Journal of Chromatography A Volume 1152, Issues 1–2, 8 June 2007, Pages 234–246
http://dx.doi.org/10.1016/j.chroma.2007.02.046

USE OF COMPRESSED FLUIDS FOR SAMPLE PREPARATION: FOOD APPLICATIONS

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ABSTRACT

This review attempts to provide and updated overview (including works published till June 2006) on the latest applications of compressed fluids as sample preparation techniques for food analysis. After a general revision of the principles of supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE; also called accelerated solvent extraction, ASE or subcritical water extraction, SWE), the principal applications of such techniques in the mentioned fields of food and natural products and environmental analysis are described, discussing their main advantages and drawbacks.

KEYWORDS: supercritical fluid extraction; pressurized liquid extraction; subcritical water extraction; foods; pollutants; environmental; natural products; functional foods.

Journal of Chromatography A Volume 1152, Issues 1–2, 8 June 2007, Pages 234–246
http://dx.doi.org/10.1016/j.chroma.2007.02.046

Contents

1. INTRODUCTION.

The demand on new analytical techniques in food and environmental sciences is strongly related to the higher demand of information on processing, quality control, adulteration, contamination, environmental and food regulations, etc. Consequently, faster, more powerful, cleaner and cheaper analytical procedures are required by chemists, regulatory agencies and quality control laboratories to meet these demands. In this regard, the progress in modern analytical techniques has led to significant improvements in the quality of analysis, however, the importance of sample preparation has been often under-estimated.

Traditionally, several techniques have been used for sample preparation involving extraction with organic solvents, column fractionation, etc. These are usually time-consuming and labor-intensive, introducing potential quantitative errors and using large volumes of organic solvents, with the associated risks for the human health and the environment. Consequently, several alternative techniques for sample preparation have been developed to solve these problems. The techniques discussed in the present review are based on the use of compressed fluids as extracting agents; examples of such techniques are: Supercritical Fluid Extraction (SFE), Pressurized Liquid Extraction (PLE) or Accelerated Solvent Extraction (ASE) and Subcritical Water Extraction (SWE). Thus, the scope of the present review is the discussion of the principles and main applications of such techniques in food, natural products and environmental fields, compared to conventional techniques commonly used for sample preparation.

Among these techniques SFE is the most well-known. SFE experienced a rapid development in many areas of application including analytical sample preparation in the

mid and late 1980s. Several reviews of its applications have been written since then [1-7].

One of the main advantages of SFE in sample preparation is the reduced use of organic solvents (zero in many cases) allowing performing the extractions with nonpolluting, nontoxic supercritical fluids, such as carbon dioxide. The most widely used supercritical fluid is CO_2 (critical conditions=30.9ºC and 73.8 bar). Carbon dioxide is cheap, environmental friendly, generally recognised as safe by FDA and EFSA. Also, important for food and natural products sample preparation, is the ability of SFE to be operated at low temperatures using a non-oxidant medium, which allows the extraction of thermally labile or easily oxidized compounds. Other solvents are or have been under study but, most of them presented several drawbacks such as the high critical temperature and pressure for water, the high flammability of nitrous oxide, ethers and hydrocarbons and the chemical reactivity and corrosiveness of ammonia [8]. It is possible, therefore, to substitute a variety of conventional solvents with a single supercritical fluid. For instance, supercritical carbon dioxide at 7.5 MPa and 80ºC (density, d= 0.15 g/ml) is characterised by a solvating strength similar to gases, such as pentane, while at 38.2 MPa and 40 ºC (d= 0.95 g/ml) its solvating strength resembles liquids, such as methylene chloride, carbon tetrachloride, toluene or benzene [9].

An important drawback of SFE is that predominantly a non-polar extraction fluid, such as CO_2, is used. Therefore, a logical trend to widen the application range of this technique is the study of new methods to decrease analyte polarity to make them more soluble in non-polar supercritical fluids. In this sense, chemical in-situ derivatization has been applied [10, 11]. Nevertheless, a more common practice in SFE is to increase the polarity of the supercritical fluid used by employing modifiers (co-solvents). For

example, the addition of relatively small percentage (1–10%) of methanol to carbon dioxide expands its extraction range to include more polar analytes [12].

A different approach has to be used if solid or liquid samples have to be extracted; for solid samples, a previous step of drying [13], freeze drying [14], grounding and/or mixing with an inert agent like sea sand [15] or alumina should be included. For liquid samples, two different strategies have been used such as the absorption of the sample onto a porous and inert substrate [16] or the co-injection of the sample with the supercritical fluid in the extraction vessel (as supercritical antisolvent precipitation or in a countercurrent packed column [17, 18].

In terms of amount of CO_2 consumed it's important to select a good extraction strategy, it means, dynamic vs static. Dynamic extraction use to provide higher extraction yields but using higher amounts of CO_2 (in case that it isn't recirculated). On the other hand static extraction wi

Some reviews dealing with the on-line coupling of SFE with different separation techniques are suggested [19, 20]. No discussion will be included in this revision on this point.

2.2. Pressurized Liquid Extraction (PLE).

In pressurized-fluid extraction techniques pressure is applied to allow the use as extraction solvents of liquids at temperatures greater than their normal boiling point. Among them, accelerated solvent extraction (ASE) (which can be considered a new version of the Soxhlet apparatus but operating at high pressures and temperatures), pressurized hot water extraction (PHWE) or subcritical water extraction (SWE), near-critical fluid extraction and enhanced fluidity extraction are the most promising techniques in food and environmental sample preparation. Following there is a

description of the principles and applications of Pressurized Liquid Extraction (PLE), including under PLE denomination all the above mentioned techniques.

The main principles of PLE are relatively simple. As mentioned, PLE is based on the extraction at high temperatures with solvents submitted to high pressures to maintain the liquid state. When these conditions are employed, faster extraction processes as well as higher extraction yields can be obtained compared to traditional extraction techniques and, moreover, in an automatic way [21]. The increase on the extraction temperature can promote higher analyte solubility by increasing both, solubility and mass transfer rate. Besides, the high temperatures decrease the viscosity and the surface tension of the solvents, which helps to reach areas of the matrices more easily, improving the extraction rate. Moreover, the simultaneous use of high pressures and temperatures will have an effect on the surface of the matrix, allowing a deeper penetration of the solvent in the matrix, therefore, having a positive influence in the extraction rates [22]. The extraction pressure has widely been reported to have little effect on the extraction process considering that its value is enough to maintain the solvent on liquid state [23-29]. However, theoretically, a raise in the extraction pressure could lead, depending on the matrix studied, to an increase on the extraction yield, since the pressure would push the solvent through the matrix pores or could help to the breakage of the matrix particles (e.g. cell walls).

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

The instrumental requirements to carry out extractions using PLE are relatively simple (Figure 2). Briefly, the extraction system includes a solvent reservoir and a high pressure pump to introduce the solvent in the extraction cell. The stainless steel extraction cell is placed inside an oven which allows the selected extraction temperature to be reached. Besides, these instruments normally have several valves to maintain the desired pressure into the cell during the extraction process. Once the extraction is finished, the extract is pushed out of the extraction cell and placed in a collection vial. Additionally the instruments can have a nitrogen circuit to purge the system. Temperatures up to 200ºC and pressures up to 3000 psi are generally used. Additionally, lab-made instruments can have several pumps (for different solvents or for cleaning the system after extraction), different preheating devices, and/or cooling devices at the end of the circuit.

The combined use of high pressures and temperatures provides faster extraction processes that require small amounts of solvents (e.g., 20 minutes using 10-50 ml of solvent in PLE can be compared to a traditional extraction step in which 10-48 h and up to 200 ml are required) decreasing in this way the dilution of the sample. Besides, PLE is broadly recognized as a green extraction technique due to the low organic solvent consume. Furthermore, the use of water as extraction solvent in PLE, in the so-called subcritical water extraction (SWE), can undoubtedly enhance even more this consideration. Water is a non-flammable, non-toxic, readily available and environmentally clean solvent. For these reasons, water could be the optimum solvent to carry out pressurized extractions. SWE is based on the extraction with liquid water at high temperatures between 100 and 374ºC (i.e., below its critical temperature). The main parameter influencing these extractions is the dielectric constant (ε) of water. At room temperature its dielectric constant is around 80, i.e., a highly polar solvent.

However, the chemical structure of water provides unique properties, which allow a huge decrease in the magnitude of this parameter when the temperature is increased and its liquid state maintained. Thus, at 250ºC the dielectric constant of water is ca. 27, which is similar to that shown by several organic solvents at room temperature such as methanol (ε = 33) or ethanol (ε = 24) [30]. Therefore, at these conditions water can be potentially employed to extract less polar compounds [31] instead of using toxic organic solvents. Readers interested in a deeper physico-chemical description of PLE and SWE extraction techniques can found it elsewhere [1, 22].

3. APPLICATIONS OF SFE AND PLE IN FOOD ANALYSIS AND NATURAL PRODUCTS ANALYSIS.

3.1. Functional compounds.

Nowadays, the growing interest in the so-called functional foods has raised the demand of new functional ingredients that can be used by the food industry [32]. These functional ingredients are preferred to have natural origin and to be obtained using environmentally clean extraction techniques. As expected, the complexity of the natural ingredients with biological activity is very high; this fact has lead to the development of new methodologies to extract and characterize them. In order to preserve the activity of such ingredients and to prevent changes in the chemical composition of the functional compounds and/or mixture of compounds, sample preparation techniques based on the use of compressed fluids have widely been developed.

3.1.1 SFE applications.

Table 1 shows a summary of the applications of SFE in food and natural products analysis. SFE has been used to obtain extracts with antioxidant activity from microalgae

[33, 34]; by using the combination of SFE and HPLC with both, DAD and ESI-MS, several functional compounds were identified corresponding to different carotenoids along with chlorophyll a and some chlorophyll degradation products. These compounds could be associated to the biological activity of such extracts. Supercritical CO_2 has also been used to extract and characterize antimicrobial compounds and food preservatives from microalgae. Mendiola et al [35] correlated the antimicrobial activity of *Chaetoceros muelleri* supercritical extracts with its content in DPA and tryglicerides, analyzed by HPLC-ELSD. A common important thing to remark of these works is the low yields obtained. In case of Chaetoceros these low extraction yields are due to its siliceous cell wall, a common characteristic to the diatomaceous microalgae [35]. In case of Spirulina [33, 34]], and many other microalge [36], the main problem in order to obtain higher extraction yields is its high proteins and carbohydrates content, which are almost insoluble in supercritical CO_2.

Carotenoids are a group of compounds of great importance to human health since they can act e.g., as potent antioxidants; however, due to their chemical characteristics they are easily degraded by temperature or oxygen, so, the use of SFE has been suggested to minimize risks of activity lost being thus applied to the extraction of carotenoids from different matrices. Sun et al. [37] carried out the extraction of carotenoids from carrot and compared the traditional solvent extraction method with the SFE using canola oil as co-solvent. Results showed that not only hydrocarbon compounds such as α- and β-carotene were recovered by supercritical CO_2 but also oxygenated carotenoids such as lutein. The authors suggested that the use of canola oil as co-solvent greatly increased the extraction yield of carotenoids. Similar results were obtained for the SFE of lycopene from tomato [38]; in this application, a vegetable oil was also used as co-solvent showing an improvement in the extraction yield as well as in the stability of the

pigment. In both cases the use of oils as co-extracting agents present an important drawback: the elimination of oil. It helps to improve the extraction but the extract is a mixture of the extracted components of the oil and the "pure" extract. On the other hand, Lopez et al. [39] developed a highly selective automated SFE method for the isolation of carotenoids from crustaceans by using 15% ethanol as cosolvent. In this case it was necessary to use an ODS (C18) trap wich was flushed with acetone.

Another compound with antioxidant properties that has also been extracted with supercritical carbon dioxide has been squalene from different matrices such as olive oil [18, 40], oil rafination by-products [41, 42] and from different plants [43]. In all the cases the extract could be directly analyzed using liquid chromatography with no other fractionation step. An important point in favour SFE in the obtention of squalene is the relatively short extraction times, mild pressures (~200 bar) and temperatures (~50ºC) used.

Simó et al [17] developed a method to extract and characterize antioxidants from orange juice based on SFE followed by a chemical characterization of the extracts using Micellar Electrokinetic Chromatography (MEKC) and HPLC-MS. The main advantage of MEKC, that is, its high separation speed, can facilitate the rapid optimization of CC-SFE conditions. In this case SFE provided three fractions with different composition in each extraction, which lasted only 20 minutes. No other clean- ups were needed at present work where a full characterization of antioxidants in oranges could be achieved in less than 1 hour.

SFE has been widely used as sample preparation method to analyze essential oils from foodstuffs like onions [44], or from different herbaceous materials like oregano [15], rosemary [45], laurel (bay leaves) [46], cinnamon [47], cumin [48], horsetail (*Equisetum giganteum L.)* [49] or St John's wort (*Hypericum sp.*) [50, 51]. Essential oils

are not only valuable as aroma but also some of them are highly appreciated as functional ingredients with different activity (antioxidant (oregano), antimicrobial (rosemary), antidepressant (St. John's wort). In general terms, the use of SFE allows the analysis of essential oil preserving its integrity, without the formation of off-flavors that could interfere in the characterization of the sample as could be demonstrated by Statshenko et al [52].

As mentioned, to widen the range of application of SFE to relatively polar compounds, small amounts of modifiers (≤15%) are added to carbon dioxide allowing the extraction of more polar substances. Examples of functional compounds of relatively high polarity that have been extracted using SFE with polar modifiers are: polyphenols from grape skin [53], from grapefruit (*Citrus maxima*) [54] and from other fruits like *Forsythia koreana* [55]. There are mainly two different ways to use modifiers, mixed with the CO_2 flow is the most common way to work with modifiers as [39, 53, 56, 57] for example. Some authors prefer to add it mixed with raw material [58, 59]. This way should only be used in case a static extraction step is employed. In case of dynamic extraction the CO_2 flow would be saturated with entrainer instead of analytes.

Other examples of the extraction of valuable compounds from foods using SFE are the isolation of cholesterol from cattle brains [60, 61] and fat soluble vitamins from parmigiano regiano cheese [13]. The main problem with cattle brains, as well as many other raw food matrices is its high content in water. It can interefere in the extraction process in two ways: lixiviation and acting like as entrainer. In order to avoid this situation the most common strategy is drying [49, 59, 62] or freeze drying [43, 60, 61, 63] the sample prior to extraction. Some authors mix the sample with any kind of water absorbent inside the extraction cell, for example magnesium sulphate [64]. But the problem when trying to isolate compounds from foodstuff is not always water but fat.

The most commonly used fat retainer materials are basic alumina, neutral alumina, forisil and silica. Two main approaches have been used; one where the fat retainer is placed in a separate chamber downstream from the extraction thimble, and one where the fat retainer is added inside the extraction cell.

3.1.2 PLE applications.

As for the pressurized liquid extraction, numerous applications have been developed in the last few years involving the extraction of interesting compounds from foods and natural matrices; Table 3 shows a summary of the most remarkable applications of PLE in this area. Probably, the main reason of the extremely important development of PLE-based techniques is the possibility of its automation along with the reduced extraction time and solvents required.

One important group of compounds with biological activity that can be found in several plants and food by-products are phenolic compounds. They are widely distributed in plants [65] and possess different functional activities mainly associated to their antioxidant properties . PLE has been widely employed as sample preparation technique to obtain phenolic compounds from grape seeds and other winery by-products [66-68]. Water has been used to perform these extractions [66, 69] as well as organic solvents such as ethanol and methanol [67, 68]. Palma et al. [68] developed a new method based on the use of solid phase extraction (SPE) combined with PLE. The configuration consisted in a solid phase placed at the bottom of the extraction cell and covered by a cellulose filter; the sample was deposited on top of the filter. Using this new procedure, it was possible to obtain an in-line clean-up of a grape extract that could be directly analyzed by HPLC to determine its phenolic content. Other matrix that has been often used is soybean, mostly to obtain isoflavones, a kind of phenolic compounds highly

appreciated because of their functional properties. Different approaches have been applied to extract isoflavones from soy by PLE [70, 71]. In general, mixtures of organic solvents and water are selected at high extraction temperatures to provide good extraction efficiencies. Klejdus et al. [70] tested the effect of the distribution of the sample into the extraction cell in the reproducibility of the extraction method. They placed subsequent layers of a filter paper-absorbent cotton-commercial matrix-sample (in a filter paper envelope)-matrix and absorbent. Using this distribution, it was possible to obtain clean extracts without other undesirable interferences. The extracts were only evaporated and re-dissolved before LC-MS analysis. Other phenolic compounds have also been extracted using PLE from different raw materials such as parsley [72], rosemary [73], brewing products as hops [74] and malt [75] or other different plants [76, 77]. The possibility of performing two sequential PLE extractions to partially clean-up the sample was implemented by Papagiannopoulos et al. [74]. With the aim to analyze polyphenols from hops, a two cycle pressurized pentane extraction was carried out followed by a pressurized acetone extraction. In this way, several interfering compounds (mainly hop oils, resins and chlorophylls) could be eliminated and the final acetone extract could be on-line extracted by solid phase extraction before HPLC analysis. On the other hand, Ibañez at al. [73] studied the selectivity of subcritical water to extract the most active compounds from rosemary by means of a home-made PLE device. In this work [73], HPLC monitoring of the relative amounts of several interesting compounds was carried out. The study showed that the selectivity of subcritical water towards the extraction of antioxidants could be easily tuned considering small changes in the extraction temperature. Similar results were found using a commercial PLE instrument characterizing the extracts in this case by capillary electrophoresis-mass spectrometry (CE-MS) [78]. Besides rosemary, the antioxidant

activity and chemical composition of subcritical water extracts, obtained form other plants such as sage [79] and oregano [80] have been studied. From the latest work [80] it could be concluded that subcritical water efficiency to extract antioxidant compounds is better than that of other organic solvents or hydro-organic mixtures.

The vitamin content of certain foods and natural products frequently needs to be correctly determined to assess their nutritional value. PLE has been the technique chosen to analyze the content of vitamin E in different matrices [81-83]. For example, Sivakumar and Bacchetta [83] optimized the extraction of vitamin E from hazelnuts using hexane at 60 ºC and adding 0.01% BHT to the solvent to prevent tocopherol oxidation. Likewise, the extraction of β-carotene (vitamin A precursor) as well as other carotenoids from different sources has been studied [81, 84]. For this purpose, in general, low polarity solvents were used (hexane, light petroleum).

Dunaliella salina is a green microalga that is generally used as a natural source of carotenoids. Several works have been carried out to extract carotenoids from this microorganism using PLE [85, 86]. In these works authors demonstrated the possibility to obtain this kind of compounds from *Dunaliella salina* in a fast an efficient way by means of PLE. Namely, the direct extraction of the lyophilized material was possible filling the extraction cell with successive layers of sea sand-microalga-sea sand to avoid the clogging of the system [85, 86]. Regarding microalgae, other species have been also studied for their interest as potential source of functional compounds. For example, the microalga *Spirulina platensis* has been also investigated as natural source of different functional compounds together with PLE using different solvents such as hexane, petroleum ether, ethanol and water [65, 87]. One of these applications used CE-MS to monitor the optimization of the extraction of phycobiliproteins from this microalga [87]. Different extraction parameters were studied and the optimized conditions included a

distribution of the sample inside the extraction cell in 9 packings and the use of glass beads between them as supporting material. Using this configuration for the sample distribution, it was possible to carry out 7-cycle pressurized extractions without clogging the system. The final extraction yield after the optimization process was increased more than 5 times.

Essential oils from different plant materials have been extracted using pressurized hot water [88-91]. By combining dynamic extractions and high temperatures (150ºC), recoveries obtained using SWE were comparable to those provided by traditional extraction techniques, such as steam distillation and Soxhlet extraction, but in a much faster and environmentally clean way [91]. Moreover, other less polar compounds have been extracted under pressurized liquid conditions, such as different fatty acids and other lipids from different food products [92, 93] and plants [94]. In general, low polarity solvents such as hexane and chloroform/methanol as well as several static extraction cycles are used; once the extraction process is finished, the extracts are ready for GC analysis. Toschi et al. [93] compared the extraction of lipids from poultry meat considering PLE and different traditional methods, and determined that the use of PLE allowed recoveries around 98%, using one third of solvent volume and reducing the extraction time in more than one hour (compared to traditional methods).

Other compounds, such as anthraquinones [95, 96], as well as other active compounds from foods [97, 98] and medicinal plants [99-104] have also been extracted by PLE prior to their analysis.

3.2. Food safety.

At present, food safety includes many different issues such as detection of frauds, adulterations, contaminations, etc. Among these topics, detection of food pollutants is

important not only for consumers but also for administrations, control laboratories, and regulatory agencies. In order to protect consumers' health, regulations establish strict limits to the presence of pollutants in foods that must be carefully observed and determined. Generally, the analysis of food pollutants is linked to long extraction and clean-up procedures commonly based on the use of e.g., soxhlet and/or saponification. These procedures are laborious and time consuming and, besides, usually employ large volumes of toxic organic solvents. With the objective of reducing both, the sample preparation time and the massive use of organic solvents, techniques based on compressed fluids such as SFE and PLE have been developed.

3.2.1 SFE applications.

One of the main areas of application of SFE in the last few years has been in food pollutants analysis, mainly pesticide residues and environmental pollutants as can be deduced from the summary shown in Table 4. Rissato et al. developed several methods [105, 106] for the analysis of multiple pesticides (organochlorine, organophosphorus, organonitrogen and pyrethroid) in potatoes, tomatoes, apples, lettuces and honey with a single clean up step using supercritical CO_2 modified with 10% of acetonitrile. Similar works have been carried out for the analysis of multiresidues of pesticides, using SFE as a clean up step, in cereals [107], fish muscle [108], vegetable canned soups [64], vegetables [62] or infant and diet food [109]. A common characteristic of these works is the extremely high selectivity of SFE in the isolation of the low polarity pesticides; this fact makes SFE probably the technique of choice to isolate pesticides from low fat food. A common strategy is the use of traps. As have been seen previously [39], these traps must consist on a phase compatible with the analyte an must be flushed away with any compatible solvent. The most common is C18 [109] traps, but Rissato et al used florisyl

cartridges [105, 106, 110] to trap pesticides. The trapping step is very important in SFE methods (though often overlooked) and extra selectivity can easily be introduced, especially in the case of solid-phase trapping, with an accompanying decreased sample preparation time, due to the fact that post-extraction clean-up is not necessary [111].

Jerry King et al developed a method to derivatize carbamate pesticides in supercritical CO_2 media [112]. In this work, authors dissolve derivatizing agents in CO_2 that acted as modifiers. The derivatized carbamate pesticides were then analyzed by GC–ECD or GC–MS with excellent sensitivity. Extraction and conversion of the carbamates was complete, as indicated by HPLC with post-column hydrolysis and o-phthalaldehyde derivatization then fluorescence detection. GC-MS (ion trap) was also used to confirm the formation of the carbamate derivatives. Compared with the same HFBA reaction in an organic solvent the derivatization reaction time was considerably shorter in SC-CO_2. The described approach, combining both extraction and derivatization, simplifies the analysis of carbamate pesticides and eliminates the use of organic solvents associated with the derivatization step. The combination of extracion-reaction prior to analysis is not something new, Turner et al [113] reviewed the state of art of enzyme reactions in supercritical media to form useful analytical derivatives for gas chromatography, liquid chromatography, or SF chromatography analysis.

Another strategy to improve the isolation of pesticides is the use of supercritical fluid mixtures [114]. Excellent recoveries were obtained for incurred organochlorine and -phosphorus pesticides from a variety of food products at ppb levels using either CO2/N2 or CO2/HC-134 mixtures. Results from these and additional experiments suggest that binary fluid mixtures can significantly reduce the need for additional sample cleanup prior to Chromatographic analysis

As well as pesticides, veterinary drugs are widely spread in the primary sector, but none of them should reach the consumers or, at least, should not reach them over the maximum allowed limits. Matabudul et al [115] developed a rapid method for the determination of lasalocid in poultry feed using SFE and HPLC; lasalocid is widely used as a coccidiocidal drug in poultry to increase feed efficiency and for weight gain in ruminants. The actual lasalocid analysis method [116] involves several steps that were reduced to a single one by using SFE. Only 20 ml of low toxicity solvent mixture (ethanol/ethyl acetate/NaOH) are required for the complete extraction and determination of lasalocid; thus, the new SFE method is fast, economic and represents little hazards from exposure to solvents.

But not only non-polar drugs are susceptible to be isolated using SFE. One example is the extraction of sulphonamides, which are commonly used in subtherapeutic doses in drinking water but also as bacteriostatic in chicken, beef and pig grown. Arancibia et al [16] optimized the isolation of sulphonamides by using SFE at high temperatures (between 120-160 ºC), in only 33 minutes of extraction (30 static + 3 dynamic), and avoiding the use of further clean up steps prior to HPLC injection. In this case, authors mix the sample with Celite in order to absorb the moist. Due to the polar nature of sulphonamides its necessary to use a modifier, in this case Arancibia et al optimized the analytical procedure to use only 3ml of methanol per gram of raw meat.

Supercritical carbon dioxide extraction can advantageously be used to extract non-polar pollutants, such as polyaromatic hydrocarbons (PAH), from foods [117, 118]. Different extraction and clean-up methods have been used, but the extracting conditions turned to be very similar (around 300 bar and 100ºC) to optimize the PAH extraction. Yusty et al [117] used octadecylsilane (ODS) beads in the SFE extraction cell to adsorb lipids from the sample (fish muscle) while extracting the PAH. This significantly reduced lipid

interference in subsequent GC-MS analyses. On the other hand, Yeakub et al [118] used a different strategy for the analysis of PAH in vegetable oils, consisting in the extraction of the raw material by SFE (without previous clean-up step) and the used of HPLC coupled to fluorescence detection to avoid lipid interferences.

Other environmental pollutants that can be found in food samples, and therefore should be quantified and controlled, are halogenated dioxins and biphenyls [111, 119, 120]. In this field SFE has proved its effectiveness as sample preparation method previous to GC-MS. The isolation of these kind of compounds is relatively easy using SC-CO_2 due to their low polarity. A reasonable approach would be to combine moderate SFE conditions (reducing coextracted lipids) with £orisil present in the extraction thimble as fat retainer. The extracted PCBs should then be trapped on a solid phase packed with £orisil and eluted with n-heptane. Additionally, it might be possible to determine also the fat content in the same extract by applying methanol as modi¢er after the PCB extraction step, breaking the interactions between the lipids and the fat retainer, as recently demonstrated for a model fat sample [121].

In many countries legislation limits or bans the use of ionizing radiations of foodstuff, therefore, the detection of radiolytic products must be improved. Among these products, alkylcyclobutanones are formed in very low doses and, as a consequence, it is necessary to develop efficient extraction methods. In this sense, Gadgil et al [122], developed a method to assess the content of alkylcyclobutanones in 20 min working at 340 atm and 75ºC, even in high fat content samples. In this case the strategy to avoid interferences with fat is load the sample with sand and florisil in order to trap the fat, after extraction cyclobutanones were adsorbed in glass wool.

Other important area of application of SFE has been in the assessment of food frauds. Karásek et al. developed an study for wine variety certification [123]; in this work they

compared a direct countercurrent SFE with a two step SPE-SFE and found that the direct SFE resulted in a more specific and representative gas chromatographic fingerprint of the wine sample.

SFE has been also used to identify adulteration of black pepper powder with ground papaya seed [124] combining SFE with thin layer chromatography (TLC) allowing the detection of 20 g of papaya seed per kg of mixture. Adulteration can be detected on the basis of the presence of a fluorescent band in TLC at Rf 0.172 at 366nm in an SFE extract of the sample. Bhattacharjee et al studied model blends of papaya and pepper and then analyzed marker compounds present in papaya but not in pepper β-elemene, α-murolene and β-bisabolene, were found exclusively in pepper, they cannot be used as markers of adulteration. 3-Eicosene was found in papaya seed extract alone.

3.2.2 PLE applications.

As mentioned, analysis of pesticides is an important issue in food safety. Pesticides are widely employed in agriculture and frequently are regarded as toxic; thus, their presence in vegetables and fruits has to be limited. Due to the characteristics of PLE, this technique has been successfully applied to the extraction of several pesticides simultaneously; this is an important advantage over other sample preparation methods since frequently different kind of pesticides are employed at the same time. In fact, the applicability of PLE as a routine technique for the extraction of pesticides in vegetables has been demonstrated [125]. In that work [125] a PLE method, using a commercially available instrument, was developed to extract simultaneously 100 pesticides of different polarity from food commodities using a mixture of ethyl acetate and acetone (3:1, v/v) as extraction solvent. The whole extraction procedure, consisting on two static cycles, took only 15 minutes. By comparing the PLE extraction of these pesticides with

other traditional extraction methods (such as solvent shake extraction), it can easily be seen that PLE is less time and solvent consuming while providing at the same time acceptable values of recoveries, precision, quantification limits and selectivity, corroborating the effectiveness of PLE for routine pesticide analysis. This technique has also been evaluated for the extraction of 25 pesticides from rape seed [126]. Although in this particular case, the application of PLE implied the necessity for a lipid removal clean-up procedure, authors considered PLE as a good alternative to the conventional liquid-liquid extraction procedure. Different fruits have also been studied to determine their content in pesticides. The studies employed PLE (with commercial instruments) combined with diverse analytical techniques [127, 128]. Adou et al. [127] determined the presence of different pesticides in several fruit samples with appropriate recoveries while minimizing environmental concerns and time. To carry out this study [127], they introduced the homogenized sample inside the extraction cell between sand layers in a sandwich-like format. This strategy has been extensively employed to obtain a better dispersion of the sample while keeping it into the extraction cell. Using acetone/dichloromethane (3:1, v/v) as extraction solvent at 110ºC and 1500 psi, using 2 static extraction cycles, it was possible to extract 24 pesticides and to determine them by GC with different detectors [127]. Similarly, PLE has been employed to extract trace pesticides from oranges and peaches [128]. In that work [128], the extraction conditions were optimized for different pesticides and the best recoveries were achieved at 75ºC and 1500 psi as extraction conditions using ethyl acetate as solvent. Higher temperatures led to the co-extraction of other organic compounds present in fruits such as carotenoids and flavonoids. The extraction time was set at 7 minutes, which was considered sufficient due to the high solubility of the target compounds in ethyl acetate. Using these extraction conditions, the authors found that the efficiency of PLE to

extract pesticides from fruit was comparable to that obtained using traditional extraction techniques, while using smaller solvent volumes and significantly less time. LOQ below European legislation requirements were achieved.

Veterinary drugs are used in breeding animals and they can be easily found in foods, if no good manufacturing practices are used. The presence of such residues in foods should be assessed due to their negative effects on human health. Sulfonamides are a group of compounds used to promote animal growth. 13 sulfonamides could be detected in raw meat and infant foods using PLE [129]. To extract these compounds, water at 160ºC was used for 15 min of extraction time (including 5 min of static extraction time). To get proper extracts, homogenized meat was mixed with C_{18} particles, and the extraction cell was filled then with diatomaceous earth. Direct analysis of the extracts was possible using this strategy.

Several extraction and clean-up methods have been developed using PLE to analyze polychlorinated biphenyls (PCBs) in food and food-related materials [130]. The use of sorbents in the PLE extraction cell has been studied by Gómez-Ariza et al. [131]; using mild extraction conditions (40ºC for 10 minutes and two static cycles) and a dichlorometane/pentane (15:85, v/v) mixture as extraction solvent, the sorbents allowed the extraction of PCBs from natural materials retaining the co-extracted lipids from the matrix and enabling for a direct analysis of the extract collected in a single-step procedure. In a previous work, Björklund et al. [132] demonstrated the possibility of obtaining fat-free extracts from naturally contaminated fish meal using sulphuric acid-impregnated silica as fat retainer. In this way, an on-line cleanup of fat-containing matrixes was possible prior to their analysis to determine the PCBs content. A similar procedure was used to obtain fat-free extracts ready for PCBs analysis from several food and feed matrices [133]. In these applications the dispersion of the sample into de

extraction cell proved to be critical. To obtain appropriate extracts, a layer of fat retainer was placed above two filter papers in the bottom of the extraction cell. Then, other filter paper was introduced with the sample dispersed and with sodium sulphate above it. The rest of the extraction cell volume was filled with sodium sulphate and two more filter papers on top. Similar packing was employed to achieve an integrated extraction, clean-up and fractionation of the different analytes to proceed with the determination of dioxins in foods [134]. In this case, the fat retainer was replaced by a carbon/celite mixture.

Polycyclic aromatic hydrocarbons (PAHs) are other kind of pollutants that are considered dangerous because of their known carcinogenic effects. Also, it seems that some processing operations that are carried out in the food industry, such as smoking of the food, can generate these compounds. A commercial ASE instrument was employed to extract this kind of compounds from smoked food [135]. The extraction conditions consisted on 100 ºC and 1500 psi with hexane, and using two static cycles of 10 min each. The homogenized sample was placed at the bottom and the extraction cell was filled with sand. Although a clean-up procedure was needed after the extraction, the results showed similar or better recoveries for different PAHs than using Soxhlet extraction. Besides, only 20-30 ml of solvent were employed for a total extraction time of 15-20 minutes. Interestingly, up to 12 different PAHs were found in different smoked meat products. Similar conclusions were reached by Morales-Muñoz et al. [136].

Several works concerning the optimization of the extraction of toxins in contaminated foods have been published [137-142]. Specifically, zearalenone is a mycotoxin produced by the fungi of the *Fusarium* species that can be found in cereals and that has potential negative effects on humans. An experimental design was used to optimize the extraction of this compound from cereals [143]. The parameters optimized were

temperature, time and type of extraction solvent. The selected values (80ºC, 5 min and methanol/acetonitrile (1:1, v/v), respectively) allowed the recovery of zearalenone from wheat and corn with results comparable to those obtained with the conventional extraction techniques. Slightly different extraction conditions were obtained by Urraca et al. [142] being, after optimization, equal to 50 ºC, 5 min, using methanol/acetonitrile (1:1 v/v) as extraction solvent.

5. CONCLUSIONS AND FUTURE OUTLOOKS.

Today, there is a real need for new methods for preparation of samples that can help to determine an increasing number of compounds (with biological activity or with high toxicity) with low solvent consumption in a fast, reproducible and automatic way. Even if an increase in the sensitivity, reliability and speed of analysis has been fulfilled with new and costly laboratory instruments, there is a lack of standard methods for sample preparation able to provide good recoveries of the target compounds in a short time, with very low or no consumption of organic solvents. Sample preparation methods based on the use of compressed fluids, such as SFE and PLE, can meet these requirements providing fast, reliable, clean and cheap methods that can be used for routine analysis. On the other hand, there is a clear need of validation of these new techniques and procedures towards their evolvement as official methods (*e.g.*, AOAC protocols) substituting the most laborious, time consuming and classical procedures.

Miniaturized analytical procedures, based on chip technology, coupled with sophisticated detection systems and bioinformatics, could provide in the future high sample throughputs minimizing sample and solvents consumptions.

As for the sample preparation methods discussed in the present work, the discover of more selective compressed fluids and the development of new strategies based on the employment of highly selective ligands will greatly improve the extraction and quantification of target compounds to meet the actual requirements of regulatory agencies and control laboratories.

6. ACKNOWLEDGEMENTS

This work has been financed by Spanish Ministry of Education (Project AGL2005-06726-C04-01/02 and Project AGL2005-05320-C02-01) and Comunidad Autónoma de Madrid (S-0505/AGR/000153) projects. JAM and MH thank the Spanish Ministry of Education for a grant.

Table 1. Applications of SFE in foods and natural products analysis.

Compound of interest	Sample	Analysis post-SFE	Extra clean ups	SFE conditions	Reference
Fat	Infant formula powder	GC-MS and Gravimetry	No	465bar/100ºC	[56]
Lignans and cinnamic acid	*Schizandra chinensis*	HPLC-UV	No	20-27bar/40-60ºC	[144]
Identification of adulteration of black pepper with papaya seeds		TLC GC-MS	Ground	165-355 bar/45-80ºC	[124]
Polyphenolic compounds	Grape skin	HPLC-DAD	No	250bar/60ºC	[53]
Turmerones	*Curcuma longa Linn*	NMR HPLC	Ground	100-340bar/35-83ºC	[145]
Fat	Fermented Cupuaçu seeds *Theobroma grandiflorum*	HPLC	No	250-350bar/50-70ºC	[146]
Sterols, vitamin E, squalene	Olive oil	HPLC-DAD	No	200bar/40ºC	[40]
Volatile components	Wine	GC-FID	SPE	200bar/50ºC	[123]
Isoflavones	Red clover and soy bits	Ultra fast HPLC-UV-MS	Ground	35-75bar/10-40ºC	[147]
Squalene	*Terminalia catappa* Leaves and Seeds	GC-MS HPLC-UV	Freeze dry	137-275 bar/40-60ºC	[43]
Pesticidas	Apple, green bean, and carrot	GC-MS	Dry	320bar/60ºC	[62]
Astaxantin	Crustaceans	HPLC-UV	Ground	200bar/60ºC	[39]
Carotenoids and chlorophills	*Spirulina platensis*	HPLC-MSMS	No	80-360 bar/55ºC	[33]
Essential oils	Horsetail (*Equisetum giganteum L.*)	GC-MS	Dried, and homogenized	120/300bar/25-35ºC	[49]
B-carotene	Cyanobacterium *Synechococcus*	HPLC	Freeze dry and sonication	200-400bar /40-60ºC	[34]
Aurentiamide acetate	*Patrinia villosa* Juss	HPLC and high-speed counter-current chromatography -(UV, MS, 1H NMR and 13C NMR)	No	150-350bar/45-65ºC	[57]
Total fats and fat-soluble vitamins	Parmigiano cheese and salami	HPLC	Dry and blend	53.57 Mpa/100ºC	[13]
Volatile components	*Bunium persicum* Boiss.	GC-MS	Ground	200bar/45ºC	[48]

	(black cumin)				
Onion oil	Oinon	GC-MS	Ground and filter	100-280bar/37-50ºC	[44]
Antioxidant and antimicrobial compounds	Bay leaves	GC-MS	Ground	250bar/60°C	[46]
Antimicrobial compounds	Oregano	GC-MS	Ground	151 bar 40ºC	[15]
Antimicrobial compounds	Rosemary	GC-MS	Ground	251 bar and 60°C	[45]
Hypericin, hyperforin	St John's wort	GC-MS, HPLC-DAD and HPLC-DAD-MS	Separation, lyophilization, homogenization,	--	[50]
Antioxidant compounds	Orange juice	MECK LC-MS	No	160bar/40ºC	[17]
Essential oil	St John's wort	GC-MS	Ground	80-100 bar / 15-40ºC	[51]
Carotenoids	Carrots	HPLC-DAD	Freeze dry, ground compara con extraccion SL	270-550bar/40-70ºC	[37]
Sterols, vitamin E, squalene	Olive oil	TLC GC-MS	No	75-200bar / 35-50ºC	[18]
Coumarins	Citrus maxima fruit	HPLC	No	276bar/50ºC	[54]
Lycopene	Tomato	HPLC-UV	Dry and ground	335-445bar/45-70ºC	[38]
Colesterol	Cattle brain	GC-FID, NMR, IR	Freeze-dried	250 bar/ 60 °C	[38]
Colesterol	Cow brain	GC-FID, NMR, IR	Freeze-dried	230-250 bar/ 50-70 °C	[38]
Oils	Oilseeds	GC	Ground	660bar/40ºC	[148]
Cinnamon oils	*Cinnamomum cassia*	GC-MS	Ground	225bar/50ºC	[47]

Table 2. Applications of PLE in foods and natural products analysis.

Product	Compounds of interest	Solvent	T(ºC)/P(psi)	Mode / Cycles	Sample dispersion	Extraction time (min)	Analytical technique	Ref.
Phenolic compounds								
Grape skin	Anthocyanins / Phenolic compounds	Acidified water	80-100/ 1500	Static – 3		5	HPLC	[66]
Sambucus nigra	Flavonols	Ethanol/water 80:20 (v/v)	140 / 870	Static – 1	neutral glass	10	HPLC	[98]
Hops	Polyphenols	Pentane	60 / 1500	Static – 2	diatomaceous earth	10	HPLC-UV	[74]
		Acetone/water 4:1 (v/v)	60 / 1500	Static – 1		10		[75]
Soybean	Isoflavones	Ethanol/water 70:30 (v/v)	100 / 1500	Static – 3	Sea sand	7	HPLC-DAD-MS	[71]
Soybean foods	Isoflavones	Methanol/water 9:1 (v/v)	145 / -	Static – 2	Filter paper wrapping and SFE matrix	5	HPLC-MS	[70]
Grapes	Phenolic compounds	Methanol	100 / 600	Static – 3	Sea sand	10	HPLC	[68]
Grape seeds	Catechins	Ethanol	130 / 1500	Static – 1	Sea sand	10	HPLC-DAD	[67]
Winery by-products	Catechin, proanthocyanidins	Water	50-100 / 900-1000	Dynamic (1 ml/min)		30	HPLC-DAD-MS	[69]
Rosemary	Flavonoids	Water	25-200 / 600-1000	Dynamic (1 ml/min)		30	HPLC	[149]
Curcuma sp.	11 sesquiterpenes	Methanol	120 / 1500	Static – 1		5	GC-MS	[104]
Parsley	Phenolic compounds	Methanol/water 65:35 (v/v)	100 / 1000	Static	Ottawa sand	10	HPLC	[72]
Cimicifuga	Phenolic acids	Methanol/water 60:40	90 /1000	Static –	Celite 545	5	HPLC-	[77]

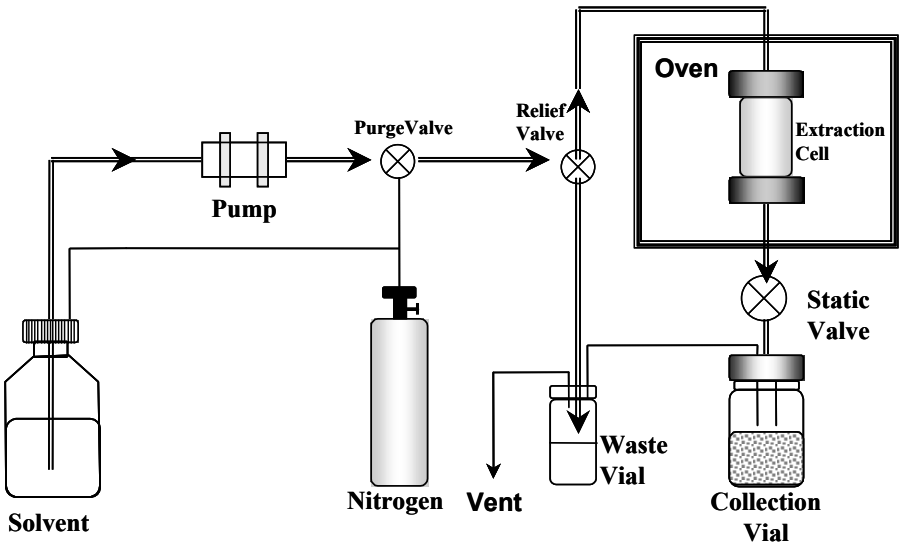
Racemosa		(v/v)		2			MS	
Malt	Proanthocyanidins	Acetone/water 80:20 (v/v)	60 / 1500	Static – 1	diatomaceous earth	10	SFE-HPLC	[75]
Rosemary	Antioxidants	Water	100 / 1500	Static – 1		25	CE-MS	[150]
Sage	Phenolic diterpenes	Water	100 / 1500	Dynamic (1 ml/min)	Sea sand	60	HPLC-ESI-MS	[79]
Carotenoids and tocopherols								
Different foods	Carotenoids	Methanol/ethyl acetate/ light petroleum 1:1:1 (v/v/v)	40 / 1000	Static – 3	Hydromatrix	2	HPLC-DAD	[84]
Palm oil	Carotene, tocopherols	Hexane	80 / 1500	Static – 2		10		[81]
Seeds and nuts	Tocopherols	Acetonitrile	50 / 1600	Static – 2		5	HPLC-ED	[82]
Hazelnut	Tocopherol	Hexane (0.01% BHT)	60 / 1500	Static – 1	Hydromatrix	15	HPLC-UV	[83]
Dunaliella salina	Carotenoids	Ethanol	160 / 1500	Static – 1	Sea sand layered	17	HPLC-DAD	[32]
Microalgae	Carotenoids	Acetone	20 / 1500	Static – 2	Sea sand layered	5	HPLC	[86]
Essential oils								
Achillea monocephala	Essential oil	Water	150 / 870	Dynamic (2 ml/min)	Glass wool	20	GC-TOF-MS	[89]
Thymbra spicata	Essential oil	Water	150 / 870	Dynamic (2 ml/min)	Glass wool	15	GC-TOF-MS	[90]
Origanum onites	Essential oil	Water	150 / 870	Dynamic (2 ml/min)	Glass wool	30	GC-GC/TOF-MS	[103]
Fructus Amomi	Essential oil	Water	150 / 725	Dynamic (1 ml/min)		5	SPME-GC-MS	[88]
Ziziphora taurica	Volatiles	Water	150 / 870	Dynamic	Glass wool	30	GC-	[91]

				(2 ml/min)			GC/TOF-MS	
Lipids								
Ziziphus jujube	Saponins and fatty acids	Methanol/ethyl acetate 95:5 (v/v)	140 / 1200	Static – 2	Diatomaceous earth	15	HPLC-ESLD	[94]
Poultry meat	Lipids	Chlorophorm/Methanol 2:1 (v/v)	120 / 3000	Static – 2	Hydromatrix	10	TLC, GC	[93]
Wheat germ oil	Fatty acids	Hexane	105 / 1500	Static – 3		5	GC	[92]
Medicinal plants								
Medicinal plants	Active compounds	Water	100 /200-500	Dynamic (1 ml/min)		40	HPLC	[102]
Morinda citrifolia	Anthraquinones	Water	220 / 1000	Dynamic (4 ml/min)		180	UV-Vis	[96]
Ligusticum chuanxiong and *Angelica sinensis*	Ligustilides	Water	150 / 600	Dynamic (2 ml/min)		10	SPME-GC-MS	[99]
Piper gaudichaudianum	Different compounds	Petroleum ether	85 / 1500	Static – 1		10	GC-MS	[100]
Rubarb	Anthraquinones	Methanol	140 / 1500	Static – 1	Diatomaceous earth	5	CZE	[95]
Cortex Dictamni	Limonoid derivatives	Methanol	150/ 1500	Static – 1	Diatomaceous earth	5	HPLC-DAD	[101]
Other food and natural matrices								
Spirulina platensis	Phycobiliproteins	Water	25 / 1500	Static – 7	Glass beads	15	CE-MS	[151]
Spirulina platensis	Antioxidants	Ethanol	111 / 1500	Static – 1		15		[24]
Peppers	Capsaicinoids	Methanol	200 / 1500	Static – 1	Sea sand	5	HPLC-MS	[97]
Green tea / Coffee	Caffeine	Water	100 / 900	Static – 1		10	HPLC	[104]

Table 3. SFE applications on the analysis of food pollutants.

Compound of interes	Sample	Analysis post-SFE	Extra clean ups	SFE conditions	Reference
Organohalogen and organophosphate pesticides	Vegetable soup (gazpacho)	GC-PFD-ECD-MS	Dry magnesium sulphate	300-500bar/50-90ºC	[64]
Organohalogenate pesticides	Fish muscle	GC-ECD col DB5	Dry and freeze dry	100-240bar/36-64ºC	[108]
Sulfonamides	Chicken liver, beef liver, and pig kidney	Hplc-uv y HPLC-amperometric	No	70–207bar/40-160ºC	[16]
P,p[1]-DDE and PCB	Sardina and chicken liver	GC-ECD and GC-MS	Freeze dry	300bar/110ºC	[119]
Organohalogenate pesticides	Infant food	GC MS	Deshidratado	170bar/70ºC	[109]
2-dodecylcyclobutanone as an irradiation dose indicato	Ground beef	GC-MS	Ground	375bar / 75ºC	[122]
PAH	Vegetable oil	HPLC fluorescencia	No	283bar/110ºC	[117]
Lasalocid (veterinary drug)	Poultry feed	HPLC fluorescencia fase normal	No	275bar/50ºC	[115]
Organophosphorus, Organohalogen, Organonitrogen and Pyretroids Pesticides	Cereals, cereal products, vegetables and fruits	GC-ECD GC-NPD	Dry	–	[107]
Organophosphorus, Organohalogen, Organonitrogen and Pyretroids Pesticides	Potatoes, tomatoes, apples and lettuce	GC-ECD and GC-MS	Ground, dehydrated	200-700 bar/70ºC	[105]
Pesticide multirresidue	Honey	GC-MS	Freeze dry pre SFE and SPE post SFE	200-600bar/40-90ºC	[106]
Polychlorinated and polybrominated contaminants	Aquaculture fish feed and cultured marine species	GC/MS/MS	Ground , Freeze dry and SPME	165 bar/60ºC	[152]
PAH/anti-cancer agents	Smoked fish/milk	GC-MS	Mix with C18 and dry	300bar/100ºC	[118]

1



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3 Figure 2. Scheme of a device for PLE.

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Journal of Chromatography A Volume 1152, Issues 1–2, 8 June 2007, Pages 234–246
http://dx.doi.org/10.1016/j.chroma.2007.02.046

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