids, chlorophyll	Antioxidant	CO ₂ , 300 bar, 50 °C, 3h (dynamic)	UV spectrometry (665, 480 nm)	[161]
	Carotenoids, chlorophyll	Antioxidant	CO ₂ , 300 bar, 50 °C, 3 h (dynamic) - carotenoids CO ₂ , 500 bar, 60 °C, 3 h (dynamic) - chlorophyll a	-	[162]
<i>Dictyoperis membranacea</i>	Volatiles	Antimicrobial	CO ₂ , 91 bar, 40 °C, 30 min (dynamic)	GC-MS	[163]
<i>Chlorella pyrenoidosa</i>	Lutein	Prevention macular degeneration	CO ₂ + ethanol, 400 bar, 32 °C, 3 h (dynamic)	-	[164]
	Lutein	Antioxidant	CO ₂ + ethanol (50%), 250 bar, 50 °C, 4 h (dynamic)	HPLC-DAD	[165]
<i>Chlorella vulgaris</i>	Carotenoids, fatty acids	Antioxidant	CO ₂ + ethanol, 300 bar, 40 °C	GC-FID; HPLC	[166]
<i>Spirulina platensis</i>	Vitamin E	Antioxidant	CO ₂ , 220 bar, 83.3 °C (dynamic)	HPLC-FD (ex. 295 nm, em. 350 nm)	[167,168]
	γ -linolenic acid	Antimicrobial	CO ₂ + ethanol, 400 bar, 40 °C, 1 h (dynamic)	GC-FID	[169,170]

Table 4.- Summary of the works published on the extraction of toxic compounds by SFE in the period 2007-2009

Compound	Matrix	Extraction conditions	Other features	Analytical technique	Ref.
Dioxins and PCB	Sediments and soils	CO ₂ +H ₂ O, 300 bar, 130 °C	Compared with soxhlet	GC-MS	[221]
Polycyclic and nitro musks	municipal wastewater	CO ₂ , 358 bar, 80 °C	Silica sorbent trapping	GC-MS	[222]
15 organohalogenated pollutants	Turbot, clam, mussel and cockle from aquaculture	CO ₂ , 210 bar, 60°C	Clean-up involved different adsorbents, complete factorial design	GC-MS/MS	[223]
Fluoroquinolones	Pig tissues	CO ₂ +Methanol, 300 bar, 80 °C	Na ₄ EDTA inner matrix	HPLC	[224]
Organochlorine pesticide and heavy-metal	Epimedium brevicornum Maxim	CO ₂ , 150 bar, 60 °C	Compared with soxhlet	GC, HPLC, AAS	[225]
Radiolitic hydrocarbons	Irradiated cheese	CO ₂ , 150 bar, 80 °C	Tenax trap	GC-MS	[226]
PAH	earthworms in manufactured-gas plant site soils	CO ₂ , 200 bar, 50 °C	Compared with soxhlet	Headspace GC-MS	[227]
Insecticides	Melon	CO ₂ , 300 bar, 50 °C	Hydromatrix and anhydrous magnesium sulfate sorbents	GC-MS	[228]
PAH	<i>Lepidium sativum</i>	CO ₂ +Methanol, 80-200 bar, 40-120 °C	--	HPLC- ^{3D} FLD (Ex. 260 nm, Em. 350, 420, 440, and 500 nm)	[229]
PAH	Aged industrial soils	CO ₂ , 300 bar, 50 °C	Study of efficacy of myco-remediation	HPLC-DAD (282 nm)	[230]
PCB	Sediments	CO ₂ , 120-365 bar, 40-100 °C	Extraction completed with PLE	TOC, GC	[231]
15 pesticides	River sediments	CO ₂ , 350-400 bar, 60-100 °C	H ₂ O and acetone as modifiers, toluene collection	GC	[232]
PAH	River floodplain soils	CO ₂ , 200 bar, 50 °C	C18 trapping	GC-MS	[233]
Artemisin	Soils	CO ₂ +Ethanol, 300 bar, 50 °C	--	HPLC	[234]
PAH	Seaweed before and after oil spill	CO ₂ , 256 bar, 100 °C	C18 trapping	HPLC-FLD (Ex. 250-296nm, Em. 302-456nm)	[235]
Orbifloxacin	Milk and plasma	CO ₂ +Methanol, 250-320 bar, 60-150 °C	--	HPLC-FLD (Ex. 278 nm, Em. 450 nm)	[236]
Dioxins and PCB	Fish oil	CO ₂ , 300 bar, 70 °C	Countercurrent extraction, coupled to activated carbon	GC-MS and GC-FID	[237]

Table 5.- Summary of the works published on the metal recovery by SFE in the period 2007-2009

Metal	Matrix	Extraction conditions	Chelating agent	Ref.
Pd, Pt, Rh	Spent automobile catalytic converters	CO ₂ , 300 bar, 40-80°C	<i>Terc</i> -butylphenoxo (TBP) ligands	[238]
Cr, Cu, As	Treated wood	CO ₂ , 200 bar, 60 °C	organophosphorus Cyanex 302	[239]
Th	Tissue paper residues	CO ₂ , 200 bar, 60 °C	organophosphorus reagents (TBP, TOPO, TPP, TPPO, TBPO)	[240]
Ur	Radioactive metal wastes	CO ₂ , 200 bar, 40 °C	surfactant (NP-4)	[241]
Cu	Copper(0) foil	CO ₂ , 240 bar, 60 °C	dialkyldithiocarbamate lithium salts	[242]
In(III)	Acidic aqueous solution	CO ₂ , 138 bar, 70 °C	β-diketone (AcAcH), fluorinated β-diketone (TTAH), thiopyridine (PySH), and piperidinyldithiocarbamic acid	[243]
Ga(III)	Acidic aqueous solution	CO ₂ , 205 bar, 70 °C	AcAcH, TTAH and PySH	[244]
Nd(III), Eu(III)	Acidic aqueous solution	CO ₂ , 200 bar, 50 °C	Oxa-diamides (TBODA)	[245]