Modern Extraction Techniques

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Modern Extraction Techniques

Food and Agricultural Samples

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Foreword

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Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previously published papers are not accepted.

ACS Books Department

Preface

This book is based on the symposium entitled "Modern Extraction Techniques for Food and Agricultural Samples," which was arranged by the Division of Agricultural and Food Chemistry during the American Chemical Society's 227th national meeting in Anaheim, California, held March 28–April 2, 2004.

One of the biggest challenges facing the chemical and pharmaceutical industry today is the need to replace current environmentally unsustainable and often polluting processes with cleaner ones. The transition to these new "green" processes will be easier if these technologies are more efficient and less costly than the current ones. In their book, *Green Chemistry: Theory and Practice,* Anastas and Warner (Oxford University Press, 1998) describe the 12 principles of green chemistry thus:

1. It is better to prevent waste than to treat or clean up waste after it has been created.
2. Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.
3. Wherever practicable, synthetic methods should be designed to use and generate substances that possess little or no toxicity to human health and the environment.
4. Chemical products should be designed to effect their desired function while minimizing their toxicity.
5. The use of auxiliary substances (e.g., solvents, separation agents, etc.) should be made unnecessary wherever possible and innocuous when used.
6. Energy requirements of chemical processes should be recognized for their environmental and economic impacts and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure.

7. A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable.
8. Unnecessary derivatization (use of blocking groups, protection/deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste.
9. Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.
10. Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment.
11. Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.
12. Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.

Although these principles are mainly for chemical synthesis applications, they can also be applied to other processes, such as extraction.

The extraction techniques described in this book fulfill many of Anastas and Warner's principles. For example, the use of supercritical carbon dioxide (SC-CO_2) as the sole extraction solvent results in a nonpolluting process (prevention of waste and safer solvents and auxiliaries). Other beneficial properties of supercritical CO_2 include fast diffusivity and nearly zero surface tension, which lead to extremely efficient extractions. In Chapters 2–4, applications of SC-CO_2 as an extraction solvent are described. Ethanol and water are also environmentally friendly solvents that can be used as extraction media in many applications (see Chapters 5–7). Pressurized hot water (~100–200 °C) in particular is a safe and nonpolluting solvent that has a similar dielectric constant to polar organic solvents, such as ethanol or acetone. Hence, pressurized hot water is a viable green alternative to many current extraction processes that use toxic organic solvents. Similarly, pressurized hot ethanol is an excellent solvent for the extraction of most medium polar to nonpolar organic molecules. Some of the techniques, such as membrane-assisted solvent extraction, described in Chapter 10, use organic solvents but in much smaller amounts compared to classical extraction techniques. Other techniques, for instance solid-phase microextraction and stir-bar sorptive extraction, described in Chapter 11, use no solvents.

Chapters 2–5 and 7 describe an important aspect of green chemistry: the use of plants and biomass waste as feedstock for extraction of various high-value compounds. Hence, instead of chemically synthesizing pharmaceutical drugs, food-grade antioxidants, or other high-value compounds of industrial and consumer interest, these compounds are extracted from renewable natural sources or from industrial wastes or byproducts using the green solvents and processes described in this book. This rather novel approach fulfills most of the green chemistry principles, including prevention of waste, atom economy, less hazardous chemical syntheses, use of renewable feedstocks, and inherently safer chemistry for accident prevention. More new extraction plants are being built around the world based on these ecologically sustainable processes.

As the applications described in this book demonstrate, these modern extraction techniques are not only better for the environment but are also faster, easier to automate, more sensitive, more selective, and more robust than classical organic solvent-based extraction techniques. These qualities are demonstrated in Chapter 8, regarding online coupling of extraction and analysis techniques, and in Chapter 9, on extraction methodologies with integrated cleanup steps.

The first chapter gives a short description of each of the techniques described in the book. This section is mainly intended for people who are new to the field. Those already familiar with the various extraction techniques can go straight to the second chapter.

I would like to acknowledge all the contributing authors. They have put in a lot of time and effort, which has helped to make this book an excellent compilation of different areas of expertise. I am also grateful to the numerous reviewers, who also helped to improve the quality of the contributions. I would like to thank the American Chemical Society for publishing this book.

I hope you will enjoy reading the book and that you will find the information useful for your own activities.

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Modern Extraction Techniques

Chapter 1

Overview of Modern Extraction Techniques for Food and Agricultural Samples

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In this introductory book chapter, several modern extraction techniques will be described, including supercritical fluid extraction, pressurized liquid extraction, pressurized hot water extraction, microwave assisted extraction, membrane-assisted solvent extraction, solid phase micro extraction and stir-bar sorptive extraction. These are techniques that meet many of today's requirements in terms of environmental sustainability, speed and automation. Basic principles of operation as well as method optimization will be discussed and compared for the different techniques. Both analytical and industrial applications will be discussed, together with commercial instruments available on the market. Key references will be given, and conclusions regarding applicability of the different techniques with respect to sample type, target-molecules and analytical vs. large-scale applications.

Introduction

This first chapter is a simple overview of existing "modern" extraction techniques, which were discussed at the ACS symposium in 2004 "Modern Extraction Techniques for Food and Agricultural Samples".

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What can be demanded from a "modern" extraction technique today? Prefereably, it should be safe to the environment, non-toxic, fast and automated, robust, highly sensitive and cost-efficient. Unfortunately, there is no such technique that fulfills all of these criteria. However, some of the more promising ones are supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), pressurized hot water extraction (PHWE), microwave assisted extraction (MAE), membrane-assisted solvent extraction (MASX), solid phase micro extraction (SPME) and stir-bar sorptive extraction (SBSE). These techniques have been used to extract minor (or major) components from food and agricultural samples, and they are more deeply discussed in the various chapters of this book. In this chapter, descriptions of the principles behind these extraction techniques are presented, which apply to both analytical and process-scale applications. For more thorough information about basic theory and applications, please consider the excellent review articles on SFE *(1,2)*, PLE *(3,4)*, PHWE *(5)*, MAE *(6,7)*, MASX *(8)*, SPME *(9)*, SBSE *(10)* and a review discussing all of the techniques *(11)*.

Supercritical Fluid Extraction (SFE)

A liquid (or a gas) becomes supercritical as you increase the temperature and the pressure above its critical point (defined by T_c and P_c, see figure 1). For CO_2, this point occurs at a T_c of 31°C and P_c of 72 bar. Several supercritical fluids (SFs) can be used as extraction solvents in SFE *(12)*, but only CO_2 will be discussed here because of its low toxicity, low critical temperature and pressure, and wide applicability.

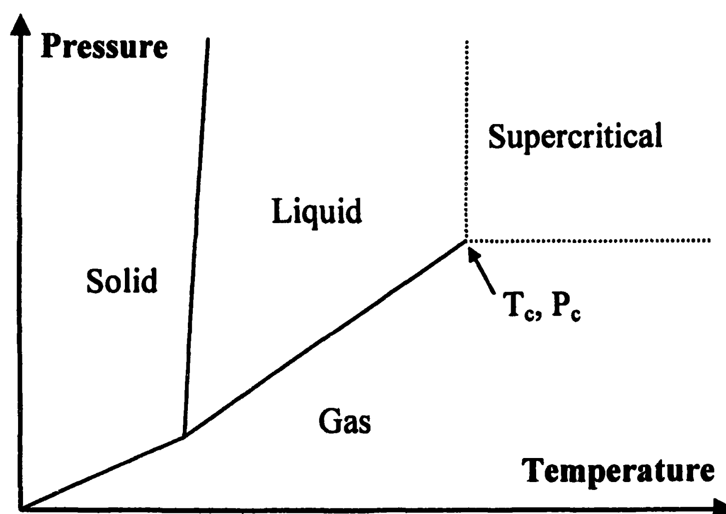

Figure 1. *A general phase diagram.*

$SC-CO_2$ is attractive as extraction medium because of its high diffusivity combined with its high (and easily tunable) solvent strength. Another advantage of $SC-CO_2$ is that it is gaseous at room temperature and ambient pressure, which makes product (or analyte) recovery fairly simple. Furthermore, the CO_2 can be easily recycled as the solutes dissolved in the $SC-CO_2$ will precipitate upon depressurization.

SFE is simply performed by pumping the SF through a vessel filled with sample, and further down the line depressurize the SF for collection of extracted components. Hence, a SF instrument consists of one or two high-pressure pumps for delivery of SF and if necessary also a polar cosolvent such as ethanol, a high-pressure vessel for holding the sample, a restrictor and a collection device (for example an empty vessel or a glass tube containing a collection solvent). The sample vessel is placed in an oven for control of the extraction temperature. See figure 2 for a simple schematic of a SFE instrument.

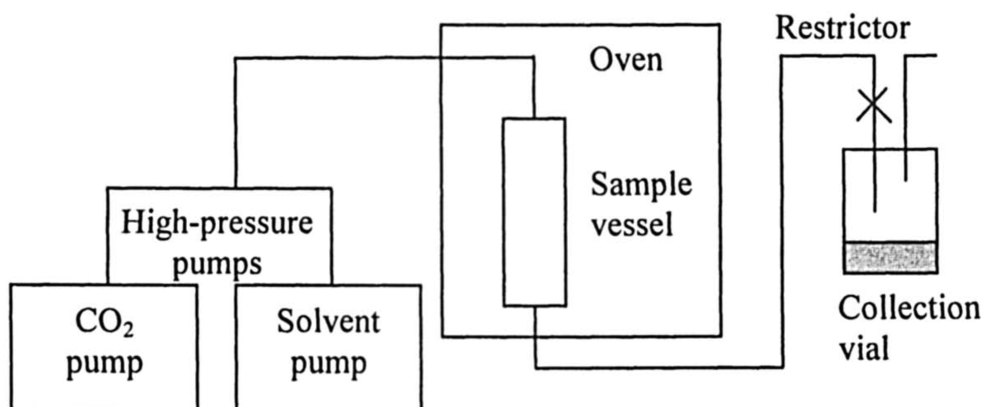

Figure 2. *Illustration of a SFE instrument.*

Method development in SFE is not so simple since several parameters have to be optimized, including temperature and pressure of the SF, extraction time, flow rate, addition of cosolvent (type of solvent and amount) and finally collection mode (e.g. in a solvent, in an empty vessel or on a solid-phase trap). Furthermore, the methods are generally matrix-dependent, i.e. a method developed for a particular target-molecule(s) cannot be directly applied to other types of samples than the one(s) it was optimized for.

Regarding available SFE instrument on the market today, there are several options. Applied Separations (Allentown, PA) has various-sized systems, including analytical (one-, two-, or four-parallell vessels of volume 0.5 mL to 1.0 L), pilot-scale (2 or 5 L vessels), process development systems (500 mL vessels) as well as large-scale industrial plants. Pressure Products Industries (Warminster, PA) offers single-vessel units from analytical-scale (100 mL vessels) to pilot-plant scale. Thar Technologies (Pittsburgh, PA) has extraction systems ranging from analytical (100 mL vessels) to process-scale (1500 L vessels), and they also provide high-pressure components such as pumps, vessels, view cells and back

pressure regulators. Thar Technologies as well as others manufacturers make phase monitoring systems that can be used for determining solubilities of solutes in SF under varying conditions. Unfortunately, there is a lack of fully automated analytical SFE equipment on the market as Isco (now Teledyne Isco Inc. Los Angeles, CA) stopped their production of SF extractors in 2004 (although they are still manufacturing high-pressure syringe pumps) and Hewlett Packard discontinued their production of SF extractors several years ago. LECO (St. Joseph, MI) manufactures an analytical "total fat/oil determinator" (TFE2000), which has three 10-mL parallel extraction vessels and three collection vials. However, it is of course possible to design your own equipment using high-pressure parts from for example Baskerville (Manchester, UK), Autoclave Engineers (now part of Snap-tite Inc., Erie, PA) or Thar Technologies. Industrial-scale (as well as pilot-scale) equipment is available from Chematur Engineering (Karlstad, Sweden), Uhde High Pressure Technologies (Hagen, Germany), Natex (Ternitz, Austria), Separex (Champigneulles, France) and to some extent also Thar Technologies and Applied Separations.

Some existing examples of industrial plants around the world are John Haas hops extraction plant in Yakima, Washington, Aromtech berry seed oil extraction plant in Torneo, Finland, NaturNorth Technologies birch bark extraction plant in Duluth, Minnesota, and several decaffeination plants in the USA and Germany. There are also some analytical-scale monitoring programs running on SF equipment, for example pesticide analysis in food and agricultural samples in Japan and persistent organic pollutants in lake/river sediments in the USA. In terms of research applications about SFE of food, agricultural and plant samples, there are several hundred published papers, including the analysis of pesticides *(13-15)*, PAHs *(16)*, fats and oil *(17,18)*, antioxidants *(19,20)*, fat-soluble vitamins *(21-23)*, sterols *(24)* and other bioactive compounds *(25,26)*. Another interesting class of applications is to use wastes or byproducts from industry as raw material for extracting valuable and health-beneficial compounds such as antioxidants, phytosterols and essential fatty acids *(27-33)*.

For more information on basic principles of SFE as well as potent applications, please see chapters 2-4 of this book. There are also several excellent review articles on the topic *(2,13,25,34-37)*.

Pressurized Liquid Extraction (PLE)

The principle of PLE is somewhat simpler than that of SFE. In PLE, fast extractions are obtained because of higher diffusivity, improved solubilization capability and more efficient disruption of analyte-matrix interactions in a liquid solvent at temperatures above its boiling point *(4)*. PLE is performed by filling a sample-vessel with solvent and then heat the vessel to desired temperature and

pressurizing the vessel to maintain the solvent as a liquid. Several static extractions are commonly performed with nitrogen-purging in-between. Less commonly, dynamic extractions can be obtained using pumps and restrictors. The solvent containing the extracted solutes is collected in an empty vessel. See figure 3 for a schematic of a typical PLE instrument.

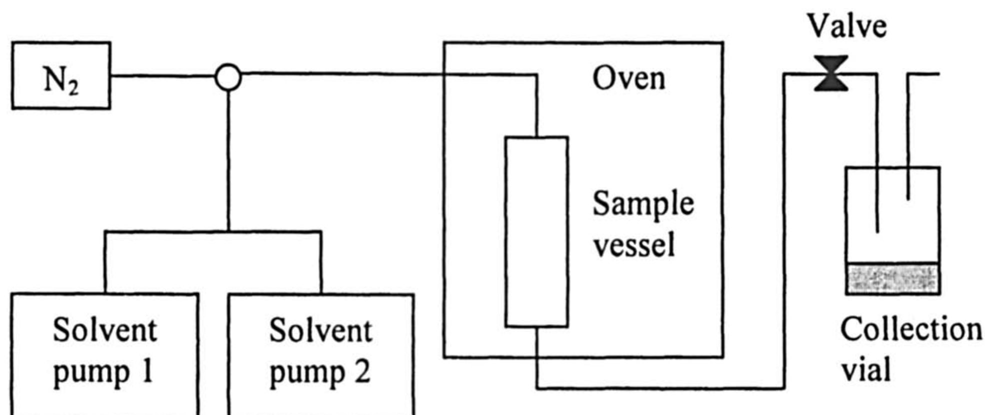

Figure 3. *Schematic of a PLE instrument.*

An advantage of PLE is that the same solvents can be used as the ones typically used in conventional solvent extraction methodologies, i.e. it is fairly easy to replace old methods with PLE. In addition, method development in PLE is simple since only two parameters are important to optimize once a solvent has been selected; temperature and extraction time (usually given by static extraction time and number of extraction cycles). Typically, analytical-scale extractions take between 10 and 30 min at a temperature between 60 and 200°C. Regarding the selection of an appropriate extraction solvent, it is advicable to use one that is generally recognized as safe (GRAS), for example ethanol. Ethanol is readily available at high purity and low cost and is also produced from biomass materials in many large chemical industries.

There are several analytical-scale equipment available on the market, for example Dionex (Salt Lake City, UT) has three different types of units; ASE100 (single cell, 10-100 mL), ASE200 (24-cells carrousel, 1-33 mL) and ASE300 (12-cells carrousel, 34-100 mL). Applied Separations has two versions; onePSE (single cell, 11-33 mL) and fastPSE (six parallel vessels, 11-33 mL).

There are lots of research applications published regarding PLE and analysis of various compounds in food and agricultural materials, for example pesticides in rapeseed *(38)*, babyfood *(39)* and strawberries *(40)*, lipids in corn and oats *(41)* and in egg-containing foods *(42)*, acrylamide in food samples *(43)*, carotenoids in processed food *(44)*, xanthones and flavanones in root bark *(45)*, tocopherols in seeds and nuts *(46)*, antioxidants in microalgae *(47)* and anthocyanins and total phenolics in dried red grape skin *(48)*. More discussions

on various interesting PLE applications can be found in this book (see chapters 5 and 7). There are also many excellent review articles available *(49-51)*.

Pressurized Hot Water Extraction (PHWE)

For polar to medium polar target compounds it may be feasible to use water as a solvent at elevated temperatures. Subcritical water is obtained in a pressurized system at temperatures between 100 and 374°C, and above 374°C (and 218 atm) water is supercritical. Here we will use the terminology pressurized hot water extraction (PHWE) for liquid water above 100°C and fairly low pressure, around 20 atm. PHWE is based on the same principles as PLE, but using only water as extraction solvent. Water is a particularly interesting solvent because its physical properties dramatically changes with increasing temperature. As shown in figure 4, the dielectric constant of water decreases from 74 at room temperature to around 36 at 200°C and 22 at 300°C. Hence, water behaves more like an organic solvent at higher temperatures. Again, the extraction vessel is pressurized to maintain water as a liquid.

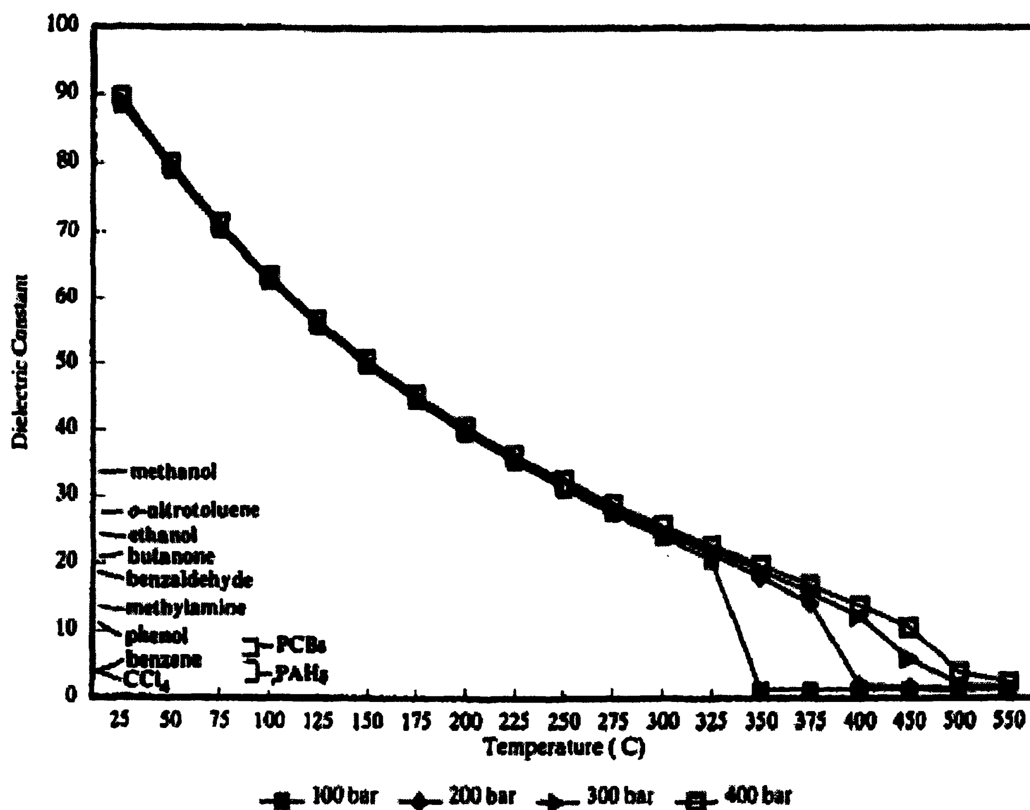

Figure 4. Diagram of the dielectric constant vs. the temperature of water. Reproduced with permission from ref (52).

Since water is perhaps the most environmentally friendly solvent available at high purity and low cost, it has been exploited for the extraction of avoparcin in animal tissue *(53)*, fungicides in agricultural commodities *(54)*, fragrances from clove *(55)*, antioxidative compounds from sage *(56)*, anthocyanins and total phenolics from dried red grape skin *(48)*, and other bioactive compounds from plants *(57)*. See also chapters 5 and 6 in this book and a few review articles *(51,58)*.

The same equipment can be used as in PLE, but for temperatures above 200°C, there is no commercial equipment available and specially designed or home-built instruments are therefore used. Chematur in Sweden manufactures industrial-scale supercritical water oxidation plants operating at temperatures above 400°C (Aqua Critox®). It is possible that such equipment can be modified to subcritical water extraction processes. Uhde in Germany probably also has industrial-scale solutions for PHWE.

Microwave Assisted Extraction (MAE)

Microwave assisted extraction (MAE) is also an extraction technique based on heating an organic solvent. The principle is roughly that a sample and an appropriate solvent (or solvent mixture) are put in a vessel, which is then pressurized and heated by microwaves. After typically 5 to 20 min the extraction is complete, and the vessels are allowed to cool down before removing the sample/solvent mixture. The solvent must be filtered to remove sample particles prior to analysis of the extracted components. See figure 5 for a schematic of a MAE equipment.

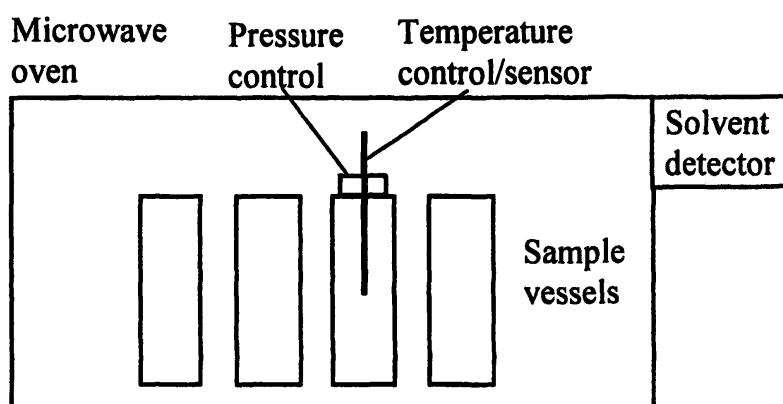

Figure 5. *Simple schematic of a MAE instrument.*

MAE is a more manual technique than PLE as it is performed in batch mode. However, many samples can be processed at the same time. Another feature of MAE is that the heating of the solvent is fast, it goes from inside the

sample and outwards, and the heating capability depends on the microwave-absorbing properties of the solvent. Polar solvents such as acetone will absorb microwave energy efficiently, as they have molecules with permanent dipole moment that can interact with microwaves. Non-polar solvents such as hexane will not be heated when exposed to microwaves, but can instead be used in mixtures with polar solvents in order to obtain the desired heating properties. Some common solvent mixtures that have been used in MAE are acetonitrile/methanol, hexane/acetone, ethylacetate/cyclohexane and iso-octane/acetone.

MAE applications in the literature include for example extraction of capsaicinoids from capsicum fruit *(59)*, PBDEs from marine biological tissues *(60)*, pesticides from vegetables *(61)* and pigments from paprika powder *(62)*.

In general, method development in MAE involves optimization of solvent composition, solvent volume, extraction temperature and extraction time. The temperature of the solvent/sample mixture is usually well above the boiling point of the solvent(s). Hence, MAE utilizes in a similar manner as in PLE the enhanced solvent strength and faster diffusivity of a heated solvent. For more discussions on optimization of MAE and various applications, please consider chapter 9 of this book as well as several review articles *(6,7,49,50,63)*.

There are a few different brands of equipment on the market; CEM (Matthews, NC) has the MARS-X™ that can process up to 14 samples simultaneously with optional sample stirring. Milestone (Shelton, CT) manufactures instruments named Ethos™ (6 to 24 vessels of volumes 100-270 mL, with sample stirring). Radient Technologies (Burlington, ON, Canada) has developed a large-scale MAE technology that they call MAP™ and they design pilot plants for natural-product customers. There are several MAE pilot and industrial plants around the world. For example, Archimex (Vannes, France) is doing contract research on pilot-scale MAE (as well as SFE) for isolation of bioactive/high-value compounds from plant origin.

Membrane-Assisted Solvent Extraction (MASX)

Non-porous membranes can be used for extraction of polar and non-polar compounds from liquid samples using only minimal amount of organic solvent. A non-porous membrane is a liquid or a solid (e.g. polymeric) phase sandwiched between two other phases, usually aqueous but can also be gaseous *(8)*. One of these two phases contains the components to be extracted, i.e. the donor phase. On the other side of the membrane is the acceptor phase, i.e. where the extracted components are collected. Usually, the membrane unit is made of two blocks of inert material with a machined groove in each. The membrane is placed in-between these blocks and clamped together, so that a channel (typically 10-1000

μL) is formed on each side of the membrane. A schematic of a MASX unit is shown in figure 6.

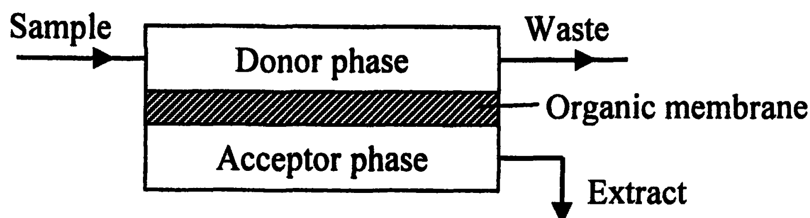

Figure 6. *Simple schematic of a MASX unit.*

The driving force for the extraction process over the membrane is in most cases a concentration gradient *(64)*. High enrichment-factors of the extracted components can be obtained if a small-volume, stagnant acceptor phase is used. Hence, the advantages of MASX compared to conventional liquid-liquid extraction (LLE) are that much less organic solvent is used, significantly higher enrichment factors can be obtained and the set-up can be easily incorporated in a flow system and automated for on-line coupling to HPLC, GC or capillary electrophoresis *(65)*. Disadvantages are that the organic solvent used often is rather hazardous (although small volume) and that only liquid or gaseous samples can be processed.

There are different variations of the MASX technique, including supported liquid membrane extraction (SLM), microporous membrane liquid-liquid extraction (MMLLE), polymeric membrane extraction (PME) and membrane extraction with a sorbent interface (MESI). These techniques will be briefly described below.

In SLM, both the donor phase and the acceptor phase are aqueous, and the membrane is an organic solvent such as n-undecane or dihexyl ether, held by capillary forces in the pores of a supportive hydrophobic porous membrane. Different pH, i.e. below and above the pKa of the target molecules, is commonly used in the donor and the acceptor phase respectively, in order to enable extraction of uncharged compounds and trapping by making the target molecules charged in the acceptor phase. There are also other ways of trapping target molecules in the acceptor phase, as described in several review articles *(8,64,66,67)*. Common applications include the extraction of small organic acids and pesticides from agricultural and food samples (see chapter 10 of this book).

In MMLLE, the acceptor and membrane phases are both organic, while the donor phase is aqueous. This technique is more suitable than SLM for extracting non-polar compounds. The chemical principle of MMLLE is the same as that of LLE, but performed in a dynamic flow-system, which makes it easy to hyphenate MMLLE with other separation techniques such as GC *(68)*. For example, Hyötyläinen and colleagues coupled PHWE on-line with MMLLE and GC for

the determination of pesticides in red wine *(69)* and grapes *(70)*. More information on hyphenations including MMLLE, PHWE, SFE and MAE can be found in chapter 8 of this book.

In PME, a solid hydrophobic membrane (usually silicon rubber) separates the aqueous or organic donor and acceptor phase. PME applications are described in chapter 11 of this book.

In MESI, target-compounds are extracted into a membrane (usually a silicon rubber hollow fiber) from its surrounding aqueous or gaseous donor phase. The acceptor phase, which flows through the lumen of the hollow fiber, is however always gaseous for better compatibility with GC analysis *(71,72)*. The extracted components from the membrane are transported into a cold sorbent trap. The analytes are desorbed from the trap by heating and thereafter transported into the GC. For example, Liu et al. *(73)* used MESI combined with a portable GC system for continuous on-line monitoring of biogenic volatile organic compounds emissions from Eucalytus dunnii leaves in a greenhouse.

Membrane units can be purchased either from Global FIA Inc. (Fox Island, Washington), or from the mechanical workshop at the Chemical Center at Lund University (Lund, Sweden). Process-scale membrane units can be designed by Membrane Extraction Technology Ltd (London, UK).

Solid Phase Micro Extraction (SPME)

Solid phase micro extraction (SPME) is a widely used extraction technique that was developed by Pawliszyn and co-workers in 1990 *(74)*. SPME uses a fused silica fiber that is coated on the outside with an appropriate stationary phase (a 5 to 100 μm thick coating of different polymers, e.g. polydimethylsiloxane, PDMS). The small size of the SPME fiber and its cylindrical shape enables it to fit inside the needle of a syringe-like device. Target molecules from a gaseous or a liquid sample are extracted and concentrated to the polymeric fiber coating. SPME has been used coupled to GC and GC-MS *(75)*, as well as to HPLC and LC-MS *(76)*. Figure 7 shows a commercially available SPME device.

SPME can either be performed by head-space extraction (HS-SPME) by placing the fiber in the vapour above a gaseous, liquid or solid sample, or by direct immersion extraction (DI-SPME), by immersing the fiber in a liquid sample. After a certain extraction time, the SPME needle is removed from the septum and inserted into the injection port of the GC or into the desorption chamber of the SPME-HPLC interface. The desorption is performed by heating the fiber in the GC inlet, or by pumping a solvent through the desorption chamber of the SPME-HPLC interface. The main advantages of SPME compared to LLE and solid phase extraction (SPE) are that no or little solvent is

needed and that the technique is easily automated and coupled to chromatographic techniques such as GC and HPLC. The main disadvantage of SPME is the lack in sensitivity due to the extremely small amount of sorbent that can be coated onto the SPME fiber in combination with the low analyte capacity of the SPME sorbents compared to many other typical adsorbents *(10)*.

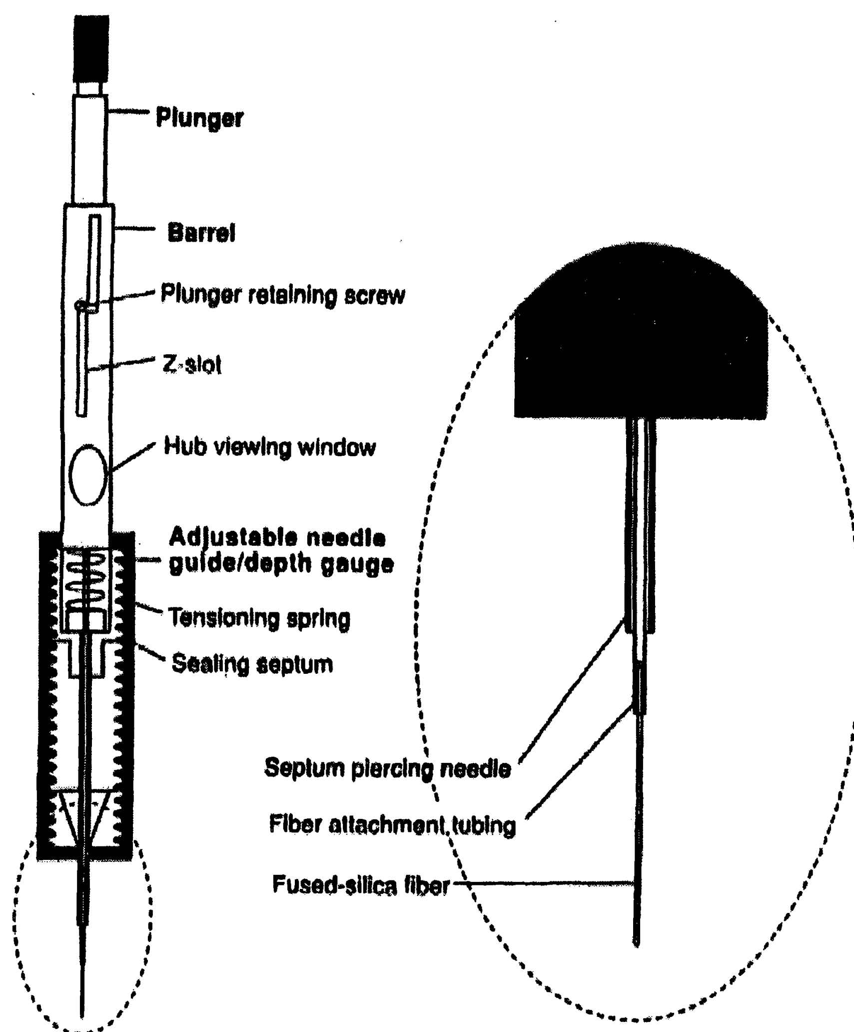

Figure 7. *Commercial SPME device made by Supelco. (Reproduced with permission from reference 9. Copyright 2000 Elsevier.)*

There are several parameters to consider in SPME, such as extraction mode (HS-SPME or DI-SPME), type and thickness of fiber coating, extraction time, sample properties (analyte concentration, pH, buffer, temperature, agitation) and analyte desorption. Applications and optimization of SPME for food and agricultural samples are discussed in the excellent review articles *(9,77)*. SPME fibers are commercially available from Supelco (Bellafonte, PA).

Stir-Bar Sorptive Extraction (SBSE)

SBSE has many similarities to SPME, as it is also a solventless sample preparation technique and it uses similar sorbents (based on PDMS). SBSE was first described by Baltussen and co-workers in 1999 *(78)*. In SBSE, an aqueous sample is extracted by stirring for a certain time with a PDMS-coated stir-bar. The stir-bar is thereafter removed from the sample and the absorbed compounds are then either thermally desorbed and analyzed by GC-MS, or desorbed by means of a liquid for interfacing to a LC system. Heat-desorption gives higher sensitivity while liquid desorption provides higher selectivity.

Headspace sorptive extraction (HSSE) is a similar technique developed by Bicchi et al. in 2000 *(79)*. In HSSE, a PDMS stir-bar is used for head-space sampling of volatile organic molecules. This technique also has similarities to HS-SPME. There are several review articles discussing and comparing HS-SPME, DI-SPME, SBSE and HSSE *(10,80-82)*.

Figure 8 shows a simple schematic of a PDMS stir bar and a HSSE sampling-device.

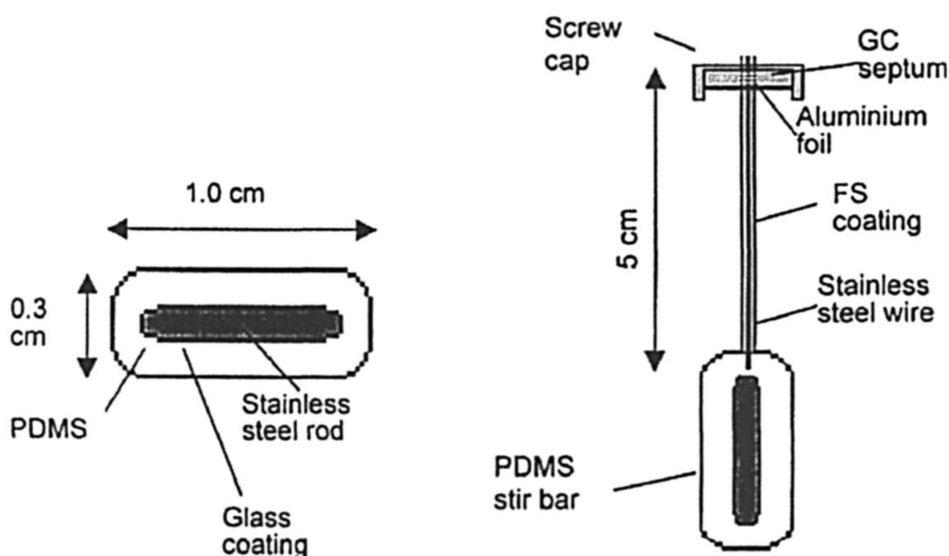

***Figure 8.** A schematic of a PDMS stir-bar (A) and HSSE-device (B). Reproduced with permission from ref (80).*

A stir-bar in SBSE is coated with up to 125 µL PDMS, which enables quantitative extraction of many organic compounds from aqueous samples of 10-100 mL in volume *(10)*. This should be compared to the situation in SPME, where the maximum volume of PDMS that can be coated onto the fiber is around 0.5 µL (100 µm thick coating). Hence, compared to SPME, SBSE offers a favorable alternative for quantitative analysis with much higher sensitivity and repeatability. Another advantage is that just as with SPME, no organic solvent is

required for the extraction. However, SBSE is not as easily coupled on-line to other separation techniques such as GC and HPLC.

Common applications for SBSE (and HSSE) are the analysis of PAHs in drinking water *(83,84)*, flavors and off-flavors in food samples *(80,85-88)*, and benzoic acid and other preservatives in beverages *(89,90)*. The technique has also widely been used for pesticide analysis in sample matrices like wine *(91)*, grapes *(92)*, honey *(93)* and other food matrices *(94)*.

The parameters to optimize in SBSE are similar to those in SPME (see above). For more information on optimization and advantages and disadvantages with SBSE, see chapter 11 of this book as well as several review articles *(10,82)*. Commercial SBSE devices can for example be purchased from Gerstel GmbH & Co.KG (Twister™, Mülheim an der Ruhr, Germany).

Conclusions

All of the techniques discussed here and in the following chapters in this book have their advantages and disadvantages. What they all have in common is that they are part of a sustainable development towards the use of less (or no) organic solvents, and they are faster, less toxic, more automated, sensitive and easier to use than classical extraction techniques (e.g. Soxhlet, Folch, Bligh & Dyer and LLE). There are however a few variations regarding applicability to different types of samples and target-molecules that will be pointed out here. Solid and semi-solid samples are preferably processed by SFE, PLE, PHWE or MAE, whereas liquid and gaseous samples are easier to process by MASX, SPME or SBSE. There are of course ways of processing liquid samples by SFE, PLE, PHWE and MAE as well, for example in SFE by using a counter-current column. MASX, SPME or SBSE can only be used with solid samples if they are first dissolved in a liquid solvent. Another difference between the techniques is the type of target-molecules that can be extracted. SFE is usually used for the extraction of non-polar to medium-polarity compounds, whereas PLE and MAE can be used for a wide range of different-polarity compounds. PHWE is mostly useful for polar compounds (or medium-polarity compounds at higher temperatures). MASX can be used for extracting polar compounds using SLM or non-polar compounds using MMLLE. SPME and SBSE are usually applied to extract volatile organic analytes, but can also be used for other, less volatile small organic compounds.

Not all of the techniques are suitable for large-scale industrial processes. SFE is clearly the most attractive technique of the ones discussed here, with more than 100 SFE productions plants and over 500 pilot plants worldwide. However, we will probably see more pilot plants based on PLE, PHWE and maybe also MASX in the future. SPME and SBSE are mostly developed for

analytical applications. To summarize, the future appears promising with respect to continued "green and clean" technologies on both an analytical as well as an industrial scale.

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Chapter 2

Supercritical Carbon Dioxide Extraction of Agricultural and Food Processing Wastes and Byproducts

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The agricultural processing industry generates a large variety of by-products and wastes ranging from manure to packing residuals. Researchers are working to develop new technologies and new uses for these materials in order to reduce environmental costs of the agricultural processing industry. New processes are developed to recover components producing value added products, such as novel foods, food ingredients, bioactive molecules for cosmetic or pharmaceutical industry. Supercritical carbon dioxide has been tested for the extraction of lipids and lipophilic components, where this technology has advantages over conventional solvents. For example, high extraction rates are achieved at lower temperatures, preserving many of the thermolabile extracted components. In this chapter, the composition of by-products and wastes, and extraction of useful components is discussed.

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Sustainable development is an important issue at the beginning of the third millennium and every research conducted in the direction of reducing the use of toxic chemicals, saving energy and managing wastes and by-products is welcomed on an International level *(1, 2)*. Sustainable development is a blending of environmental, social, and economic opportunities that try to meet the needs of the present without compromising the ability of future generations to meet their own needs. The research world is committed to this area, asking that each of its researchers follow a sustainable development strategy geared specifically to its mandate and integrated into its day-to-day and long-term decision making processes in the agriculture and agri-food sector. It is a way of producing and processing agricultural products that can be carried out over the long term, in a manner that supports or enhances the high quality of life.

High-quality food products and a commitment to environmental protection have contributed to the successful marketing of agri-food products *(3)*. The agri-food sector could capitalize further on this opportunity and contribute to achieving economic sustainability by differentiating and commercializing products on the basis of environmentally sustainable production, and by marketing technologies and expertise with demonstrated environmental benefits.

There are a great variety of putative value-added products in wastes and by-products from biological origin. Most of them are the consequence of food processing operations such as the peeling and coring of fruits and vegetables; other sources are animal and fish wastes, carbohydrate-rich grain, agricultural crops (wheat, corn, sugarcane and molasses, dairy whey, potatoes, etc.), fermented industrial wastes, etc. *(4, 5)*. Laufenberg *et al.* *(6)* recently reported a literature survey covering more than 160 articles, bringing together occurrence, quantities and utilization of vegetable residual products all over the world.

Supercritical Carbon Dioxide as a Clean and Mild Technology

There are several ongoing activities within the food-processing community in the areas of economic recovery, pollution prevention and clean technology implementation. Companies will continue to look at novel approaches to reduce solid waste generation, use less or reusable packaging, and use biodegradable packing products, but they will also show increased consideration for using mechanical methods for food processing (mild technology). Emerging mechanical technologies can indeed be used to perform many of the same functions as chemical processing. An example of a mild/selective technology is the Supercritical Fluid Technology (SFT) *(7)*.

SFT is a sophisticated technology that can be used to perform many of the same functions as traditional chemical processing when proper machinery, material, and scientific knowledge are used. A pure component is considered to

be in a supercritical state if its temperature and pressure are higher than critical values, and the liquid and gaseous states are indistinguishable from one another and the meniscus disappears. The term Supercritical Fluids (SF) has been coined to describe these media, and in recent years these have been widely exploited in many industries. SFT can be used in many different ways in the industry, e.g. for extraction (SFE), fractionation (SFF) and chromatography (SFC).

For the extraction of high value compounds, a suitable solvent for protection of thermolabile compounds must be used. In SFE, a widely studied clean extraction technology, the use of carbon dioxide (SC-CO_2) satisfies these needs because of a low critical temperature (31°C). In many ways carbon dioxide can be regarded as a typical non-polar solvent. Solubility in this solvent is a function both of the molecular weight of the solute and its polarity *(8)*.

Several classes of non-polar compounds could be recovered from by-products and wastes, but the use of SC-CO_2, a relatively high-cost extraction technology, should primarily be exploited for high-value products design. The most important classes of high value non-polar natural compounds are antioxidants, vitamins, flavors and fragrances, lipids, pigments, and pharmaceuticals.

The interdependence of volume, temperature and pressure are of extreme importance for SFE, since properties of SFs compounds change significantly when varying these parameters, and these variations are the basis for obtaining selectivity in many applications *(9)*. The physical properties of a SF are intermediate between those of a typical gas and a liquid. By varying the pressure and/or temperature of the fluid, the density changes. Hence, a SF can have a density, which ranges from those exhibited by gases to liquid-like values.

SC-CO_2 extraction has a potential to shorten the extraction time and improve the recovery of bioactive molecules in wastes and by-products. Generating solubility isotherms of extractants in SFs is essential for the development of potential commercial SFE processes. To date, no data exists for solubility of several bioactive molecules in SC-CO_2. This data alone would be a valuable addition to the existing literature concerning the solubility of compounds in SC-CO_2. SFE of bioactive molecules from various sources have shown potential for commercialization *(10, 11)*.

SFT can be applied to produce novel products including essences, fragrances and other derivatives of essential oils. These techniques include extraction processes acceptable in food use, such as SFE. One of the main objectives of our research is to investigate the extraction of bioactive molecules using SC-CO_2. We are identifying experimental parameters for optimal extractions leading to the design of suitable equipment. Other objectives include the development of novel products.

SFE of Valuable Compounds from Rice By-Products

We have studied products and by-products of the rice processing chain because they contain natural antioxidants *(12)*. SFT was employed to extract and enrich tocochromanols and oryzanols from an SC-CO_2 extracted rice oil taken from the rice processing chain. The amount of rice and sampling of products and by-products are reported in Figure 1.

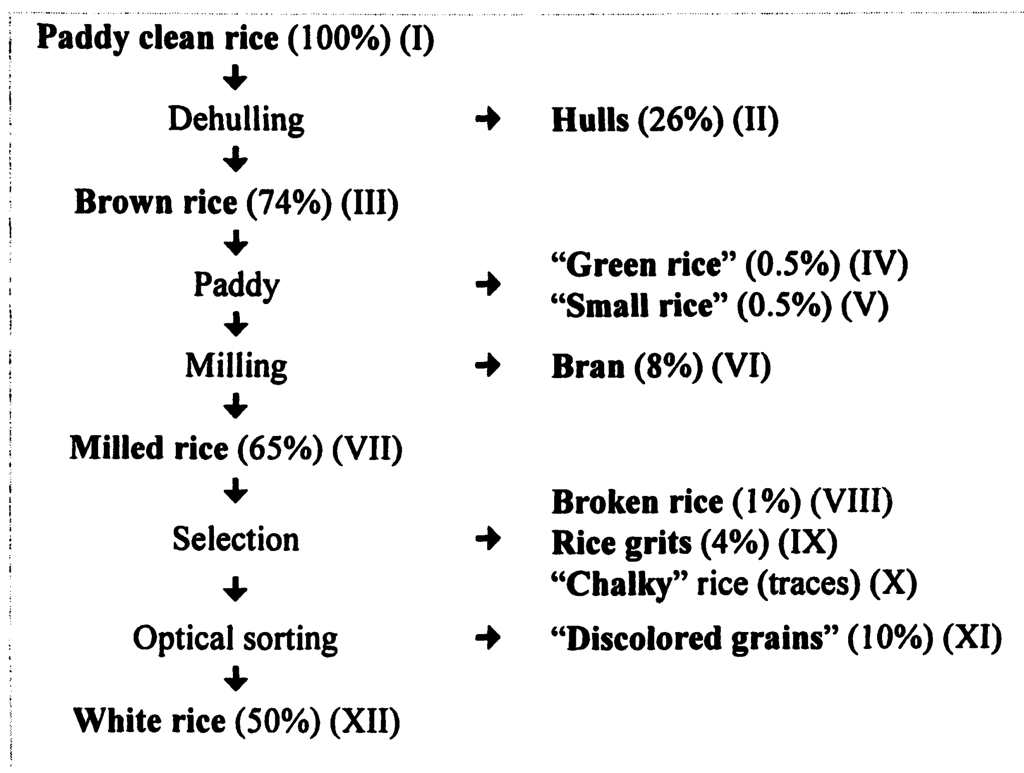

Figure 1. *Rice milling process: flow sheet, amounts of products and byproducts, and coding of samples. (Reproduced with permission from reference 12. Copyright 2003 Elsevier.)*

The broken rice goes into animal feed (to pet food manufacturers) and the hulls (or husks) are sold for plant (mulch) and animal bedding. Rice hulls are also used as fuel in power plants and rice mills, and as abrasives. Rice bran is rich in color, flavor, fiber and nutrients. It is used in cereals, mixes and vitamin concentrates, as it is rich in niacin, thiamin, vitamin B_6, iron, phosphorus, magnesium, and potassium *(13)*. It has been an extremely popular source of dietary fiber because of the hypocholesterolemic property of its oil fraction (15-30%). Stabilized rice bran has been made available by extrusion to stabilize the raw bran by inactivating its lipase. Non-food grades of rice bran are used as livestock feed *(14, 15)*. The rice bran is mostly used to feed pigs. The remaining part is split between use as a supplementary feed for scavenging poultry and for fish. Oil is commercially extracted from rice bran by hexane *(16)*; in the year of

2002 the world rice bran oil production was 1,079,000 t according to FAO data bank (17).

The aim of this study was to evaluate SFT for the exploitation of all the rice by-products, and novel processes to manufacture value-added food products. Conditions were studied to extract and, eventually, enrich tocochromanols and oryzanols from a SC-CO_2 extracted rice oil taken from the rice processing chain (raw material, intermediate products, by-products). To study the extraction conditions (pressure, temperature, extraction time, flow rate, etc.) and oil solubility for rice samples, extractions were initially conducted on rice bran because of its high oil concentration (generally 15-30%).

The pressures were 690, 520 and 345 bar, and temperatures were 40, 50 and 60°C. Extracts were analyzed for fatty acid composition, fat-soluble vitamin and γ-oryzanol content. Extractions were then conducted on all the processing samples for mass balancing (H_2O, N and lipids) at the highest extraction conditions (690 bar, 80°C). The proximate analysis of water, total nitrogen and total lipids along the process was conducted. 54% of the total nitrogen was still in the white rice, while 72% of the oil was in the bran. Figure 2 shows the extraction curves of rice bran oil collected as a function of the amount of CO_2 used. The extraction conducted at 690 bar and 80°C gave the highest extraction yield. 4.93 g of rice bran oil (24.65%) were extracted with only 100 g of CO_2. With a decrease of the temperature to 40°C and the decrease of the pressure to 520 bar, at least 300 g of CO_2 was required to obtain 4.80 g of oil. At 345 bar and 40°C, more than 400 g of CO_2 was required to obtain 4.23 g of oil.

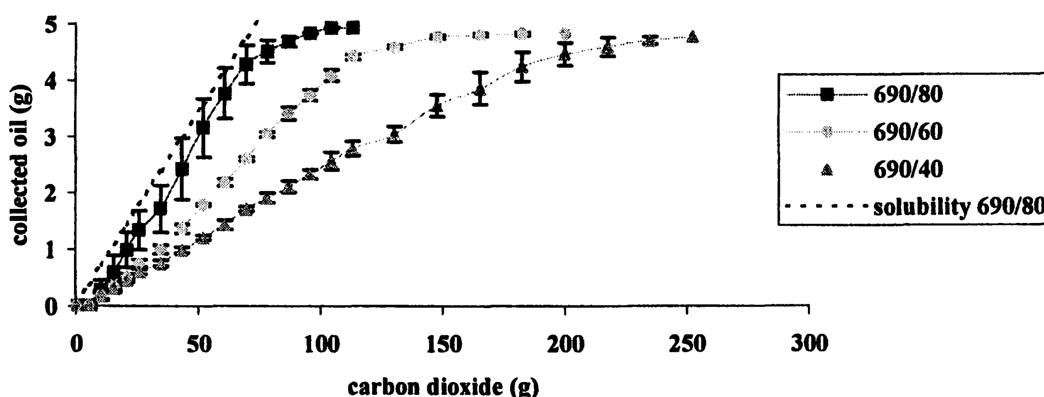

Figure 2. Extraction curves for 20 g of rice bran at different temperatures and 690 bar. (Reproduced with permission from reference 12. Copyright 2003 Elsevier.)

Oryzanols are natural antioxidants having phenolic hydroxyl groups similar to tocopherols. The γ-oryzanol content in the by-products is reported in Table 1. The highest amount of γ-oryzanol (18 mg per g of rice bran oil, sample XI) was

obtained at 690 bar and 80°C. This was followed by green rice (sample IV) and discolored rice (sample XI). These quantities were comparable with results obtained by Shen *et al.* *(18, 19)*. Moreover, since SC-CO_2 extracted oils generally need only purification (reduction of free water and removal of solid impurities through centrifugal separation) after the extraction, the effect of refining, as studied by Yoon and Kim *(20)*, is inapplicable. Also, the result of Xu and Godber *(21)*, stating a higher yield for γ-oryzanol extraction by SC-CO_2 than obtained by organic solvent extraction, allow us to consider our samples nutritionally better than traditionally extracted rice oils. Moreover, by changing the extraction conditions, the selective ability of SC-CO_2 can be yet again exploited to enrich the oil with these compounds.

Table 1. γ-Oryzanol content in SC-CO_2 extracts of rice by-products (12)

Sample	*γ-oryzanol (mg/g oil)*	*Sample*	*γ-oryzanol (mg/g oil)*
II	n.d.	VIII	9
IV	18	IX	8
V	n.d.	X	n.d.
VI	18	XI	11

n=1. n.d.: not determined

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We concluded that SC-CO_2 can be used to efficiently and rapidly extract oil from all by-products of rice processing. The initial analyses indicated that the quality of the SC-CO_2 extracted oil is as suitable for human consumption as the traditionally extracted one. The exhausted cake is still rich in high quality proteins and phosphatides *(22)*. The possibility of obtaining a SC-CO_2–extracted rice oil rich in γ-oryzanol and other antioxidants (e.g. vitamin E) indicates that this rice oil can be considered a nutraceutical product. Saito *et al.* *(23)* and Dunford and King *(24, 25)* used SC-CO_2 to fractionate rice bran oil by SFF. Their results are extended by the present study to take advantage of the effect of SC-CO_2 extraction eventually combined with SC-CO_2 fractionation to over again increase the value of rice by-products.

Other Examples of SC-CO_2-Extracted Wastes and By-Products

Szentmihalyi et al. (26) studied a rose waste material, and a valuable oil was obtained for medicinal use. Traditional Soxhlet extraction was compared with ultrasound, microwave assisted, and sub- and SC-CO2 extraction. The high

concentration of some metals that they found, particularly iron in oil obtained by microwave assisted extraction, is undesirable from the aspect of oxidation stability. Subcritical CO2 extraction at 28°C and 100 bar appeared to be the best method to recover from rose wastes the highest oil yield, including β-carotene, and linoleic acid. SC-CO2 extracted oil on the other hand was rich in MUFA and PUFA and contained the lowest amount of carotene and pheophytin. Oil yield in most new extraction methods was higher than in traditional Soxhlet extraction.

Baysal et al. (27) extracted lycopene and β-carotene from tomato paste waste using SC-CO2. To optimize the SFE procedure for the isolation of lycopene and β-carotene, a factorial designed experiment was conducted. The factors assessed were the temperature of the extractor (35, 45, 55, and 65°C), the pressure of the extraction fluid (200, 250, and 300 bar), addition of cosolvent (5, 10, and 15% ethanol), extraction time (1, 2, and 3 h), and CO2 flow rate (2, 4, and 8 kg/h). The total amounts of lycopene and β-carotene in the tomato paste waste, extracts, and residues were determined by HPLC. A maximum of 54% of lycopene was extracted by SC-CO2 in 2 h (CO2 flow rate = 4 kg/h) at 55°C and 300 bar, with the addition of 5% ethanol as a cosolvent. Half of the initially present β-carotene was extracted in 2 h (flow rate = 4 kg/h), at 65° C and 300 bar, also with the addition of 5% ethanol.

Favati et al. (28) extracted lycopene and β-carotene with neat SC-CO2 from products of the tomato industry (skins and seeds). The extractions were conducted at 200 to 700 bar and 40 to 80°C. At 700 bar and 80°C more than 80% of the lycopene and 70% of the β-carotene could be removed from by-products in only 11 minutes. A procedure was proposed for SFE of all-trans-lycopene from tomatoes using different CO2 densities. Lycopene extractions were carried out using CO2 densities ranging from 0.25 to 0.90 g/ml for 30 min at 40°C. A CO2 density of 0.25 g/ml did not enable significant amounts of carotenoids to be extracted. The amount of the trans-form extracted increased with increasing extraction pressure (due to increased solvating power). The extract contained 88% of all-trans-lycopene and 12% of cis-lycopene. It was suggested that SFE of all-trans-lycopene may be a potential method for the production of high value added food ingredients from, for example, surplus tomatoes and tomato processing wastes (29). In a similar study, Rozzi et al. (30) applied SFE for the extraction of various phytochemicals from tomato processing by-product.

Application of SC-CO2 extraction to waste powder derived from sweet potato roots with orange flesh in a food processing factory was examined by Okuno et al. (31). Contents of antioxidants (β-carotene, α-tocopherol and chlorogenic acid) remaining in the waste powder after SC-CO2 extraction under different conditions (40-80°C, 100-350 bar) were determined by HPLC. At a constant extraction pressure, the extraction yield of β-carotene from the waste powder was highest at 40°C; in contrast, the α-tocopherol yield did not vary

under different temperature conditions. At a constant extraction temperature, increasing extraction pressure increased the extraction yields of both β-carotene and α-tocopherol. Under the conditions tested, the yields of β-carotene (95.1%) and α-tocopherol (76.8%) were highest at 40°C and 350 bar. Little chlorogenic acid was extracted from waste powder. The β-carotene remaining in the waste powder after SC-CO2 extraction correlated well with the color value of redness of the powder.

There are several more studies exploring the use of SC-CO2 for the extraction of carotenoids. Hartono et al. (32) used six different cubic equations-of-state to predict the solubility of β-carotene in SC-CO2. Yang et al. (33) developed a model to predict the lycopene SC-CO2 extraction process using conventional and soft computing approaches. Doker et al. (34) investigated the modeling of β-carotene extraction from industrial waste product of apricot bagasse at the production of fruit juice. The shrinking core model was selected as the best mathematical model, which characterizes the extraction process, after taking into consideration mass-transfer mechanisms such as adsorption, diffusion, solubility and desorption. Extraction of β-carotene was performed in a semi-continuous flow extractor using SC-CO2 as a solvent to measure the effects of flow rate, particle size, temperature and pressure on the extraction yield.

We studied the extraction of lipids from algae (Isochrysis galbana Parke) to verify the possibility to enrich EPA, DHA or other omega-3 fatty acids for healthy products or pharmaceutical application (35). We found that the SFs allow the extraction of lipids from microalgae with interesting fatty acid composition and good extraction yield in comparison with traditional solvents.

Seong-Sil-Kang et al. (36) studied wastes from squid processing factories because they contain high levels of EPA and DHA. Extractions were performed with and without 3% ethanol as entrainer in a semi continuous flow extractor with pressures in the range of 83-138 bar and temperatures of 25-50°C to improve the extraction efficiency. The extracts they obtained contained high levels of DHA, EPA and other PUFA. Highest extraction yield was achieved at 124 bar and 40°C with or without ethanol. Major fatty acids detected in the extracts (in addition to DHA and EPA) were myristic acid, palmitic acid, palmitoleic acid, oleic acid and arachidic acid.

Mendes et al. (37) studied the use of SC-CO2 for vitamin E concentration in deodorizer distillate of soybean oil. Lee et al. (38) applied a SFE method to test the feasibility of tocopherol concentration from soybean sludge with SC-CO2. Shishikura et al. (39) separated tocopherols from soybean sludge by SC-CO2 and nitrous oxide (SC-N2O). Lecithin and other compounds can be concentrated in liquid wastes with continuous/semicontinuous SC-CO2 operations; production costs will be substantially reduced in comparison with batch operations.

Ibanez et al. (40) studied the SFE of tocopherols from olive by-products; while De Lucas et al. (41) investigated the extraction of high-quality oil from

olive pomace. Le Floch et al. (42) tested SFE for isolation of phenols from olive leaf samples.

SC-CO2 extraction of tamarind seed coats was investigated for the extraction of antioxidative components by Tsuda et al. (43). In another study,

pressure and solids load were studied as important factors affecting astaxanthin extraction from crab shell wastes (44). A mixture of SC-CO2 and ethanol was used and the maximum extraction yield was obtained at 340 bar and 45°C. In yet another study aimed to optimize the astaxanthin extraction yield from crawfish (Procambarus clarkii) tail shells by SC-CO2, with respect to temperature, pressure and moisture (45).

Marsal et al. (46) studied the use of SC-CO2-extraction to recover natural fat present in the fatty waste obtained from the residual bath of sheepskin degreasing.

Processing of Coenzyme Q10 was studied by Nunes et al. (47) using SC-CO2. Some other studies were conducted on the extraction by SC-CO2 of polar compounds such as phenols, with the addition of polar modifiers. Chepe et al. (48) demonstrated the possibility to obtain the concentration of the isoflavones biochanin A and formononetin continuously from a red clover EtOH/water extract by means of SC-CO2-fractionation using a column.

SC-CO2 extraction of essential oils from fruit and various other parts of citrus trees was investigated in an EU-sponsored project (AGRE-0058). Experimental parameters were identified for optimal extraction leading to design of suitable equipment (49). SC-CO2 extraction of β-cryptoxanthin from citrus press cake was investigated by Sangbin-Lim et al. (50).

The amount of "feed grade egg" that the industry generates from processing operations is estimated to be approximately 2% of the total egg production. With nearly 80% of the total phospholipids present in egg being phosphatidylcholine, inedible eggs constitute an inexpensive source of an ingredient with high nutritional and functional properties. A phospholipid-rich fraction was successfully extracted with a unique two-step process, consisting of a first step with SC-CO2 and a second step with ethanol as co-eluent (51). In an effort to optimize the extraction conditions of the first step, pressure, CO2 flow rate and temperature effects were investigated. Various times of extraction were also employed to obtain the highest concentration of phosphatidylcholine in the residual egg powder with the highest elution of unwanted neutral lipids. Optimal conditions to achieve the highest retention of phosphatidylcholine in the dried egg were found to be 415 bar pressure, CO2 flow rate of approximately 5 l/min and 45°C extraction temperature. A second step with SFE using CO2 containing ethanol as modifier resulted in elution of a fraction rich in phosphatidylcholine. The yield was estimated to be approximately 49 g phosphatidylcholine /kg of dried egg (51).

Aresta and Dibenedetto (52) studied the use of selected Mediterranean macro-algae as a source of biofuel. The extraction of oil was carried out using different technologies under mild energetic conditions. It was shown that SC-CO2 as well as solvent extraction could be used to efficiently extract the oil for fuel.

Raynie et al. (53) studied the development of value-added products from distillers' dried grains, a by-product of ethanol fermentation via the dry grain milling process. The aim was to produce syngas, a mixture of carbon monoxide and hydrogen, which could work as a feedstock in anaerobic fermentation. The interest was focused in polyhydroxyalkanoates (PHA) with potential applications in the manufacture of bio-based plastics, fibers, and films. SFE was used prior to gasification for the selective separation of lipophilic material. Following fermentation, SFE was again used for the recovery of PHA from the cellular biomass.

Conclusions

Wastes and by-products from agricultural and food processing contain many potential useful bioactive compounds. It is important to determine the presence and solubility of these compounds in SC-CO_2 before designing an industrial extraction plant. The objective is often to use the extracts for food/feed, pharmaceutical or cosmetic purposes. Sample preparation is particularly difficult because of the heterogeneity of the possible discarded materials in terms of chemical, physical and (micro)biological composition.

However, SC-CO_2 appears to be a promising extraction tool for the exploitation of wastes and by-products mainly because of the minimized impact on the sample (and on the environment) and its great selectivity. Partial confirmation of this ability is already available in literature, and this review has tried to report some examples.

The cost of extraction plants is the ultimate limit for the acceptance of this technique. Evaluation of wastes and by-products availability throughout the year is one of the requisites for minimization of fixed costs. The seasonal variations in availability is a common problem in the agricultural sector, and industrial costs are a consequence of plant use.

The most important feature of useful compound recovery from discarded materials is that the cost of raw material amount to zero, and sometimes using these materials represents a positive income that can balance the expensiveness of the extraction technique, considering the high cost of the equipment required for the handling of supercritical fluids. Also, the exploitation of selectivity (i.e. purification ability) of the SF technology, can give the extract products a higher value and this can outweigh the higher cost of extraction.

Potential novel products can be affordable in a market in which the consumer is willing to spend more money to justify its higher production cost.

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Chapter 3

Analysis of Supercritical CO_2 Extractables from Cranberry Seeds

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A supercritical fluid extraction (SFE) method for analysis of CO_2 extractables in cranberry seeds was investigated. The SFE operating conditions were optimized to maximize the extraction yields. Extraction yields obtained by SFE were comparable to conventional Soxhlet extraction. The extracts were derivatized and then analyzed by GC-MS. The extracts obtained via hexane and CO_2 mostly contained methylated fatty acids. Linoleic acid and palmitic acid were the major compounds extracted.

Introduction

The cranberry belongs to the genus *Vaccinium* along with other berries such as blueberry, bilberry, huckleberry, and gooseberry. The small cranberry (*oxycoccus*) is mostly found in Europe; whereas the large cranberry (*macrocarpon*) is grown in the northern parts of America such as New England and Oregon. *Vaccinium macrocarpon* was a part of North America long before the Europeans first established settlements. Native Americans have used cranberry as food, medicine, and dye. The commercial American cranberry

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production is approximately 580 million pounds. Thus, cranberry is an economically important fruit in the United States.

Cranberry seeds are the waste that is left behind during processing of cranberries for juices and related products. After the cranberry is pressed to extract its juice, the remaining pulp (e.g. waste) is air-dried, and the seeds are separated. Cranberry seeds are believed to contain high levels of unsaturated omega 3 fatty acids that are associated with lowering of total cholesterol and inhibiting the oxidation of low density lipo-proteins.[2,3] Cranberry seeds are thus a valuable source of nutraceuticals which are primarily associated with improving the quality of life and prevention/cure of disease.[4] According to the Dietary Supplement Health and Education Act, the United States Food and Drug Administration (FDA) has 75 days to approve a new dietary supplement.[5] This situation has contributed to a rapidly growing nutraceutical market. To protect the consumer and to ensure the quality of these dietary supplements, standardization and validation of the analytical methods for evaluating these products is vital.

The conventional methodology for extracting fatty acids from matrices such as grape seeds involves Soxhlet extraction with either hexane[6] or diethyl ether[7]. However, there have been only two published reports that describe the extraction of "high value" compounds from cranberry seeds. A method for performing extractions[2] of large quantities of cranberry seeds (1450-1850g) using hexane as the extracting solvent has been reported. Fatty acids were reported to be the major components of the hexane extract, while sterols, triterpenic compounds, and tocochromanols were the minor components. Croteau and Fogerson[3] extracted lipids from cranberry seeds with boiling isopropanol, followed by $CHCl_3$-isopropanol, and finally $CHCl_3$. The three extracts were combined and concentrated under vacuum and later were purified by elution through Sephadex with $CHCl_3$-methanol (1:1) which was saturated with water. The lipids were found to constitute 23.3% (w/w) of the total isopropanol/$CHCl_3$ extract. The lipids were than fractionated by column chromatography on a silica column. Neutral lipids (95.5%) were eluted with $CHCl_3$, glycolipids (3.4%) were eluted with acetone and phospholipids (1.1%) were eluted with methanol. The extracts were derivatized with BF_3/MeOH and bis-(trimethylsilylacetamide) then analyzed by GC-FID. The neutral lipid extract showed the presence of fatty acids, sterols, hydrocarbons, and triterpenic compounds.

Supercritical CO_2 has been considered as a potential alternative to conventional solvents due to its relative non-toxicity and non-flammability, as well as its low critical temperature and pressure. Supercritical fluid extraction (SFE) has been used for example in the extraction of fatty acids from diverse matrices such as grape seeds[6,7], ginseng seeds[8], wood pulp[9], and infant formula[10]. The absence of oxygen and light during the supercritical extraction process helps prevent degradation of the extract. For example, Tipsrisukond, et al.[11] found

higher anti-oxidant activity in supercritical fluid (SF) extracts than extracts obtained by conventional means. Furthermore, due to the higher diffusivity of supercritical fluid compared to liquid solvents, extraction times were shorter for SFE than for corresponding liquid/solid extraction.

The objective of this research was to determine the quantity and chemical composition of supercritical CO_2 extractables from cranberry seeds. SFE parameters such as CO_2 pressure, extraction temperature/time, and CO_2 flow rate were optimized to obtain the best possible extraction conditions. The amount and type of SF extractables have been compared with Soxhlet extractables to evaluate the feasibility of SFE as an alternative extraction method. The SF and Soxhlet extracts were compared by gravimetric determination followed by derivatization of the fatty acids into their methyl esters with subsequent determination of their compositions by GC-MS.

Experimental

Materials

Cranberry seeds were obtained from Ocean Spray Cranberries, Inc. (Lakeville-Middleboro, MA) and stored in polyethylene containers at 10°C. The seeds were freshly ground prior to each extraction. HPLC grade methanol, water, and toluene were obtained from Burdick & Jackson Laboratories, Inc. (Muskegon, MI), acetyl chloride was obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI), and sodium carbonate and Ottawa Sand Standard (20-30 mesh) were obtained from Fischer Scientific (Houston, TX). The internal standard, C_{11} triglyceride (triundecanoin), was obtained from Nu-Check Prep, Inc. (Elysian, MN). CO_2 (SFC/SFE grade) with helium headspace was supplied by Air Products and Chemicals, Inc. (Allentown, PA).

Soxhlet Extraction

Conventional extraction was carried out with 200mL of hexane on 10g of crushed cranberry seeds. The extraction was allowed to proceed for 24h in the dark at the boiling point of hexane. The extract, thus obtained was concentrated by rotary evaporation and the residue was left under continuous vacuum for 24h to remove solvent traces. The weight of this residue was considered to be 100% recovery. The experiments were performed in triplicate.

Supercritical Fluid Extraction

An Isco-Suprex (Lincoln, NE) AutoPrep (AP-44) system equipped with a variable flow restrictor was used for all supercritical CO_2 extractions. Ground cranberry seeds (1g) were mixed with Ottawa Sand standard and placed in a 5 mL stainless steel extraction vessel. Pure CO_2 was used for all extractions. The solid trap was packed with C_{18} and after each extraction the trap was rinsed with 5 mL of a mixture of dichloromethane and methanol (1:1). The fixed restrictor was heated to 55°C during the extraction, while the solid phase trap was held at –30°C and 25°C during the collection step and rinsing step respectively. All the samples were extracted in the static mode for 15 min. The extraction conditions, such as CO_2 pressure (250atm to 450atm), extraction temperature (45°C to 100°C), CO_2 flow rate (1mL/min to 2mL/min), and dynamic extraction time (45 min to 90 min) were varied to optimize the SFE. All extractions were performed in triplicate. The trapped SF extracts were rinsed into pre- weighed vials. The vials were then placed on a hot plate (50°C) and a stream of nitrogen was passed over them for 10 minutes. The yields of the dried extract, expressed as percent recovery, were defined as the percentage ratio of the weight of SF extractables to the weight of Soxhlet extractables.

Derivatization

Fatty acids can be present in cranberry seeds as free fatty acids, alkyl esters of fatty acids, or as triglycerides. In the presence of acetyl chloride/MeOH, free fatty acids are esterified to form methyl esters and the alkyl esters (expect methyl esters) are trans- esterified to methyl esters. The dried extracts (0.2 g) were transferred to 25 mL screw cap vials for derivatization[10]. The dried extracts were re-dissolved in 10 mL of toluene/MeOH (1:1) and spiked with 10 μL of 45 mg/mL triundecanoin internal C_{11} standard. Acetyl chloride was added to this solution, which reacted with methanol to provide a catalytic amount of HCl that is required to initiate acid hydrolysis (i.e. digestion). The vial was then purged with a stream of nitrogen, capped, and placed on a sand bath for 1 hour at 100°C. After the vial was cooled to room temperature, 10 mL of 6% sodium carbonate was added to quench the reaction and the resulting solution was mixed vigorously to assist phase separation. This solution was further centrifuged for 5 minutes and the organic layer that resulted was then used for GC-MS analysis.

GC-MS Analysis

A Hewlett Packard (Little Falls, DE) 5890 Series II GC was interfaced to an HP 5972 series Mass Selective Detector (MSD) for GC-MS analysis. A DB-5 MS column (0.25mm × 30m, d_f=0.25μm) from J&W Scientific (Folsom, CA) was used for separations. An HP 7673 GC automatic injector was used to introduce 1 μL of organic layer in the splitless mode. The injector and detector temperatures were maintained at 300°C. The oven temperature was held initially at 40°C for 2 min and then ramped to 145°C at 4°C/min and held for 1 min, then heated at 5 °C/min to 220°C and held for 30 min, and finally heated at 7°C/min to 300°C and held for 10 min. The extractables were identified using an HP Chem Station equipped with the Wiley library of mass spectral data. The composition of the extracts is expressed in terms of weight percent of each identified analyte in the total extract.

SPME Analysis

Solid phase microextraction (SPME) was used to determine the presence of volatiles in cranberry seed extract. The optimized SF extract was diluted with 5mL of a mixture of dichloromethane and methanol (1:1). A carbowax/divinylbenzene (CW/DVB) fiber was exposed for 60 min to the headspace of the diluted cranberry seed extract[12]. The assembly was kept at 30°C with stirring. The fiber was then desorbed for 5 min in the injection port of an HP 6890 Series GC interfaced with a HP 5973 Series MSD. The injector temperature was maintained at 220°C. The helium flow was kept at 2mL/min. The oven temperature was initially maintained at 40°C for 3 min and then ramped at 4°C/min to 100°C and finally heated to 220°C at 15°C/min.

Results and Discussion

Extraction

SFE parameters such as CO_2 pressure, flow rate, extraction temperature, and extraction time were varied in order to estimate the best extraction conditions. Figure 1 shows the relationship between CO_2 pressure and percent recovery. At a constant temperature (45°C), as the density increased from 0.87 g/mL (250 atm) to 0.97 g/mL (450 atm), the percent recovery increased dramatically from 11.5% to 87.2%. Since the density of CO_2 at 450 atm is greater than the density of CO_2

at 250 atm and the extraction time is the same, the amount of CO_2 used was greater when the extraction was done at 450 atm as compared to the extraction done at 250 atm. This dramatic increase is due to increased solvating power of CO_2 at higher densities and/or a greater amount of CO_2 used at the higher pressure relative to the lower pressure. Unfortunately, no experiment was performed at fixed pressure and variable flow rate to fully test this hypothesis.

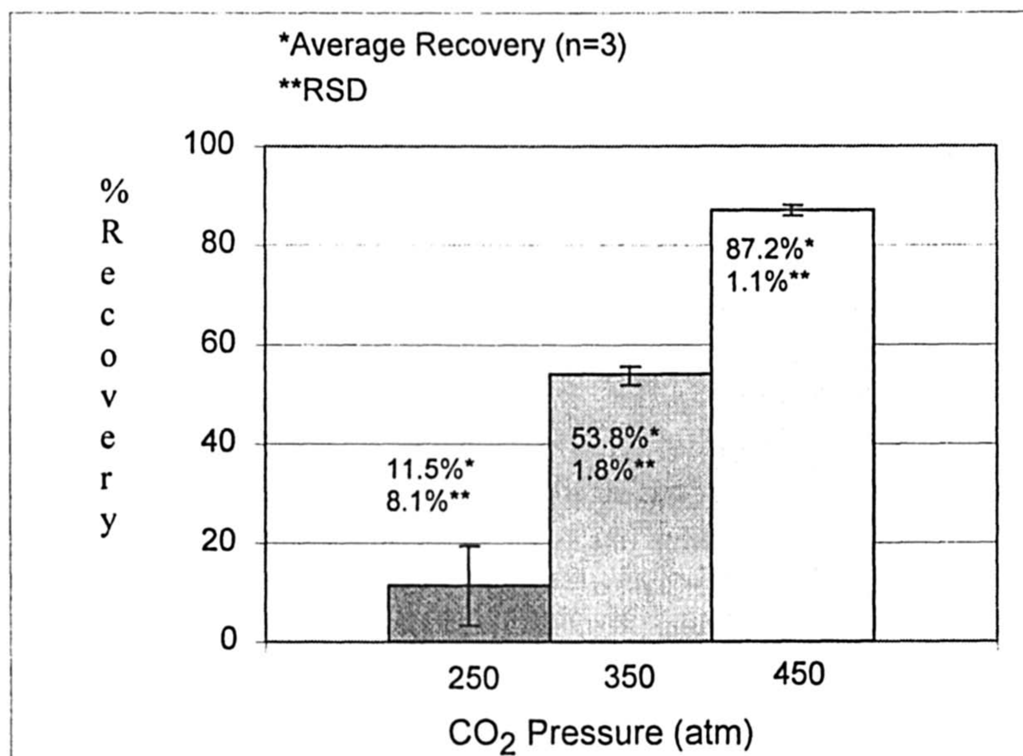

Figure 1. *Effect of CO_2 pressure on percent cranberry seed oil recovery. Extraction temp.: 45°C, Extraction time: 45 min, CO2 flow rate: 1 mL/min*

Generally, an increase in temperature at constant pressure causes an increase in the vapor pressure of the solute (which increases analyte extractability) but a decrease in supercritical solvent density (which decreases analyte extractability). So the extraction is governed by two opposing factors. Figure 2 shows the effect of extraction temperature on the percent recovery. Paired t-testing at 95% confidence limit shows that the recovery at 45°C (87.2%) was significantly higher than at 75°C (80.3%, p-value = 0.02) and at 100°C (75.6%, p-value = 0.006) and it indicates that CO_2 density is the more dominant factor. A p-value less than 0.05 for two values suggests that the two values are significantly different from each other at the 95% confidence limit.

Although no data are shown, when the CO_2 flow rate was increased from 1 mL/min to 2 mL/min recovery did not significantly change (p-value at 95% confidence was 0.057), in spite of the fact that twice as much CO_2 at fixed

density was naturally used at the higher flow rate. This failure to increase recovery with a greater amount of CO_2 could be due to the additional mechanical force at the solid phase trap generated by exiting decompressed gas at the higher flow rate (e.g. ~1000 mL/min versus 500 mL/min of decompressed CO_2) resulting in "blow-by" SFE trap loss. In other words, the yield of extractables may have increased, but the efficiency of trapping decreased in this experiment. Figure 3 shows that extraction in the dynamic mode for 45 minutes yielded recovery that was significantly less than dynamic extraction for 60 min (p-value at 95% confidence limit was 0.041); whereas the recoveries were not significantly different when the dynamic extraction time was increased from 60 minutes to 90 minutes (p-value at 95% confidence limit was 0.123). Based on these data, the optimized SFE extraction conditions for CO_2 extractables are as stated in Table I.

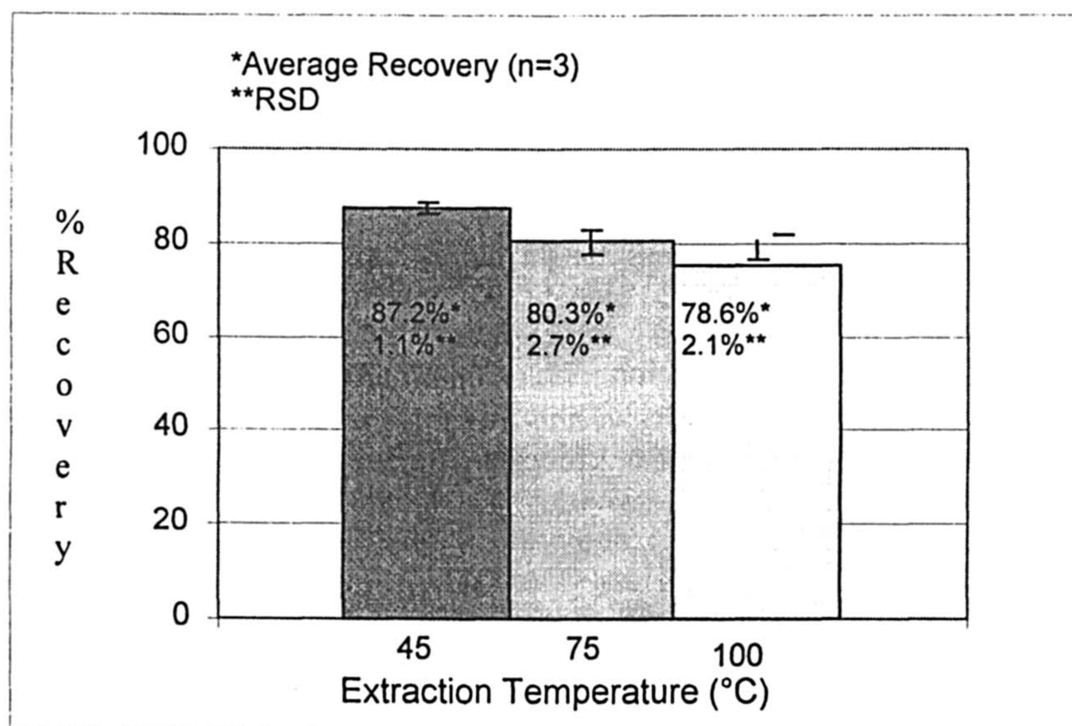

Figure 2. *Effect of extraction temperature on percent cranberry seed oil recovery. CO_2 Pressure = 440 atm, Extraction time: 45, CO2 flow rate: 1 mL/min*

Comparison of optimized SFE with a 24 hour Soxhlet extraction in terms of percent recovery, extraction time, and reproducibility shows significant differences. Both methods showed comparable recoveries (e.g. SFE = 94% relative to Soxhlet). An obvious advantage of SFE (1.5 hours) over Soxhlet (24 hours) lies in the time required for extraction. SFE (relative standard deviation, RSD = 2.5%) was also more reproducible than Soxhlet extraction (RSD= 3.4%) although the mass of the seed extracted via supercritical CO_2 was smaller than that extracted by Soxhlet (1g versus 10 g).

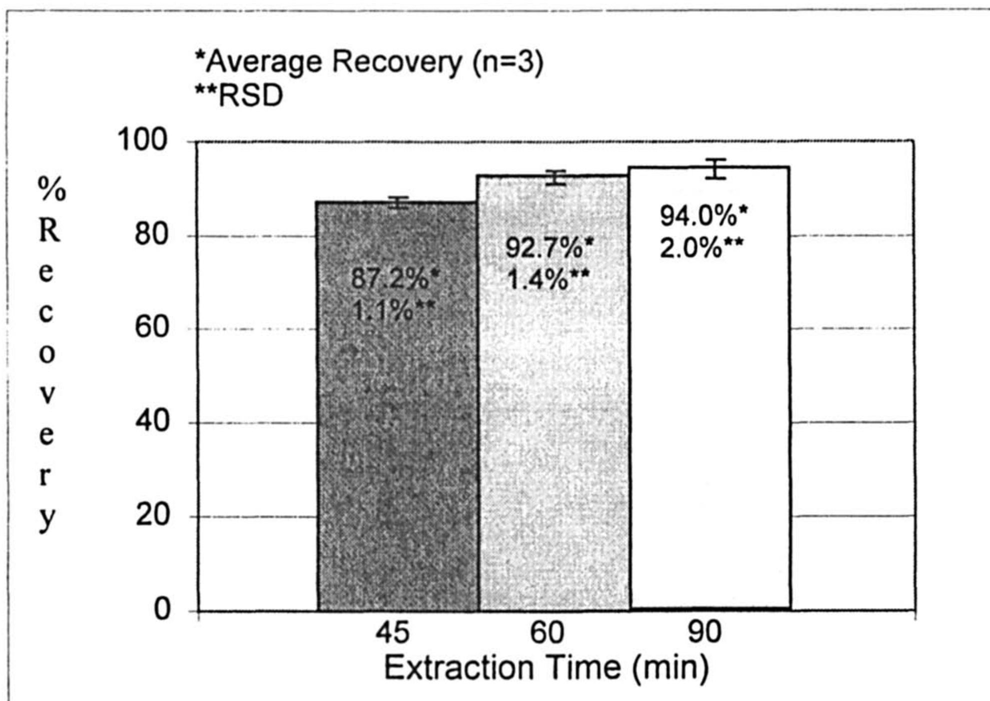

Figure 3. *Effect of eExtraction time on percent cranberry seed oil recovery. CO_2 pressure: 450 atm, extraction temp. = 45°C, CO2 flow rate: 1 mL/min*

Table I. Optimized Conditions for SFE of Cranberry Seed Oil

Extracting Solvent	*Pure CO_2*
Restrictor Temperature	55°C
Trap	C_{18}Silica
Trap Rinsing Solvent	5 mL (1:1 DCM + MeOH)
Static Time	15 min
Trap Temperature-Extraction	-30°C
Trap Temperature-Rinse	25°C
Pressure of CO_2	450 atm
Extraction Temperature	45°C
Dynamic Time	90 min
CO_2 Liquid Flow Rate	1 mL/min

Composition of the Extracts

The Soxhlet and the SFE extracts were derivatized to convert any free fatty acids in the extracts into their methyl esters because the methyl esters are more volatile as compared to the free fatty acids. When the derivatized extracts were subjected to GC-MS, 21 analytes were separated and identified (Table II). The GC-MS chromatograms (Figures 4-7) show that both methods extracted the same analytes and the composition of the extracts was similar. The extracts contained saturated fatty acids such as palmitic acid, docosanoic acid, tricosanoic acid, tetracosanoic acid, and hexacosanoic acid (peaks 4, 14, 15, 16, 17 respectively). Among the unsaturated fatty acids, the extracts contained methyl esters of omega-3 fatty acid such as α-linolenic acid (peak 9), omega-6 fatty acids such as linoleic acid and eicosadienoic acid (peak 7 and 10), and omega-9 fatty acids such as oleic acid, palmitoleic acid, and eicosenoic acid (peaks 8, 3, and 11). In addition, several hydrocarbons were tentatively identified (e.g. peaks 13 and 19). An alkyne suggested to be 4-hexadecen-6-yne (peak 13), a hydrocarbon assigned to squalene (peak 18) and a phytosterol such as β-sitosterol (peak 21) were also found. Peak 19 showed mass spectral data characteristic of tocopherols and could be either γ-tocopherol (match quality = 93) or β- tocopherol (89) or a mixture of these two tocopherols. The mass spectrum of peak #20 showed ions at masses 426, 411, 218, 203, and 189 amu which is characteristic of amyrin (pentacyclic triterpene alcohol). Surprisingly, no readily volatile material was found in either of the extracts probably because they would have been lost during post extraction treatment (i.e. solvent evaporation of soxhlet extracts) and extract collection (i.e. carried off with CO_2).

Table II. Analysis of Derivatized Cranberry Extractables using GC-MS[a]

Peak No.	*Analyte Identified*	*Quality*	*Soxhlet w/w, %*	*SFE[b] w/w, %*	*SFE[c] w/w, %*
1	Tridecanoic acid, 12-methyl-(13:0)	94	0.1	0.1	-
2	Pentadecanoic acid (15:0)	93	0.01	0.01	-
3	Palmitoleic aicd (16:1)	93	0.07	0.6	-
4	Palmitic acid (16:0)	97	9	6	5
5	Cyclopentanoic acid, 2-hexyl	78	0.05	0.04	-
6	Heptadecanoic acid (17:0)	95	0.02	0.02	-
7	Linoleic acid (18:2)	95	71	72	25
8	Oleic acid (18:1)	91	13	13	22
9	Linolenic acid (18:3)	90	6	6	22
10	Eicosadienoic acid	95	0.2	0.2	-
11	Eicosenoic acid (20:1)	93	0.3	0.1	-
12	Eicosanoic acid (20:0)	98	0.1	0.1	-
13	4-Hexadecen-6-yne	91	0.1	0.1	-
14	Docosanoic acid (22:0)	99	0.1	0.1	-
15	Tricosanoic acid (23:0)	90	0.01	0.01	-
16	Tetracosanoic acid (24:0)	98	0.04	0.03	-
17	Hexacosanoic acid (26:0)	90	0.03	0.02	-
18	Squalene	70	0.005	0.005	3
19	γ-tocopherol	93	0.02	0.01	-
21	β-sitosterol	90	1.1	1.0	3

[a]The mass spectral data of peak #20 shows ions at masses 426, 411, 218, 203, and 189 which is characteristic of amyrin, [b]This work, [c]Reference 3

In a companion study, M. Palma[12] et al. studied the extraction of grape seeds with pure SF CO_2 and analyzed the derivatized extracts by GC-MS. These extracts were found to contain volatiles such as aliphatic aldehydes in addition to fatty acids and sterols. Even though we used similar conditions for our SF extraction and GC-MS analysis, we were unable to detect any similar volatile compounds. To further investigate the presence of volatiles in the cranberry seed extract, we adapted a solid phase microextraction (SPME) method from the work of Jelen et al.[13] who had earlier developed it for the characterization of volatile compounds in different vegetable oils. SPME followed by gas chromatography was performed on the headspace of the cranberry seed extract to test for the presence of volatile compounds. The GC trace failed to show the elution of any components for either the SF or Soxhlet extract.

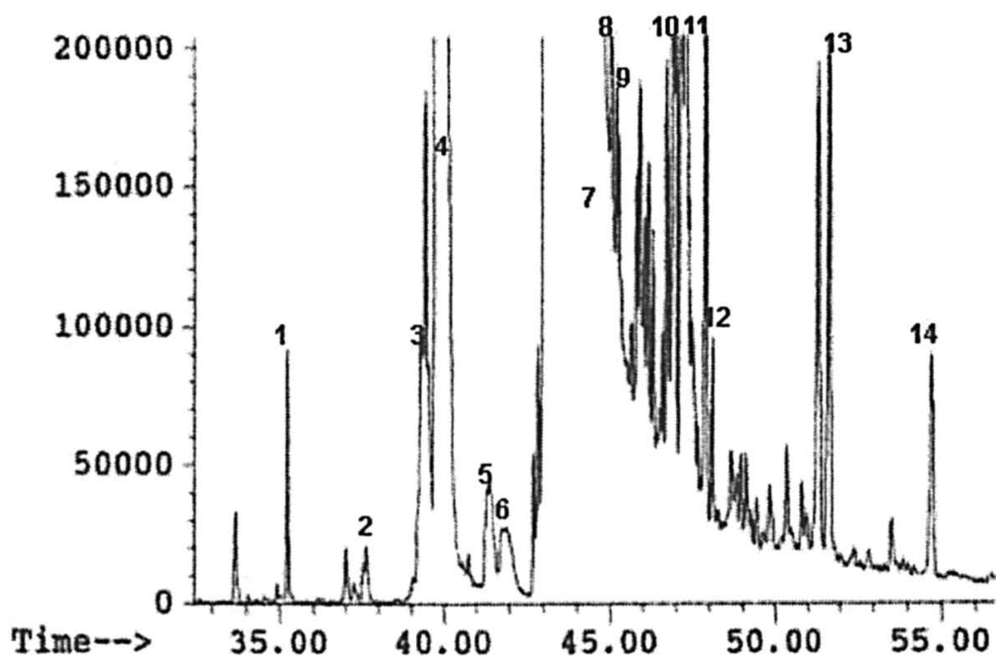

***Figure 4.** GC-MS total ion chromatograms of derivatized soxhlet extractables. (peak 1 through peak 14) Refer to Table 2 for peak assignments*

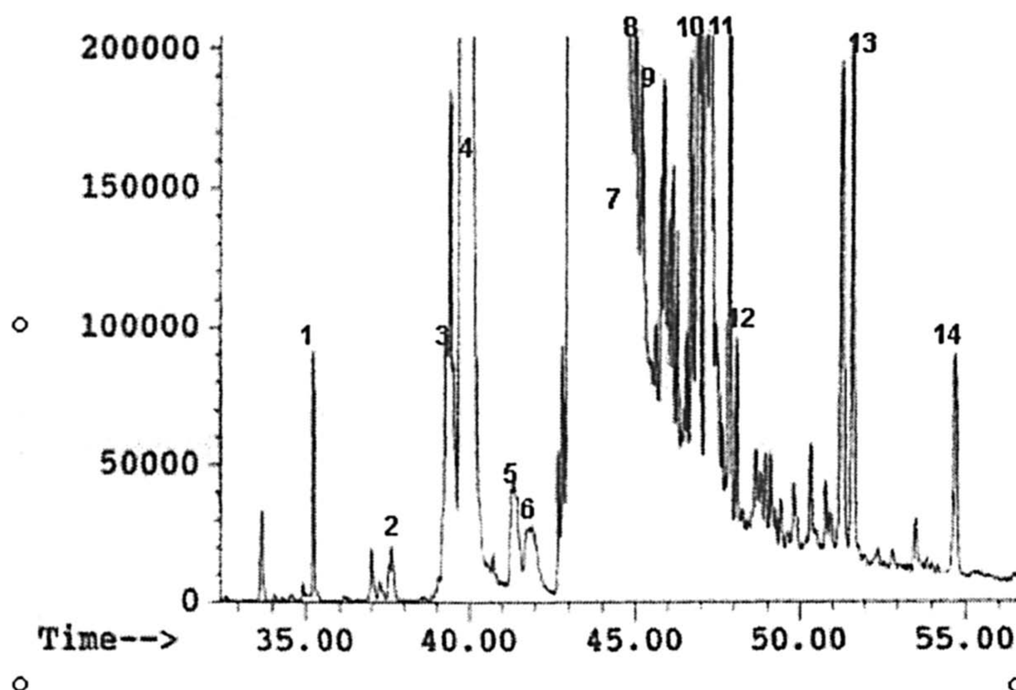

***Figure 5.** GC-MS total ion chromatogram of derivatized SF extractables. (peak 1 through peak 14) Refer to Table II for peak assignments*

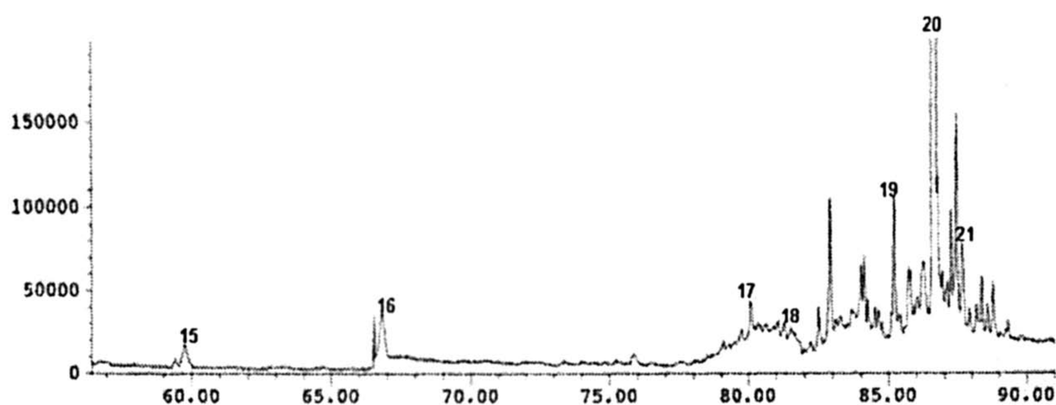

Figure 6. *GC-MS total ion chromatograms of derivatized soxhlet extractables. (peak 15 through peak 21) Refer to Table II for peak assignments*

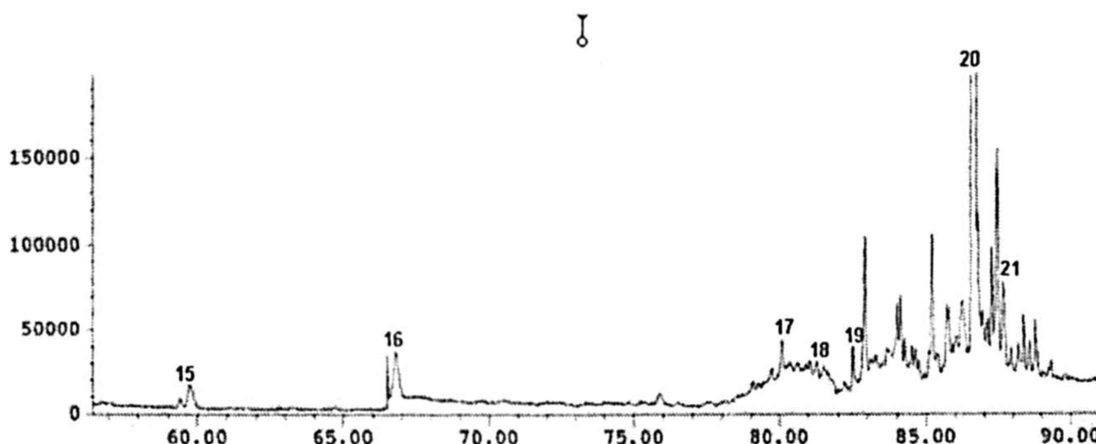

Figure 7. *GC-MS total ion chromatogram of derivatized SF extractables. (peak 15 through peak 21) Refer to Table II for peak assignments*

The major component of cranberry seed extracted via either pure CO_2 or hexane was linoleic acid (omega-6 fatty acid) as determined by total ion chromatogram and selected ion chromatogram with an internal standard. The broad intense linoleic acid chromatographic peak is believed to be a mixture of positional isomers of the C18:2 fatty acid such as 9,12-octadienoic acid 8,11-octadienoic acid, 5,7-octadienoic acid, and 9,11-octadienoic acid. Two other C18 fatty acids were identified: oleic acid and linolenic acid. The extracted ion of mass 297 $\pm$ 0.5 seen at retention time 44.30 min corresponded to oleic acid (molecular weight = 296.5, match quality = 91%). The extracted ion of mass 293 $\pm$0.5 seen at retention time 44.32 min corresponded to linolenic acid (molecular weight = 292.46, match quality = 99%).

As mentioned earlier, the composition of cranberry seed oil has been studied by Croteau[3] and Fogerson. The extracting solvent used in their method was a sequence of isopropanol, isopropanol-chloroform, and chloroform with the result

that their extracts contained more polar lipids such as glycolipids and phospholipids in addition to neutral lipids. Our SFE approach was more selective as the SF extract contained only neutral lipids, and furthermore no clean up of the extract was necessary as stated before. In the Croteau and Fogerson study, the lipids were separated from the non-lipids by gel chromatography and were further separated on a silica column into neutral lipids, glycolipids, and phospholipids. The extracts were then derivatized and the derivatized extracts were analyzed by GC-FID. The components of the extract were then identified by co-injection with authentic standards.

Table II compares the composition by weight of Croteau's and Fogerson's neutral[3] lipid extract and our SF extract. The SF extracts were richer in fatty acids as compared to the neutral lipid extract. The SF extracts contained trace amounts of docosanoic acid and eicosanoic acid which were not identified in the neutral lipid extract. Since the paper published by Croteau[3] and Fogerson has no chromatograms, it is not possible to know whether all the peaks in their GC-FID trace were identified. In addition, the analytes in the extract were identified by comparing retention times of a standard with the unknown. This method does not permit the unambiguous identification of an analyte because two different analytes can have similar retention time. In our study, the SF extracts were analyzed by GC-MS which is a superior technique compared to GC-FID because the peaks are identified by comparison of the mass spectrum of the peak component to the mass spectrum of a standard from the Wiley library.

In the Croteau[3] and Fogerson study, the triperpenic alcohols and sterols were derivatized into their trimethylsilyl ether derivatives using bis-trimethylsilylacetamide. The isopropanol-chloroform extract contained higher amounts of sterols and triterpenic alcohols such as β-sitosterol and amyrin compared to the SF extract. This could be due to the high polarity of the extracting liquid solvent as opposed to the supercritical fluid, and the increased volatility of the silylated sterols and triterpenic compounds relative to our fatty acid methyl esters. In addition to these compounds, the isopropanol-chloroform extract reported earlier also contained ursolic acid, a polar triterpenic acid, which was not extracted with pure CO_2.

Conclusions

The yield of cranberry seed oil via Soxhlet extraction was 23.1% and via SFE was 21.4%. These results are similar to the lipid composition (23.3%) of cranberry seed oil obtained by Croteau and Fogerson. The optimized SFE conditions for extracting fatty acids from cranberry seeds were: CO_2 pressure = 450 atm, extraction temperature = 45°C, CO_2 flow rate = 1mL/min, and extraction time = 90min. SFE showed better RSD's, comparable recoveries, and

shorter extraction times as compared to the Soxhlet method. GC-MS analysis showed that the extracts contained mainly (a) linoleic acid, which is an omega-6 acid, (b) oleic acid which is an omega-9 acid, (c) linolenic acid, which is an omega-9 acid, and (d) palmitic acid. The extracts also contained some other therapeutically useful omega-6 acids, omega-3 acids, omega-9 acids, phytosterols, tocopherols, and pentacyclic triterpene alcohols in minor quantities. The cranberry extracts via both CO_2 and hexane contained a higher concentration of beneficial unsaturated fatty acids as compared to saturated fatty acids. Thus, SFE seems to be a viable alternative to the Soxhlet method for extraction of fatty acids from cranberry seeds.

SFE was also more selective as compared to the liquid solid extraction (LSE) with isopropanol, chloroform, and isopropanol-chloroform. Glycolipids and phospholipids were found in the LS extract but they were not extracted by SFE. The SF extracts were also cleaner than the LS extracts and did not require the various clean up procedures required by the latter extraction method. The SF extracts were richer in fatty acids; whereas, the isopropanol-chloroform combined extracts were richer in the more polar and high molecular weight lipids.

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Chapter 4

Isolation of Alkylresorcinols: Classical and Supercritical CO_2 Extraction Methods

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This chapter focuses on the use of different methods for isolation of alkylresorcinols. Alkylresorcinols are members of a lipid group called non-isoprenoid phenolic lipids. Different aspects of the extraction by classical methods and supercritical CO_2 are discussed. Supercritical CO_2 extraction of alkylresorcinols from rye bran is discussed for the first time. As compared to the classical extraction methods, supercritical CO_2 gives higher yields and it allows the separation of the crude extrac into long- and short-chain alkylresorcinol homologues.

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Introduction

The extraction of active compounds from plant materials has attracted the attention of much research. Phenolic lipids in particular have earned special interest due to their wide range of biological activities. These give them applications in food improvement, conservation or as active medical components. In the present chapter an outline is given of the extraction and isolation methods for a particular class of phenolic lipids; the alkylresorcinols (AR). Different isolation methods are presented, including conventional organic solvent extraction but also novel ones involving supercritical CO_2 extraction. Difficulties, advantages and disadvantages as well as the challenges found during the use of these techniques, are presented.

Alkylresocinols

AR are amphiphilic phenolic lipid derivatives of 1,3-dihydroxybenzene with an odd-numbered alkyl chain at position 5 of the benzene ring *(1)* (figure 1). For example, rye AR are characterized by unbranched alkyl chains with an odd number of carbon atoms (17-25) attached to position 5 of the phenolic ring *(2)*. AR are composed of mixtures of resorcinol derivatives. Resorcinol derivatives with saturated aliphatic chains represent about 85% of the AR while other resorcinol analogues, including AR with mono- and di-unsaturated aliphatic chains and with keto groups, represent 15% *(3)*.

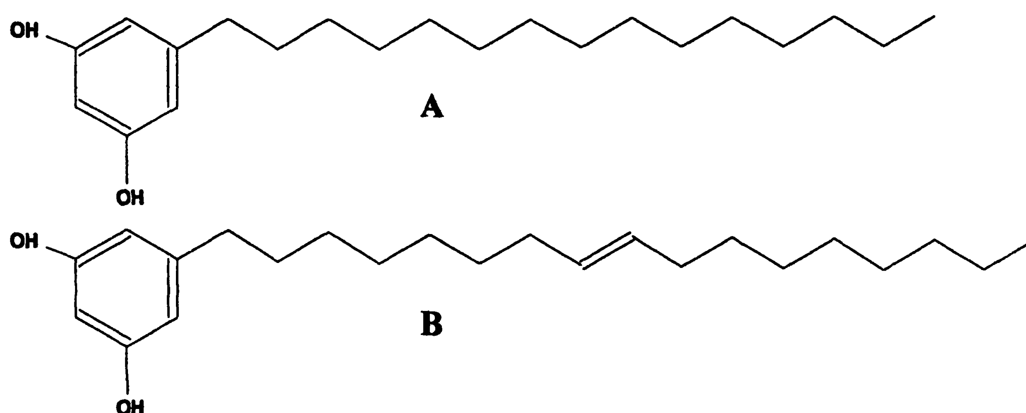

***Figure 1.** Examples of AR with saturated (A) and unsaturated (B) acyl chain(4).*

AR occur in plants from the families *Anacardiaceae*, *Ginkgoaceae* or *Proteaceae* and *Gramineae (5,6)*. They are present in high levels (>500µg/g) in the bran of cereals grains *(7,8)* such as wheat, rye and triticale. Rye is one of the

major sources of AR in the human diet. A wide range of biological activities have been reported for ARs. These include anticarcinogenic *(8)* antibiotic, antiparasitic, antitumourogenic and antioxidant effects *(1)*.

Classical Methods

Isolation

The presence of a long aliphatic chain (in most cases longer than 10 carbon atoms) makes resorcinolic lipids practically water insoluble. For the extraction of resorcinolic acids a wide range of organic solvents can be used (dichloromethane, methanol, chloroform, acetone, hexane etc.). In some cases the choice of an appropriate solvent allows extraction of smaller amounts of ballast lipids and/or impurities, as for example with rye alk(en)ylresorcinols *(9,10)*. Saponification is commonly used for separation of the phenolic fraction from the organic solvent extracts. The non-saponified material is then recovered with ether. Alternatively, the plant material can be extracted in a Soxhlet apparatus using several solvents in a sequence, some of them (e.g. hexane or diethylchloromethane) for removing triglycerides, phospholipids, sterols etc.; and others such as ethyl acetate, ethanol or acetone for removing the phenolic fraction. A procedure of this type for efficient extraction of AR was recently described by Zarnowski and Suzuki *(11)*.

Purification of the Extracts

For resorcinolic lipids, particularly those with long saturated side-chains, the use of polar solvents is important due to their amphiphilicity. The crude extracts in many cases are subjected to preliminary fractionation/purification either by solvent fractionation/partition or by application of chromatography. For pre-purification of the material and its separation from polymerized phenolics, gel filtration on hydrophobic Sephadex or TSK gel is sometimes used. Silica gel is most frequently employed for the separation and/or purification of resorcinolic lipids, notably in some studies with *Ononis* species *(12-14)*. The array of compounds reported appears partly attributable to methylation or acetylation reactions occurring during column chromatographic separation. An interesting approach for the pre-purification and selective separation of resorcinolic lipid from phenolic lipids or resorcinolic lipids from impurities has recently been reported. A selective partitioning of different non-isoprenoid phenolic lipids

between two phases of non-miscible organic solvents (e.g. diol and hexane) is mainly used *(14,15)*. Column and thin-layer chromatography on unmodified or hydrophobic (reversed phase) silica gels are used depending on preparative/analytical purposes. In general, chromatography on plain silica gel is used for the isolation and purification of the resorcinolic lipid fraction whereas partition chromatography on hydrophobic silica gel (often silica gel modified with octadecylsilane residues, RP-18) is applied for separation of individual homologues.

Separation of Alkylresorcinol Homologues

When there are only a few homologues in the isolated mixture of resorcinolic lipid, their separation into the individual fractions both according to the side-chain length and degree of unsaturation can be accomplished using a single separation on hydrophobic silica gel and elution with an appropriate mixture of the running solvent (acetonitrile or methanol) with water. To increase the efficiency and speed of the separation a gradient elution is usually employed *(16)*. However, when the mixture of resorcinolic lipids present is very complex, as in the case of gramineaceous or bacterial materials, its complexity does not allow separation of individual homologues in a single run on one type of chromatographic support. The problems encountered in such cases are similar to those found in separation of complex mixtures of fatty acids. The occurrence in resorcinolic lipid mixtures of homologues that differ, both in the length of the side-chain and in its unsaturation requires two step separation/isolation procedures. For separation of homologues according to the degree of side-chain unsaturation, argentation chromatography is used, i.e. silica gel impregnated with silver nitrate (20% load) *(5,17)*. Recent experiments have shown that for separation of cereal resorcinolic lipids high silver nitrate percentage is unnecessary, and effective separations of saturated, monoenoic and dienoic homologues can be achieved at 4-5% of silver nitrate. Fractions separated by argentation chromatography are subsequently subjected to reversed-phase column chromatography in which separation of individual homologues according to their chain length is achieved *(5,17)*. For preparative purposes HPLC is widely employed *(5,16,18,19)*. This technique is now frequently applied also for qualitative and quantitative analysis of the resorcinolic lipid composition *(20-22)*. Preparative HPLC however, has the disadvantages of using large amounts of solvent and requiring specialized equipment. In many experiments TLC is used for both preparative and analytical purposes.

Table I provides examples of classical methods used for the isolation, purification of the extracts and separation of the AR homologues.

Supercritical Fluid Extraction

Unlike classical solvent extraction where an appropriate solvent must be selected to accomplish a specific extraction task, with supercritical CO_2 this can be achieved by a suitable adjustment of the operational parameters: the temperature and pressure or by addition of small amounts of co-solvents*(48)*.

Supercritical CO_2 extraction of AR or other phenolic lipids have been only sparcely reported *(49,50)*. The solubility of a compound in a solvent depends on its physico-chemical properties. The ARs present in a crude extract, especially those with consecutive number of carbon atoms in the acyl chain (e.g. C_{15} and C_{17} homologues in rye alkylresorcinols) have very similar physico-chemical properties. Therefore, when supercritical CO_2 is used, the separation of individual homologues from crude extracts still remains a challenge. Illustrative studies on the extraction of phenolic lipids by supercritical CO_2 are given in the following sections.

Extraction of Cashew Nut Shell Liquid

AR occur in cashew nut shells as a fraction of other oil components like cardols, cardanols and anacardic acid. A comparative study on the extraction of cashew nut shell liquid (CNSL) was presented by Shobha and Ravindranath *(50)*. The study involved the extraction of the cashew nut shell by supercritical CO_2 or pentane. The pentane extraction was carried out on 50g steamed or fresh cashew nut shells in 100mL solvent. Supercritical CO_2 extraction was performed on 300g freshly broken cashew nut shells at 25 MPa and 40°C with the CO_2 flow kept at 4-5Kg/h for 17,5h with extract collection every 2.5h. The resorcinolic lipid fraction obtained by supercritical CO_2 represented 82% of the equivalent obtained by pentane extraction of fresh cashew nut shells and 70% of the extraction of steamed material. Despite this appreciable variation on the ratio of the total cardols and cardanols from one method to other, the relative proportion of the enomers in each group was very similar *(50)*. Generally, the extraction yield obtained by supercritical CO_2 was lower ($\approx$ 60%) than that obtained by the classical solvent extraction methods *(50)*, however, the product was nearly colorless. One of the major problems in the industrial application of CNSL is the very dark brown color of the solvent extracted product.

Table I. Examples of classical methods used in extraction of resorcinolic lipids (AR).

Extraction	Separation	Purification	Analysis	Reference
Hexane, Hexane-EtAc	Silica gel hexane/EtAc/AcOOH	RP18 silica, MeOH/10% H_2O	UV, IR, MS, H-NMR, C-NMR	*(23)*
Acetone	pentane extraction of dry acetone extracts	Silica gel $CHCl_3$/acetone	TLC, UV	*(24)*
Hexane	MeOH extraction of dry hexane extract, 1 M NaOH fractionation	Silica gel hexane/Et_2O/MeOH mixtures	IR, MS, H-NMR	*(25)*
$CHCl_3$, EtOH, Acetone	$CHCl_3$ extract extracted with 4% NaOH	Silica gel $CHCl_3$/MeOH	IR, UV, H-NMR, C-NMR, MS	*(26)*
MeOH, $CHCl_3$, EtOAc	Extraction with ET_2Oac, 5% Na_2CO_3, saponification and Et_2O extraction	Silica gel $CHCl_3$-MeOH, HPLC 95% MeOH	IR, NMR, MS	*(27)*
Petroleum ether (b.p. 60-80cet)	HPLC silica gel petrol/EtOAc gradient		TLC, H-NMR, MS, GC-MS, EPR	*(28)*
Petroleum,Et_2O,EtOAc, Acetone, MeOH	Sephadex LH-20, MeOH	Silica gel $CHCl_3$/EtOAc gradients	TLC, UV, MS, H-NMR	*(29)*
Hexane	Aluminium oxide, silica gel eluted with solvent mixtures of increasing polarity	RP18 silica gel, MeOH	GC	*(30)*
Ethanol	Partition between $CHCl_3$/H_2O	Silica gel petroleum/Et_2O/ AcAc HPLC RP18 MeOH/H_2O/AcAc	TLC, GC-MS	*(31)*
MeOH	Dry extract extracted with EtOAc, silica gel $CHCl_3$/MeOH	Silica gel CHCl3/MeOH; Sephadex LH 20, MeOH/EtOAc	UV, IR, NMR	*(32)*
MeOH, MeOH/$CHCl_3$, Et_2O	TLC – benzene, benzene/MeOH; Ag-Si TLC; RP-TLC Acetone/H2O		TLC, UV, IR, NMR, GC-MS	*(33)*
Et_2O	Vaccuum distillation	Preparative TLC petroleum ether/Et_2O	TLC, UV, IR, MS, H-NMR	*(34)*
Et_2O	Silica gel preparative column chromatoraphy	RP18 HPLC	H-NMR, GC-MS	*(35)*
Et_2O/Petroleum ether	Silica gel preparative column chromatography		IR, H-NMR, MS	*(36)*

$MeOH/CHCl_3/H_2O$	Silicic acid; $CHCl_3$/Acetone/MeOH	HPLC – Porasil $CHCl_3$/MeOH HPLC Bondapack C18 $MeOH/H_2O$	TLC, UV, GC, MS	*(37)*
MeOH	MeOH extract diluted with H_2O extracted with CHCl3; aluminium oxide column (benzene/AcOE)t	RP-18 HPLC; $MeOH/H_2O$	UV, IR, H-NMR, MS	*(38)*
Et_2O/petroleum ether	Silica gel, $CH_2Cl_2/C_6H_6/Et_2O$	Preparative TLC	IR, H-NMR, C-NMR, MS	*(39)*
CH_2Cl_2	Silica gel, hexane/Et_2O gradient		UV, IR, H-NMR, C-NMR, GC-MS	*(39)*
Hexane	Et_2O after saponification; aluminum oxide column (hexane/benzene/Et_2O gradient)	Repeated crystallization, vaccuum distillation	TLC, UV, IR, H-NMR, GC	*(40)*
Acetone	TLC	-	RP-TLC on paraffin impregnated silica gel	*(41)*
Acetone	Silica Sep-Pack column, stepwise elution	RP18 HPLC, $MeOH/H_2O$		*(42)*
Diethyl ether	Silica column	HPLC RP-18, AcN/H2O	TLC, IR, H-NMR	*(43)*
Petroleum ether/$CH2Cl_2$	Silica gel, petrol/dichloromethane/dichloromethanol/ EtOAc	Silica gel Lobar column; petroleum/dicholomethane; Ag-Si column	TLC,	*(44,45)*
Et_2O	Fractionation with $Pb(OH)_2$; fractional distillation	Chromatography on activated carbon/celite mixture	Chemical methods	*(46)*
Benzene, Acetone, EtOH	Repeated dissolution in Et_2O and precipitation with light petroleum		IR, UV, H-NMR, GC	*(29)*
Petroleum ether (30-60 deg C)	Counter current chromatography in hexane/nitromethane	Preparative TLC on Silica gel; argentation TLC	IR, GC-MS, GC	*(27,47)*
Et_2O	Silica gel column (light petroleum/Et_2O; MeOH; $CHCl_3$/Et2Oac)	RP18-HPLC, (AcN/H_2O)	TLC	*(27)*
Acetone	Silica gel $CHCl_3$/acetone	RP2-HPLC (MeOAC/MeOH/H_2O)	TLC, UV	*(5)*
Acetone	n-pentane fractionation. of dry acetone extract	Silica gel column (CHCl3/acetone)	TLC, GC,UV	*(21)*

Extraction of Alkylresorcinols fromRye Bran

The use of supercritical CO_2 to extract AR from cereal material was only recently presented *(49)*. Pure supercritical CO_2 was not able to extract AR even at pressures as high as 35 MPa and 55°C. This result was attributed to the amphiphilic character of the AR and the non polar character of the supercritical CO_2. With the addition of 10% of ethanol or methanol, it was possible to obtain extracts even at near the critical pressure (8 MPa). The optimal pressure was determined to be 35MPa at 55°C when ethanol or methanol acted as co-solvent. The co-solvent was added as 10%w/w of the CO_2. The CO_2 flow was kept constant at 5g/min during the experiments. A comparison of the supercritical CO_2 extraction with the addition of ethanol and classical extraction methods was made. For the classical method, pure acetone extraction at 0.1MPa and 20°C was used. Between 15 and 30 MPa at 55°C, 8 to 80%w/w higher yields of AR crude extracts for the extraction with supercritical CO_2 with co-solvents were obtained than for pure acetone extraction (refer to figure 2). However, the HPLC analysis of the extracts showed similar composition *(49)*.

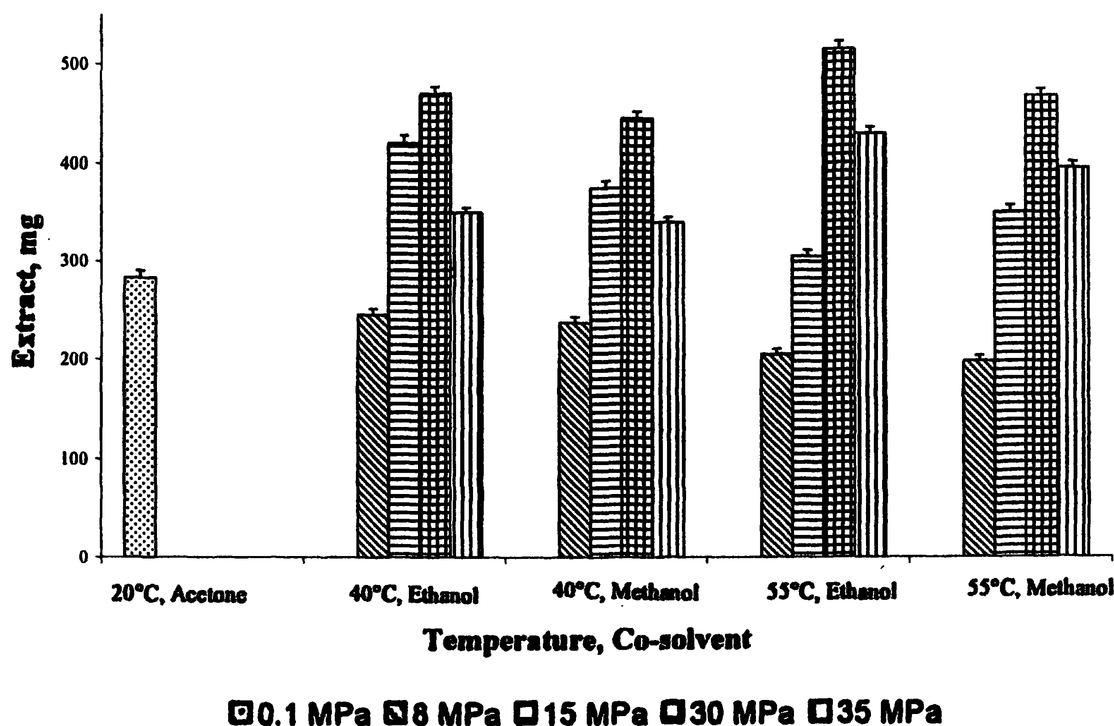

Figure 2. *AR extract amounts obtained with acetone extraction and supercritical CO_2 with the addition of co-solvents at different pressures and temperatures for two hours.*

No significant difference was achieved in the crude extract amounts by using either ethanol or methanol. Ethanol should however be preferred as it is an acceptable solvent in food preparation. Process optimization of the extraction of AR from rye and other sources, especially cereals needs still to be performed. The most common AR homologues in rye bran differ in only two carbon atoms, therefore their solubility in supercritical CO_2 is expected be very similar. The possibility of separating individual AR homolgues using supercritical CO_2 will present future additional challenges.

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Chapter 5

Accelerated Solvent Extraction: A New Procedure To Obtain Functional Ingredients from Natural Sources

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At present, there is a great interest in developing functional foods containing natural ingredients able to provide health benefits. Subcritical fluid extraction has become very popular because meets the requirements of an environmentally friendly procedure being also very selective and easy to tune. Accelerated solvent extraction (ASE) is a fast an automatic technique that fulfils the criteria of high yield and efficiency. ASE allows working with any kind of solvents but the highest interest is the use of ASE along with clean solvents, such as water or ethanol (generally recognized as safe, GRAS). The goal of the present chapter is to show the latest results obtained at our laboratory studying the selectivity of subcritical water and ethanol, using ASE at several conditions, to isolate nutraceuticals from natural sources such as rosemary leaves and the microalga *Spirulina Platensis.*

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Introduction

In the last few years, there has been a growing interest in the so-called functional foods because they can provide additional physiological benefits other than nutritional and energetic *(1)*. Usually, functional foods are obtained from traditional foods enriched with an ingredient able to provide or promote a beneficial action for human health. These ingredients are preferred by consumers to have a natural origin (i.e., non synthetic) being commonly extracted from natural sources such as plants, spices, food by-products or even algae and microalgae. Among the different compounds with functional properties, antioxidants have been the most widely studied *(2,3)*. Antioxidants are compounds that when present in foods at low concentrations, compared to that of an oxidizable substrate, markedly delay or prevent oxidation of the substrate *(4,5)*. Moreover, and even more important, the beneficial effects of antioxidants on human health have also been described *(6)*.

Among the spices with antioxidant properties, rosemary and sage have widely increased their use in many food applications. In rosemary, the antioxidant activity has been attributed to six different phenolic diterpenes: carnosol, carnosic acid, rosmadial, rosmanol, epirosmanol and methyl carnosate *(7-9)*. Some flavonoids naturally occurring in rosemary, as genkwanin and cirsimaritin, have also been described as antioxidants [10]. Among these compounds, carnosic acid is believed to possess the highest antioxidant activity *(10-12)*.

At present, different types of marine sources such as microalgae are receiving much attention mainly for their content in interesting ingredients such as polyunsaturated fatty acids *(13-14)* and β-carotene and other pigments with antioxidant properties *(3, 15)*.

The traditional extraction methods used to obtain these types of ingredients have several drawbacks. They are time consuming, laborious, exhibit low selectivity and/or extraction yields; moreover, they usually employ large amounts of organic solvents. Sub- and supercritical fluid extraction have become very popular because they meet the requirements of environmentally friendly procedures being also very selective, easy to tune and providing shorter extraction times. Among them, Supercritical Fluid Extraction (SFE), and Accelerated Solvent Extraction (ASE) are two of the most promising processes *(16)*.

ASE is a fast and automatic technique that fulfils the criteria of high yield and efficiency. ASE allows working with any kind of solvents but the highest interest is the use of ASE along with clean solvents, such as water (subcritical water extraction, SWE) or ethanol (generally recognized as safe, GRAS). The goal of the present chapter is to show the latest results obtained studying the selectivity of subcritical water and ethanol, using ASE at several conditions, to

isolate functional ingredients from natural sources such as rosemary leaves and the microalga *Spirulina Platensis.*

Materials and Methods

Samples

The rosemary samples consisted of dried rosemary (*Rosmarinus officinalis L.*) leaves obtained from an herbalist's shop (Murcia, Spain), dried using a traditional method as described previously *(17)*. Samples were ground under cryogenic carbon dioxide and stored (for two months maximum) in amber flasks at -20°C until use.

Microalgae samples (*Spirulina platensis*) consisted of air-dried microalgae with 6% moisture weight, from Algamar S.A. (Pontevedra, Spain), stored under dry and dark conditions.

Accelerated Solvent Extraction

An Accelerated Solvent Extraction system (ASE 200 from Dionex Corp. Sunnyvale, CA, USA) equipped with a solvent controller unit was used to perform all the extractions.

The rosemary subcritical water extractions were carried out at 60 and 100 °C for 25 min. The procedure was as follows: 2.0 g of sample were placed in 11 mL extraction cells; prior to each extraction a heat-up time of 5 min was completed. The extracts obtained were immediately protected from light and stored under refrigeration until dried. For this purpose, a Freeze Dryer (Unitop 400 SL, Virtis, Gardiner, NY, USA) was used. After freeze-drying, 10 mg of each extract were freshly dissolved in 1 ml of water and immediately injected into CE-MS without further purification. Water was deoxygenated by purging with He for 15 min prior its use as extraction solvent.

Algae extractions were carried out at different extraction temperatures and times and considering different solvent compositions: water/ethanol (0:100, 10:90, 40:60, 50:50, 80:20 and 100:0), see Table 1. In all cases 11 mL extraction cells were used filled with 2.5 g of microalgae. Previous to each run an extraction cell heat-up was carried out for a given time, which changed according to extraction temperature. When water was used as the extracting solvent, care had to be taken with regard to clogging of the extractor lines by the

extracted material. To avoid clogging, the microalgae were placed inside a filter paper and the extraction procedure was performed as mentioned.

Likewise, different sample pre-treatments were tested; microwaves (1 min, 900W) and ultrasounds (1 min) were applied to the sample before placing it inside the extraction cell. When ultrasounds were used, the extraction was carried out with 1,5 g of sample while the extractions with microwaves pre-treatment were performed with 2,5 g of sample.

Extractions were performed by triplicate.

Antioxidant Activity Determination (In-Vitro Assay)

Antioxidant activity was measured in all extracts obtained using a method based on a procedure described by Brand-Williams et al. *(18)*. The method consists of the neutralization of free radicals of DPPH (2, 2- Diphenil-1-pycril hydrazyl hydrate) by the extract antioxidants. The results obtained show the amount of antioxidant needed to achieve a reduction of 50 % on the initial DPPH concentration. This value is known as Efficient concentration (EC_{50}) and is utilized to describe the antioxidant activity. Therefore, the lowest the EC_{50} value, the highest the antioxidant activity. The procedure was as follows: 23.5 mg of DPPH was weighted and dissolved in 100 ml methanol. This solution was stored at 4°C. To do the measurements, the stock solution was diluted 1:10 with methanol. Different concentrations of the extracts solutions were used. 0.1 ml of these solutions were added to 3.9 ml diluted DPPH solution to complete the final reaction medium (4 ml). Due to the coloured extracts it was necessary to prepare a control (i.e. blank), which consisted of 0.1 ml of each solution added to 3.9 ml of methanol. The reaction was completed after 4 hours at room temperature, and the absorbance was measured at 516 nm in a UV/VIS Lambda 2 spectrophotometer from Perkin Elmer Inc. (Wellesley, MA, USA). Methanol was used to adjust the zero. The absorbance value was obtained by subtracting the blank absorbance measurement to the value given by the extracts solution. The method was calibrated using DPPH solutions of different concentration which allowed to know the DPPH concentration remaining when reaction was finished. This calibration curve (n=7; r=0,9999) gave the following equation: [DPPH] = (Abs + 0.0029) / 0.0247.

Assays were done by triplicate.

Capillary Electrophoresis-Mass Spectrometry

Analysis of rosemary extracts were carried out in a CE apparatus (P/ACE 5500, Beckman Instruments, Fullerton, CA, USA) equipped with a UV-Vis

detector working at 200 nm and coupled with an orthogonal electrospray interface (ESI, model G1607A, Agilent Technologies, Palo Alto, CA, USA) to the MS detector (an ion-trap mass spectrometer Esquire 2000, Bruker Daltonik GmbH, Bremen, Germany). A commercial coaxial sheath-flow interface was used (*vide infra*). Electrical contact at the electrospray needle tip was established via a sheath liquid composed of 2-propanol / water (60:40, v/v) containing 0.1% (v/v) trietylamine and was delivered at a flow rate of 0.24 mL/h by a syringe pump (74900-00-05, Cole Palmer, Vernon Hills, Illinois, USA). The mass spectrometer was operated in the negative ion mode. The spectrometer was scanned at 200-500 m/z range at 13000 m/z per s during separation and detection (target mass 350 m/z). The bare fused-silica capillary with 50 µm I.D. was purchased from Composite Metal Services (Worcester, U.K.). The detection length to the UV detector was 20 cm, the total length (to MS detection) was 87 cm. Injections were made at the anodic end using N_2 pressure of 0.5 psi (3448 Pa) for 10 s. All separations were at 20 kV as running voltage.

To analyze the algae extracts a CE apparatus P/ACE 5500 from Beckman (Palo Alto, CA, USA) equipped with diode array detector was employed. The fused silica capillary used (total length, 37 cm; detection length 30 cm; internal diameter 50 µm) was purchased from Composite Metal Services Ltd. (Ilkley, UK). The composition of the running buffer employed was: 100 mM sodium tetraborate at pH 9,0 containing 25 % ethanol and 25 % acetonitrile. The voltage was set at 15 kV. The extract was prepared at a concentration of 10 mg/mL.

Results and Discussion

Extraction of Rosemary Antioxidants by Subcritical Water Extraction

Several methods can be found in the literature to extract antioxidants from aromatic plants; among them, SFE and SWE use environmentally friendly solvents *(19-24)*. Only two works have been published dealing with the extraction of antioxidant compounds from plants (*24, 25*). In our research group we studied the extraction of antioxidants from rosemary leaves using SWE *(24)*; SWE is based on the extraction with hot water under a pressure sufficient to maintain water in the liquid state. In our previous work *(24),* the possibility of tuning the selectivity for antioxidant extraction by changing water temperature from 100 to 200°C was demonstrated.

Moreover, different methods have been used to characterize the isolated fractions obtained under SWE conditions of rosemary leaves such as high performance liquid chromatography (HPLC) *(24)* and capillary electrophoresis

(CE) *(26)*. In a previous work *(24)*, we have demonstrated the suitability of HPLC to identify the antioxidant fraction of rosemary obtained by SWE. Nevertheless, under typical reversed phase conditions, it was not possible to determine the presence of the most polar compounds (as e.g. rosmarinic acid) because they elute with the dead volume becoming unretained. In addition to the known antioxidant activity of carnosic acid and carnosol, the biological activity of other polar compounds has been already pointed out *(27-28)*. The presence of these polar compounds can reduce considerably the information about the antioxidant composition of the extracts derived from HPLC, especially when relatively polar conditions are applied (as in SWE). In order to overcome this limitation, capillary electrophoresis (CE) has been suggested as an interesting alternative *(28)*. Its utility can be greatly enhanced by electrospray ionization mass spectrometry (CE-ESI-MS). This soft-ionization technique allows the production of ions even of labile compounds, as natural antioxidants are. MS provides the high sensitivity and detectability often required for CE, in addition to its compound identification ability. Therefore, CE-ESI-MS is at present one of the most powerful analytical methodologies.

A combination of SWE and CE-ESI-MS was used to analyze extracts obtained at two different extraction conditions. Subcritical water was used at 60 and 100 °C, keeping constant the rest of the parameters. Figure 1 shows the CE-MS base peak electropherograms of the two extracts (A: 100°C and B: 60°C). It can be seen that the extraction of carnosic acid (peak 3, m/z = 331.4) is favored at higher temperatures while the peak was not detected in the extraction performed at 60 °C. These results corroborate previous studies performed in our laboratory *(24)*. The more polar compounds (such as rosmarinic acid) are preferentially extracted at lower temperatures while the less polar compounds (e.g., carnosic acid) were extracted at higher temperatures. This behaviour can be explained considering the changes of the dielectric constant for water with temperature, where high temperatures give low dielectric constant values thus favoring the extraction of less polar compounds. The result given in Figure 1 demonstrates the possibility of tuning the selectivity for antioxidant extraction by using different water temperatures, being able to yield extracts of different composition, and thus of different activity.

In conclusion, in the present work we show that SWE coupled to CE-ESI-MS is a suitable technique to characterize antioxidants from natural sources in a fast and efficient way, providing complementary information to chromatographic procedures such as HPLC. Moreover, using this methodology it was possible to determine the presence of rosmarinic acid, homoplantaginin and gallocatechin in the extracts obtained, compounds which were not detected using chromatographic techniques such as reversed-phase HPLC.

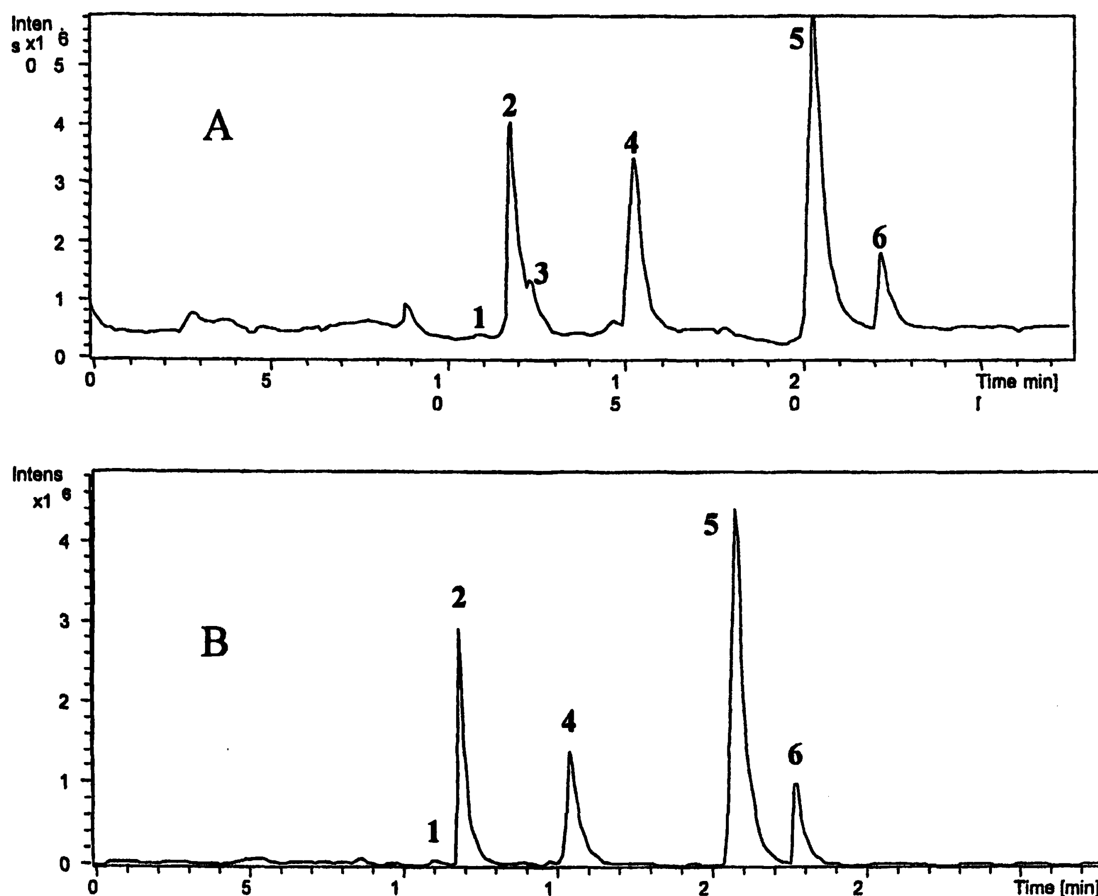

Figure 1. *CE-MS base peak electropherogram of rosemary extracts obtained with water at 100 °C (A) and 60 °C (B). CE-MS conditions: 50 µm i.d. fused silica capillary, 87 cm total length. BGE: 40 mM ammonium acetate, adjusted at pH 9.0 with ammonium hydroxide. Voltage: 20 kV. Injections: 10 s at 0.5 psi (3448 Pa). Sheath liquid: 2-propanol/water 60:40 (v/v) containing 0.1 % (v/v) triethylamine, flow rate 0.24 mL/h. Drying gas (N_2): 7 L/min, 350 °C. Nebulizing gas (N_2) : pressure 2 psi. MS analyses were carried out using negative polarity. Compound stability: 50 %. MS scan 200-500 m/z (target mass= 350 m/z). Peak assignment: 3, Carnosic acid; 4, Rosmarinic acid. Tentatively Peak assignment: 1, isoquercitrin; 2, not assigned; 5, homoplantaginin; 6, gallocatechin.*

Accelerated Solvent Extraction of Functional Compounds from Microalgae *Spirulina platensis*

In the search of feasible new sources of natural antioxidants that can be used in the food industry, algae and microalgae have been suggested as possible raw materials. Both organisms are widely known and consumed in certain countries, and numerous health benefits have been associated to their use. Different compounds with antibacterial, antiviral and antifungical activity can be found in this type of organisms (30-32), along with compounds with antioxidant activity that, as has been already mentioned, is nowadays one of the most important fields of activity in food research.

In a previous work, we studied the possibility of extracting antioxidants from microalgae *Spirulina platensis* using ASE with different solvents *(33-34)*. Likewise, other authors have studied the carotenoids extraction from microalgae *Haematococcus pluvialis* and *Dunaliella salina* using ethanol as solvent and ASE *(35)*.

In the present work an attempt has been made to optimize the parameters related to the extraction of antioxidants (with ASE) from *Spirulina platensis* using only environmentally clean solvents such as water, ethanol and mixtures. Thus, as a first approach, a study about different sample pre-treatments and how these affect both, the extraction yield and the final antioxidant activity has been performed. Moreover, a relationship between the efficient concentration (EC_{50}) and the final composition of the solvent (as a function of the dielectric constant) has been studied and a preliminary characterization of the chemical composition of one of the best antioxidant extract has been done using an optimised CE-DAD method.

In order to improve the extraction yields and antioxidant capacity of the extracts achieved under ethanol and water conditions during ASE, different sample pre-treatments previous to sample extraction were studied such as microwaves and ultrasounds. Both methods could help to improve yields due to the loss of cell membrane integrity and the exposal of the content of the cell to the extraction solvent, nevertheless, no studies have been conducted up to now to know the effect of these pre-treatments on the type of compounds extracted (thus related to the biological activity of the extracts). Figure 2 shows the effect of the different pre-treatments at different extraction conditions (using water and ethanol at different temperatures) on both, the extraction yield (% dry weight) and the efficient concentration (EC50, μg/ml). For the extraction yield, it can be seen that, for most of the extraction conditions tested, it follows the same trend increasing its value when increasing the intensity of the pre-treatment (no treatment< microwave < ultrasounds). In general, yields also increase with the extraction temperature of the water and the highest yields were obtained using ethanol. As for the EC50, the highest difference could be observed between

solvents, that is, lowest EC50 values (highest antioxidant activity) was obtained when using ethanol as extracting solvent compared to water at the different temperatures tested (25, 60, 100 and 170°C) being for ethanol less than half of the value achieved with water at any temperature. When using ethanol, the lowest EC50 was achieved without sample pre-treatment.

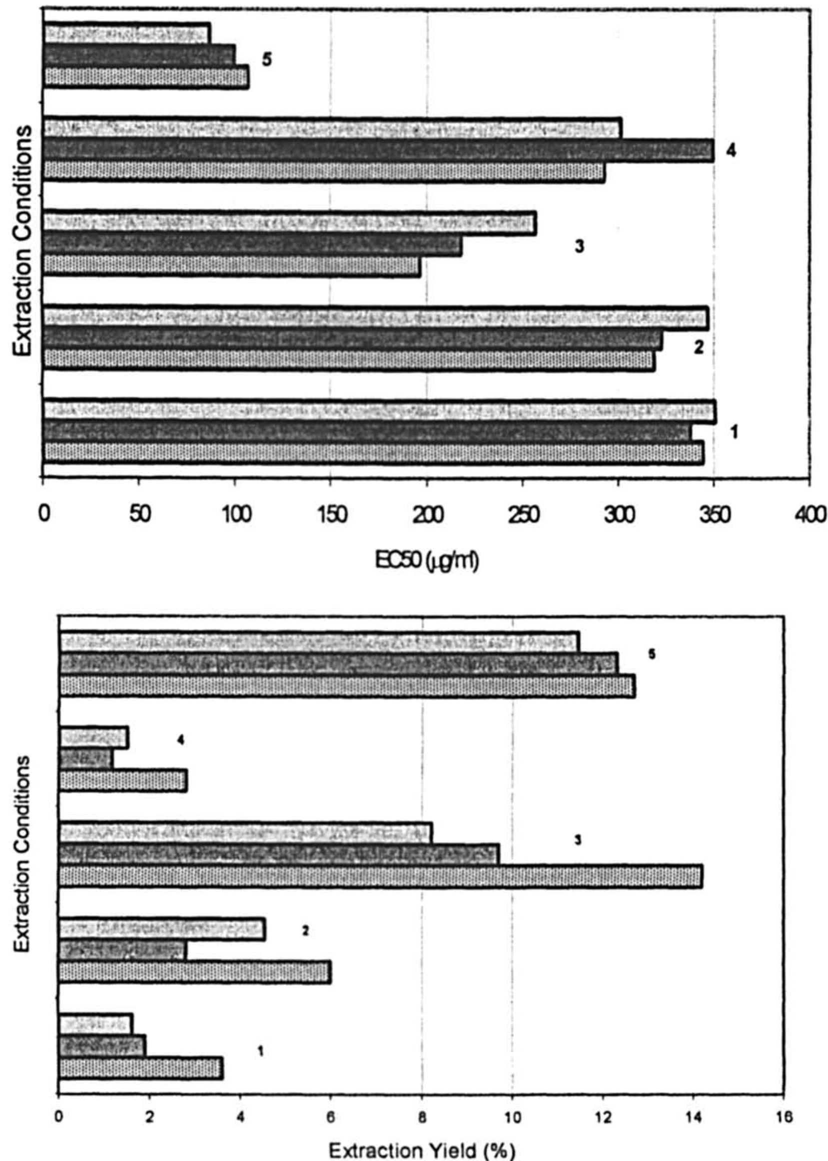

Figure 2. *Bars diagram showing top: antioxidant activity (EC50) vs sample pre-treatments, and bottom: extraction yield (%) vs sample pre-treatment. Extraction conditions: 1: water, 60 °C, 9 minutes; 2:water, 115 °C, 9 min.; 3: water, 170 °C, 9 min.; 4:water, 25 °C, 45 min.; 5: ethanol, 115 °C, 9 min. Sample pre-treatments: No pre-Treatment ▨ Microwaves ■ Ultrasounds*

For SWE experiments, small differences were observed among the different pre-treatments used. Considering the results as a whole, no pre-treatment of the sample was selected and a deeper study of the effect of the extracting agent on the antioxidant activity was performed based on the use of ethanol and mixtures ethanol:water to determine the effect of the reduction of dielectric constant in the nature of the compounds extracted.

Table 1 shows the extraction conditions selected to optimize the extraction of antioxidants from Spirulina platensis. As mentioned, different combinations between solvent composition and temperature were tested in order to cover a wide range of dielectric constants. Since dielectric constant of water drops when increasing temperature, approaching to that of an organic solvent (such as methanol), we were interested in knowing if it could be possible to achieve approximately the same antioxidant composition (and activity) using mixtures of similar dielectric constants or if the composition would mainly depend on the selectivity of the solvent used (and therefore not directly dependant on the dielectric constant). Table 1 also shows the EC_{50} values measured at all the conditions tested.

Table 1. Extractions conditions used for the extraction at different dielectric Constants

x Ehanol	Temp. (°C)	Time (min)	ε	EC50 (μg/ml)
0	4	45	86.5	309.7
0	25	45	80	301.4
0.2	25	45	69	265.1
0	60	9	60	350.7
0	115	9	50	335.5
0	170	9	40	257.2
0.5	115	3	37.5	184.1
0.6	115	9	35	185.7
0.6	170	3	31	188.8
0.9	115	9	27.5	110.8
0.9	170	3	26.5	119.2
1	25	45	25	130.4

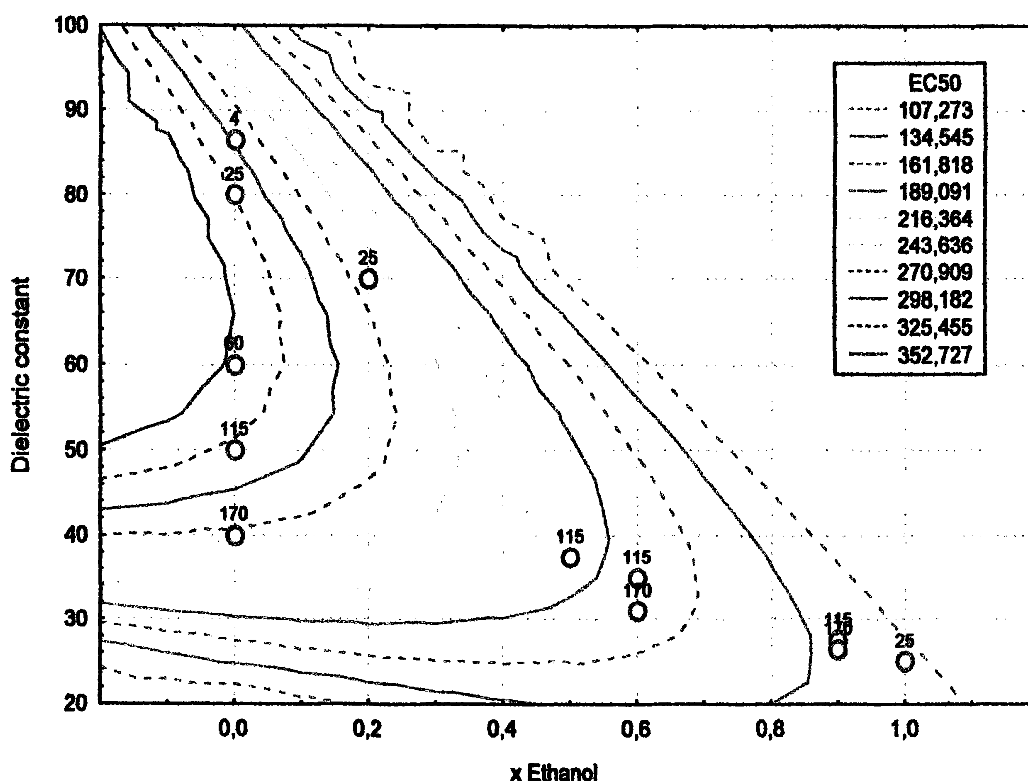

Figure 3. *Surface plot of EC_{50} vs. molar fraction of ethanol and dielectric constant. (See page 1 of color inserts.)*

Figure 3 shows the surface plot obtained by representing the EC_{50} values as a function of the dielectric constant and the molar fraction of ethanol in the mixture. By using both, data in Table 1 and Figure 3, different observations can be made:

1) The highest antioxidant activity was found in those extracts that contain ethanol in their solvent composition. Moreover, the best antioxidant activity were achieved working with 90% and 100% ethanol and temperatures of 115°C (and 170°C) and 25°C, respectively.
2) The presence of ethanol in the mixture always improves the antioxidant activity.
3) Considering experiments using only water at different temperatures, the best results were obtained either at 25°C or 170°C being the chemical composition of the two extracts different.
4) Even considering different temperatures, there is a clear relationship between the extracted compounds (and therefore, EC_{50}) and the ethanol fraction used as extracting agent. For example, better EC_{50} values were obtained when working with a mixture water:ethanol (80:20) (25°C) that implies a dielectric constant equal to 69 than when working with 100% water at 60°C (dielectric constant equal 60).

5) There is a linear relationship between EC_{50} and the molar fraction of ethanol (x) with the following equation: $EC_{50} = -190.57\ x + 297.65$; $R^2 = 0.962$.

In order to chemically characterize the extracts with the highest antioxidant activity, a procedure based on the use of CE coupled to diode array detection was used. Figure 4 shows the electropherogram corresponding to the ethanolic extract of *Spirulina platensis* that provided one of the lowest EC_{50}. In a previous paper *(32)* we analyzed the profiles obtained for ASE of Spirulina extracted with different solvents demonstrating that, when using organic solvents, a major extraction of carotenoids takes place improving the antioxidant capacity of the extracts. In the extract selected in the present work, some chlorophylls and phenolic compounds could also be detected based on their UV-vis absorption data.

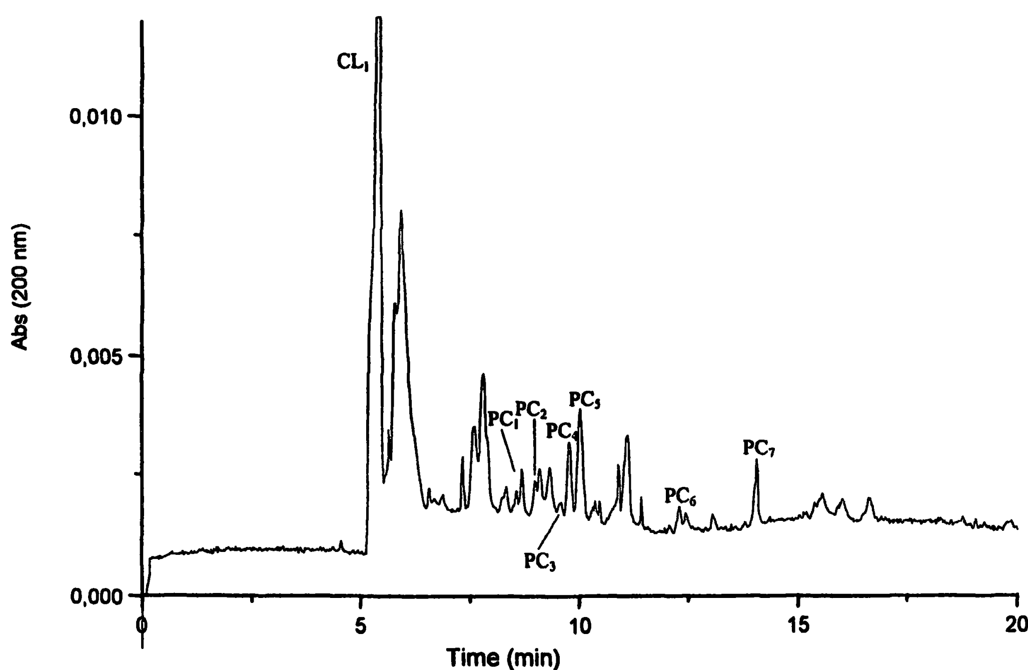

Figure 4. *Electropherogram of the extract obtained with ethanol. Extraction conditions: 25 °C, 45 min. CE method conditions: Buffer solution: 100 mM Sodium Tetraborate, pH 9.0, 25 % acetonitrile and 25 % ethanol; Fused silica capillary 50 µm i.d., total length 37 cm, detection legth 30 cm; Voltage: 15 KV; Temperature: 25 °C; Detection: UV-vis DAD detector.* CL_1: *Chlorophyll;* PC_{1-7}: *Phenolic compounds.*

In conclusion, in this work we demonstrate the excellent capabilities of using ASE to extract compounds with antioxidant activity from natural sources as rosemary leaves and the microalga *Spirulina platensis.* ASE shows several advantages compared with traditional extraction processes such as: 1) it is faster (15 min vs 2-24h in traditional extraction procedures); 2) it has less solvent volume consumption (15 ml vs 50-500 mL/10g); 3) it is more efficient (less dependant on matrix) and 4) it is automatic and allows sequential extraction of samples. The use of *in-vitro* assays and CE coupled to both, DAD and ESI-MS allows obtaining information about the biological and chemical properties of the ASE extracts.

Acknowledgements

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Chapter 6

Pressurized Water Extraction: Resources and Techniques for Optimizing Analytical Applications

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Pressurized hot water extraction above and below the boiling point of water has been demonstrated to be an effective analytical technique. However optimization of this type of extraction often is empirical in approach and fails to take advantage of experimental findings in associated fields or theoretical correlations. In this presentation it will be shown that contributions from four areas can be utilized in the design and optimization of hot water extraction processes: (1) scaled-up processing applications, (2) sub-critical water chromatography, (3) solute (analyte) solubility predictive schemes, and (4) increase of analyte flux rates from sample matrices via mass transport design. The selectivity of pressurized hot water extraction can be maximized not only by regulating the dielectric constant of water, but employing principles noted in the four above areas. Fractionation of solutes then becomes feasible using hot water alone, or with the aid of an associated technique, such as supercritical fluid extraction with SC-CO_2. Examples will be cited of pesticide, essential oil, herbal mixtures, anthocyanins, and hydrocarbon separation using hot water media. The effect of hot water on common food matrices containing lipids/oils, carbohydrates, and proteins will be cited utilizing examples from the

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literature, and how this effect can be both advantageous or a disadvantage in analyzing food or agricultural samples. Finally, several examples will be presented which illustrate the application of hot water extraction to food and agricultural analyses.

Introduction

The use of environmental compatible solvent media that is benign toward laboratory workers has received extensive study over the past decade. Such "green" solvents frequently consist of carbon dioxide in either its supercritical or sub-critical states, the use of environmental-benign solvents such as ethanol, and more recently compressed water above its boiling point under pressure (*1*). This latter medium, also referred to as subcritical water (sub-H_2O), complements the CO_2 – based technologies in providing the analyst with both a non-polar and polar solvent medium whose solvent power can be regulated by the manipulation of temperature and pressure (*2,3*) . Subcritical water is readily available, non-toxic, and inexpensive; and is generally defined as water under 250°C and pressures less than 40 atm (4MPa). However, within this temperature and pressure range its solvent power and polarity can be varied considerably, permitting control of its dielectric constant (*4*) and reduction in its cohesional energy density (*5*).

The utility of sub-H_2O extends into several areas of application, including the following:

- Selective extraction solvent (SWE – subcritical water extraction)
- Fractionating agent (SWF – subcritical water fractionation)
- Reaction medium (SWR – subcritical water reaction)
- Analytical or chromatographic agent
- Degradation agent
- Sterilization agent
- Modifying agent for materials

Many of these areas of application relate to using sub-H_2O in various processing schemes, as reported by Clifford and others (*6-8*), or as a selective reaction medium (*9*). Water at such high temperatures and pressures can affect the extraction matrix or reactants when SWE, SWF, or SWR are enacted,

causing both wanted and unwanted conversions, pasteurization, and morphological changes in the substrate matrix. In this chapter we shall focus initially on how we and others have utilized sub-H_2O for analytical purposes as well as suggest resources and techniques from allied areas that can used to optimize analytical method development.

The degree to which sub-H_2O can be used to affect the above described operations is a function of temperature and pressure as illustrated in Figure 1.

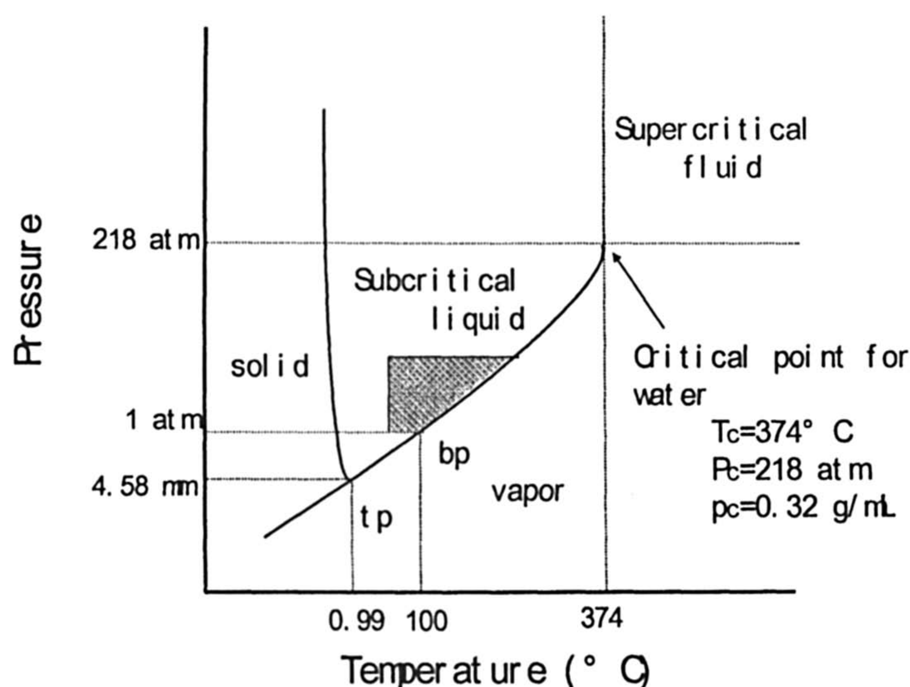

Figure 1. *Phase diagram for water.*

The subcritical liquid state that we are concerned with extends from the proximity of the boiling point to the critical point for water, which is quite high in temperature and pressure. The subcritical state of water therefore is a versatile medium depending on what the scientist or engineer wishes to accomplish. In the proximity of the critical point, water can be used to affect reactions, including the destruction of hazardous waste (*10*). As one proceeds down the vapor pressure curve between the T_c and boiling point (bp), selective reaction chemistries, such as hydrolysis (*11*), depolymerization (*12*), and acid/base-dependent reactions (*13*) become possible. SWEs are also feasible depending on the thermal stability of the solute (analyte), ranging from quite high temperatures (*14*) to a temperature range flanking the bp for thermally-labile solutes. This latter region, particularly amenable to natural products, is designated by the cross-hatched area in Figure 1. SWEs conducted in this region of the phase diagram have quite modest applied pressure requirements, e.g., at 120°C about 4

atm (0.4 MPa) will suffice to keep water in its hot liquid state. From a processing perspective, this lessens the capitalization costs associated with SWE, a factor that is often ignored in the design of analytical methods based on subcritical water.

Figure 2 illustrates the trend for water's dielectric constant (ε) as a function of temperature from ambient conditions to the bp - ε decreasing monotonically as temperature increases to values approximating the ε 's of polar organic solvents. However as noted by Hawthorne, et al. (*14*), such a trend continues well beyond water's bp, and can at the T_c become equivalent to ε values exhibited by hydrocarbon solutes.

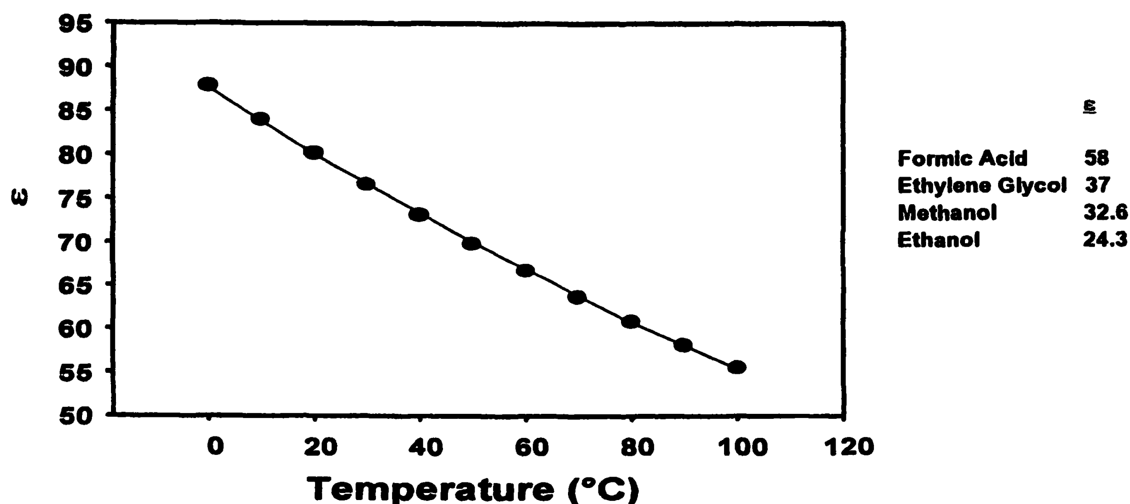

Figure 2. *Dielectric constant of water as a function of temperature (0.1 MPa).*

This change in water's polarity is reflected in the recorded enhanced solubility of solutes in water with temperature, as illustrated in Table I for atrazine between 50 – 125°C as determined by Curren and King (*15*). Here the solubility increases 25-fold over a 75°C interval, illustrating the substantial increase in solute (analyte) with increasing temperature and decreasing dielectric constant of the solvent. A more abrupt increase in atrazine's solubility occurs at the bp of water (ε = 55 from Figure 2). Note that this trend in solute solubility continues above the bp of water under pressure and has been verified for many solute types in subcritical water (*16*). Curren and King (*15*) have verified this trend for similar triazine pesticides in both neat water and water-ethanol mixtures, and shown that recorded solute solubilities can be an acute function of the molecular structure of the solute. This of course is the basis of SWF using sub-H_2O as aptly illustrated by Clifford and Hawthorne (*16*) and a proposed commercial deterpenation process.

Table I. Solubility of Atrazine in Pure Subcritical Water at 5 MPa.

T (°C)	*ε*	*Solubility (μg/mL)*
50	70	70 ± 1
75	62	210 ± 2
100	55	500 ± 30
125	49	1780 ± 70

However, thermodynamic-based solubility data and its correlation with solvent properties (sub-H2O) is only one facet in optimizing processes with sub-H2O – kinetic and mass transport factors must also be considered to receive maximum benefit in using this technique. Unfortunately, far less is known about the mass transport properties of solutes in sub-H2O, but some useful correlations and experimental data do exist. These will be considered in the next section and how they can be utilized in conjunction with analyte solubility in optimizing and designing efficient experimental conditions.

Predictive Methods for Solute – Subcritical Water Systems

Proper understanding of solute behavior in sub-H_2O systems requires evaluation of both solute-sub H_2O phase behavior (particularly in engineering scale systems where solute concentrations are finite), solute solubility trends in hot pressurized water, the diffusion coefficients of solutes in water as a function of temperature and their role in facilitating mass transport, and the potential effect of pressure – often trivialized as a major factor in SWE (*16*) – in affecting analyte extractions from sample matrices via sub-H_2O. All of these factors ultimately impact the resultant SWE, SWF, and SWR process, reinforcing one another fortuitously as temperature is increased, leading to an increase in solute flux into the sub-H_2O medium.

Correlation of Solute Solubility in Subcritical Water

The increase of solute solubility with temperature in water is well known to even the lay person, but its quantification above the boiling point of water under pressure has not been thoroughly studied or correlated. Clifford (*16*) has proposed the semi-empirical equation given below (Equation 1):

$$\ln[x_2(T)] \approx \left(\frac{T_o}{T}\right)\ln[x_1(T_o)] + 15\left[\left(\frac{T}{T_0}\right) - 1\right]^3 \qquad (1)$$

which has been used to correlate the solubility of solutes in subcritical water from experimental, or literature data taken under ambient conditions. Here a solute's solubility, x_1, at a reference temperature (T_o), is used to predict the solute's mole fraction solubility in water, x_2, at an elevated temperature, T. Results from this correlation generally agree within a factor of ten with experimentally-determined values (*17*). For example, using the above-cited triazine pesticides, agreement between experimental and calculated solubility values is quite good as tabulated in Table II. This is quite gratifying considering the subtle molecular structure differences in the solutes - and Equation 1 has been applied to a variety of solute types (*16*).

Table II. Mole Fraction Solubilities of Triazine Pesticides in Water

	323°K ($x10^6$)		348°K (10^6)		373°K (10^6)	
	Expt'l	Calc'd	Expt'l	Calc'd	Expt'l	Calc'd
Simazine	1.5	1.2	4.6	3.7	21	10
Atrazine	5.8	6.9	17	17	41	46
Cyanazine	36	27	110	65	320	150

We have used the above correlation to predict the anticipated solubilities of a number of polyphenolic solutes commonly found in berries and grapes (Table III). As can be seen from Table III, solute solubilities increase over the range of 20 to 125°C are of the order of $10^1 - 10^2$ mole fraction, a trend that is consistent

with the calculated solubilities for two model solutes, glucose and naphthalene, in Table III. Reported experimentally-measured solute solubilities recorded for these model solutes, glucose and naphthalene, are 3.569E-01 @ 90°C and 2.949E-05 @ 65°C, respectively. These values fall in between the calculated data, establishing the creditability of Equation 1 as a predictive tool.

Table III. Calculated Mole Fraction Solubilities of Flavanoids in Subcritical Water at 120°C

Solute	Exp. x_1	Cal'c x_2
Chlorogenic acid	5.077E-04	6.352E-03
Tannic acid	1.009E-01	3.283E-01
Gallic acid	1.163E-03	1.178E-02
Hesperiden	5.896E-07	4.117E-05
Rutin	3.685E-06	1.614E-04
Catechol	4.675E-02	1.850E-01
Glucose	8.325E-02	2.845E-01
Naphthalene	5.055E-06	2.044E-04

Exp x_1 = Experimental value in water@ 20°C

Cal'c x_2 = Calculated value in sub-critical water@120°C

The x_1 values used for the solutes in Table III came from a variety of sources (*2,3*) and it should be noted that solute solubilities at near ambient conditions are available in the literature for a variety of compounds (*18,19*). The recent compendium of Yalkowsky and He (*20*) contains over 16,000 data entries for 4,000 organic compounds in water – many over a range of temperatures between ambient and the bp of water. Such data provide a range of x_1 values to estimate solubilities (x_2) in sub-H_2O. Other experimental data, such as activity coefficients, Henry's law constants, and/or octanol/water (O/W) partition coefficients can be converted into x_1 values, and provide another source of estimating solute solubilities over an extended temperature range. These data bases like the O/W values can be combined with a group contribution approach

(*21-23*) to estimate the solubility of organic compounds for which no relevant aqueous solubility data exists. Other correlative methods (*24*) and prediction of vapor-liquid equilibria in aqueous systems (*25*) offer alternative approaches. The coupled approach using a group contribution or correlative scheme to find x_1 values, and then Equation 1 to predict the magnitude of solubility enhancement afforded solutes under subcritical water conditions is subject to a cumulative error or uncertainty, and must be tested against existing solute solubility values in the sub-H_2O region.

Mass Transfer Considerations in Subcritical Water

As remarked previously, knowledge of how solutes behave in terms of their mass transport properties in sub-H_2O is lacking. Experimental diffusivity data has been determined for selected solutes at approximately ambient conditions – but less so at even elevated temperatures below the bp of water. Such data show that the solute-water diffusion coefficients (D_{12}) increase as expected with temperature (*26*). For example, D_{12} for caffeine diffusion from coffee beans is 1.0×10^{-7} cm^2/sec at 50^0C while at 100°C it increases to 3.0×10^{-6} cm^2/sec, as calculated by the well known Wilke-Chang correlation (*27*). The predicted D_{12} for caffeine in water at 100°C agrees well with the experimentally-determined value of 3.5×10^{-6} cm^2/sec. The Tyn and Calus method (*28*) permits the estimation of D_{12} values by incorporating a molecular group structural contribution approach utilizing parachlor values for the various functional groups, thereby approximating D_{12} for compounds in water for which no experimental data is available. Other correlative methods for D_{12}, for example at finite concentrations of solutes in water are discussed in the well known tome by Reid, Prausnitz, and Poling (*29*). Interestingly, D_{12} has recently been measured for a polyphenolic solute, malvidin 3,5 - diglucoside, in a high pressure CO_2 – methanol system (*30*).

The combined effect of increasing solute solubility and diffusivity as the temperature is increased above or below the bp of water is to enhance the flux rate of the target solutes out of the sample matrix. The flux rate , F, is defined as:

$$F = - D_{12} \Delta c \, dx \qquad (2)$$

Δc = solute concentration gradient across the distance, x.

Hence the solute flux must increase due to the reinforcing effects of thermodynamic solubility plus mass transfer rate, and optimizing this effect above the bp of water permits rapid and efficient SWE to be achieved. The flux

equation may be recast to the form, rate = mass transfer coefficient times the driving force, to yield:

$$F = k\,(c_e - c_s) \qquad (3)$$

where k, the mass transfer coefficient = D_{12}/δ and δ is the characteristic length through which the solute mass transfer is occurring, c_e being the solute concentration in equilibrium between sub-H_2O and the sample matrix, and c_s is the solute concentration at any time, t, measured in the bulk sub-H_2O. Thus, the term $(c_e - c_s)$ is the concentration gradient or driving force through a characteristic length (in the case of interfacial mass transfer), which when combined with the characteristic mass transfer coefficient, k, determines the total solute flux rate. The important point to realize from the above discussion is that flux rates can be very rapid above the bp of water under modest pressure allowing efficient extraction to be obtained at high flow velocities of water through the extraction vessel. For example, Duan, et al. (*31*) have shown that the overall mass transfer coefficient of bio-active solutes from milk thistle extraction with hot water, doubled when going from 90 to 120°C in stirred reactor experiments. This results in rapid and differential extraction rates of the bio-active compounds in milk thistle, leading to enrichment and fractionation of the components in this biological matrix (*32*).

An important point to emphasize is that the conditions under which SWE is conducted may be deleterious to thermally-labile solutes. However, the combined effect of favorable solute mass transfer and thermodynamic solubility in sub-H_2O permits very rapid extractions to be achieved at high flow rates or face velocities through the extraction bed. King, et al. (*33*) have shown that thermally-labile anthocyanin moieties from fruit berries can be rapidly and efficiently extracted with minimal thermal degradation at linear velocities of sub-H_2O of 0.1 cm/sec through berry pomaces at 120°C. It cannot be emphasized enough that the flow rate of sub-H_2O through the extraction vessel is a critical parameter in extending the potential usefulness of SWE. Several investigators, Rogakinski, et al. (*34*) and Eller and Taylor (*35*) among them, have commented on the degradative effects SWE has on solutes, however in the former case SWE was performed in a static high pressure autoclave-type vessel, while in the latter study, an ASE (Accelerated Solvent Extraction, Dionex Corporation) was utilized. In both cases, SWE was attempted under static, non-flow conditions for varying periods of time, hence, the extraction vessel was in effect a "pressure cooker", facilitating decomposition or loss of solutes (analytes). It is interesting to note that the fluid velocity of sub-H_2O is a critical factor in affecting reaction chemistry in sub-H_2O (SFR) as shown by the hot water treatment of corn stover in a flow through reactor as studied by Liu and Wyman (*36*). It simply comes down to what the investigator is trying to achieve via the adjustment of pressure,

temperature, and flow velocity with sub-H_2O. For SWE, it is best to operate at as high a linear fluid velocity as possible consistent with good analyte recovery.

Experimental and Equipment Considerations – Subcritical Water

A variety of equipment and conditions have been employed in sub-H_2O investigations ranging from small flow cells into which water has been delivered from a variety of pumps (*17*), stirred and un-stirred autoclaves with sub-H_2O (*37*), large flow extractor systems (*33*), analytical pressurized fluid extraction systems (ASE - Accelerated Solvent Extraction – Dionex Corporation, Sunnyvale, CA); PSE – Pressurized Solvent Extraction – Applied Separations, Inc., Allentown, PA, and even columnar countercurrent contacting systems on a pilot plant scale (*16*). In the author's opinion, static systems such as high pressure autoclave vessels and ASE or PSE instrumentation introduce the possibility of solute decomposition when using sub-H_2O. This is not to say that these approaches cannot be useful depending on the ultimate goal of the experiment.

For example, ASE and/or PSE systems are widely applied in many analytical applications (*38, 39*), particularly with non-labile solutes and difficult-to-extract sample matrices. They can also be applied initially for optimizing the SWE of natural product matrices provided the experimentor distinguishes the differences between the operational basis of these instruments, and a dynamic SWE system. Wightman and King (*40*) have shown that differences can exist in terms of total anthocyanin (ACN) recovery depending on whether a dynamic sub-H_2O system is used or an ASE system. Using the laboratory-constructed dynamic SWE system shown in Figure 3 yielded lower total ACNs from elderberry pomaces than found using the ASE system. In terms of operation, the ASE system pumps the sub-H_2O solvent (also acidified water and water/ethanol mixtures were studied) into the extraction cell, where the fluid is held under pressure (~ 10 MPa) between 5-7 minutes. Then the cell contents are emptied. The above fluids in the case of the apparatus shown in Figure 3 are continuously flowed through the elderberry substrate held in the extraction cell. It should be noted that fluid velocity was not optimized in these experiments, and the sub-H_2O, etc. was delivered via applied air pressure. This is not the optimal system for this type of dynamic SWE (*40*), since the fugacity of any dissolved air in water (a N_2 sparge was employed to offset this problem) could affect the uptake of ACNs into sub-H_2O in the extraction cell proper. In retrospect, this pumping approach would best be affected using an accumulator piston.

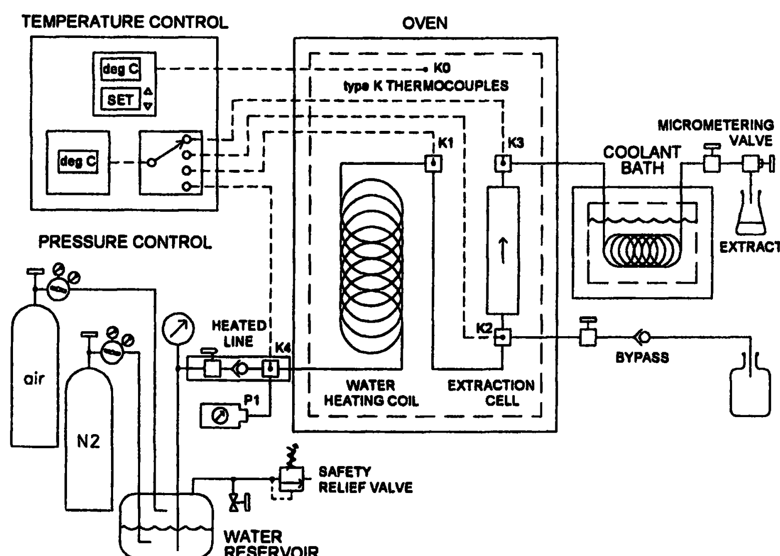

Figure 3. *Laboratory-constructed dynamic SWE system.*

Another criticism of ASE-type systems is that applied pressure on the extraction cell (applied through a pump) is set at a high pressure as noted before to accommodate a wide range of pressurized extraction solvents. This additional pressure is not warranted from the perspective of the vapor-liquid equilibrium curve on the phase diagram shown for water in Figure 1, e.g., only 0.4 MPa is required to maintain water in its hot liquid state at 120°C. The application of additional excessive pressure is energetically unfavorable from a perspective or processing costs, since energy will be expended in pumping or holding the fluid at this pressure, which in an analytical context is trivial. However it should also be acknowledged that a PSE or ASE system may be able to increase extraction efficiency by operating at a higher pressure, since this can permit the fluid, i.e., sub-H_2O to penetrate the sample matrix better, or mechanically disrupt the matrix, resulting in higher extraction efficiency. This is somewhat analogous to the expression of fluids in solid matrices as reviewed by Schwartzberg (*41*). The extra application of pressure seems to aid in the removal of adsorbed analytes in solid matrices, such as soils, overcoming adsorptive forces between the analyte and solid sample matrix (*14*). The morphological changes in bio-materials, foods, and agricultural products induced by the additional application of pressure can lead to disruption (*42*) of the sample matrix, and in specific cases, degradation of the matrix (*43*), and facilitating analyte recovery in what can best be termed SWE-SWR sample processing.

Analytical Application of Subcritical Water Extraction

Within the context of this monograph chapter, it is not possible to thoroughly review all of the applications of subcritical water extraction and processing. Already several reviews can be consulted which provide up-to-date information on the utility of this technique, including the review of Raynie (*38*), a focused review on its application in sample preparation for food analysis by Curren and King (*44*), and a recent related publication on the pressurized liquid extraction of medicinal plants (*45*) are recommended. In general, SWE requires the application of other analytical techniques mainly to concentrate the target analyte from the sub-H_2O extract. We have found techniques such as solid phase microfiber extraction (SPME), solid phase extraction (SPE), and special chromatographic materials, such as hydrophilic interaction chromatography (HILIC) to be of value in this regard. Sample dispersion aids, such as Hydromatrix (*46*), used extensively in supercritical fluid extraction, prove equally applicable in SWE applications as well as the matrix solid phase dispersion (MSPD) developed by Barker (*47*). Combinations of these techniques have been formulated into methods as reported in the literature using ASE or PSE instrumentation (*48,49*).

Typical extraction programs developed for use with the Dionex 300 ASE system are noted in Tables IV and V, for the SWE of atrazine or avoparcin from bovine kidney tissue. Each extraction requires some fine tuning to optimize for the target analyte, particularly with respect to the choice and composition of aqueous-based extraction fluid, volume of solvent used, and the flush volume percent. Details of the analytical protocol can be found in the literature (*48, 49*). Because of the complexity of pressurized fluid extracts, SPME or SPE may be required for extract cleanup, and/or specific analyte monitoring techniques, such as single ion monitoring by mass spectrometry may also be necessitated.

As remarked previously, there are numerous applications of subcritical water from non-analytical studies which may have value to the analyst, or conversely, be problematic in applying sub-H_2O for analytical purposes. Hydrolysis of fats and proteins can be affected in subcritical water, although the temperatures required are often quite higher than those employed in SWE (*50*). SWF can also be utilized for isolating flavors, such as the fractionation of spice ingredients, or deterpenation of essential oils (*16*) Depolymerization of carbohydrate moieties, i.e., saccharification can be again affected at quite high temperatures (*12*), facilitating analysis of the target analytes, or the carbohydrate matrix itself, as well as producing interfering substances that must be removed prior to analysis via additional sample preparation.

Table IV. Subcritical Water Extraction of a Non-Polar Analyte: Atrazine

Experimental Parameter	*Value*
Preheat	0 min
Heat	5 min
Static time	5 min
Purge time	60 sec
# cycles	3
Temperature	100°C
Pressure	50 atm
Solvent	30 % Ethanol /water (v/v)
Flush Volume	50%
Cell Volume	11 mL

Table V. Subcritical Water Extraction of a Polar Analyte: Avoparcin

Experimental Parameter	*Value*
Preheat	0 min
Heat	5 min
Static time	5 min
Purge time	60 sec
# cycles	3 sec
Temperature	75°C
Pressure	50 atm
Solvent	30 % Ethanol 70% 15 mM TEAP (v/v)
Flush Volume	50%
Cell Volume	11 mL

An exciting development is the combination of SWE, SWF, or SWR with SFE for the tandem processing of different sample matrices. Some of these possibilities are tabulated in Table VI below:

Table VI. Tandem $SC\text{-}CO_2$ – Subcritical H_2O Processes

- Extraction of Soils – Physicochemical Studies (*51*)
- Sequential Extraction of Heavy Metal Fractions in Sediments (*52*)
- Removal/Destruction of Dioxins from Fly Ash (*53*)
- Alkenyl Phenolic & Polysaccharides from Cashew Nut (*42*)
- Bioactive Compounds from St. John's Wort (*54, 55*)
- Extraction of Essential Oils & Boldine from Boldo (*34*)

Hawthorne and colleagues (*51*) have extensively studied the extraction of soils using supercritical carbon dioxide ($SC\text{-}CO_2$) and sub-H_2O using the difference in elutropic strengths of the two solvents to differentiate between their effects on analyte-matrix interactions. A sequential extraction was also applied to sediment samples to affect the extraction of metallic species (*52*). SFE using CO_2 was used by the Goto group (*53*) to affect the extraction of dioxins from fly ash, followed by a SWR to destroy the isolated carcinogenic analytes. Sub-H_2O has also been applied to various biological matrices, such as cashew nut (*42*), St. John's Wort (*54*), and the South American plant, boldo (*34*). Here sub-H_2O is sequenced with $SC\text{-}CO_2$ extraction to recover both non-polar lipophilic fractions from the above samples and specific solutes that are more polar.

The sequential extraction of St. John's Wort (SJW) using tandem pressurized fluid extraction is an excellent example of this coupled fluid approach (*54,55*). Here the active components in SJW are hyperforin/adhyperforin and hypercin & pseudohypercin. Hyperforin or adhyperforin can be extracted via SFE using CO_2 at 30°C and 80 atm (8 MPa) . On the other hand, hypercin can be removed using sub-H_2O containing 1%$NaHCO_3$ at pH = 7.0. Using both pressurized fluids in sequence gives hyperforin in 93 – 99 % yield, and hypercin in 80 – 93 % yield. It is also possible with this sequential use of pressurized fluids to envision an extraction step more specific for flavonoids, such as quercetin, utilizing $SC\text{-}CO_2$ and an appropriate cosolvent.

Conclusions

In this brief overview we have illustrated the application and potential of sub-H_2O for the extraction, fractionation, and reaction of various analyte types and mixtures. By careful optimization of key variables such as temperature and fluid flow rate, sub-H_2O can be used for SWE, SWF, and select SWRs. In summary then:

- Subcritical water is a unique and versatile extraction medium and compliments SC-CO_2
- Extrapolation of sub-critical water results from processing to analytical studies, and vice versa, should be done with caution since they can be experimental or equipment dependent
- Subcritical water for sample preparation can be optimized for many types of sample matrices

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Chapter 7

Pressurized Fluid Extraction of Squalene from Olive Biomass

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Pressurized fluid extraction has been used to obtain environmentally benign and fast extraction of squalene and α-tocopherol from a low-value biomass in the olive oil production; olive oil pomace. The extraction conditions, i.e., temperature (80-190°C), extracting solvent (acetonitrile, acetone, ethanol, 1-propanol, 2-propanol, 2-butanol, ethyl acetate, toluene) and extraction time (2-18 min.), were optimized through an experimental design to obtain the highest yield. Temperature was found to be the most important parameter for effective extraction of squalene from the olive biomass. The best results were obtained when 2-propanol or ethanol was used as a solvent at a temperature of 190°C, an extraction time of 10 minutes times three cycles. The content of squalene was found to be in the range of 0.2-0.5 mg/g, while the recovery of α-tocopherol was in the range of 0.01 mg/g using the same extraction parameters. The developed method can be used both in an analytical context and as a starting point for up-scaled "green" processes.

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Introduction

The olive oil pomace is a solid by-product in the extraction of olive oil. This olive biomass has a very complex composition, including components such as water, salts, proteins, hydrocarbons and triacylglycerols. Moreover, the relative concentrations of the residues can vary greatly, depending on the mechanical system employed for the extraction of the oil *(1)*. Data on chemical variations using one of the most common methods, two-phase centrifugation, is still not well known.

One of the components in this olive oil residue that may be of interest for the industry is squalene. Squalene is used as industrial lubricant, and is widely used in several cosmetic applications as a carrier for lipid-soluble components since it is easily adsorbed by the skin *(2)*. Squalene has also been claimed to enhance oxygenation of the blood, facilitate detoxification, strengthen immune system and protect against cancer *(3)*. Traditionally squalene is mainly obtained from shark liver oil *(4)*, which is an unethical use of sharks threatened by extermination and really is not acceptable.

In order to achieve long term stability in society, industry should focus on renewable sources both as raw material and as process chemicals. Therefore, renewable raw material has been searched for to give a source of squalene, for example olive oil *(5,6)* and amaranth seed oil *(7,8)*. Another component of interest in olive oil pomace is α-tocopherol, which is traditionally considered as the major antioxidant of olive oil.

The extraction of minor components in by-products from food industries, like the olive biomass, is a rather difficult task. In general, extraction of organic compounds from a sample matrix is performed by mixing/blending with organic solvents. Traditional extraction methods, such as Soxhlet extraction, have the drawbacks of being both time and solvent consuming *(9)*. To extract the target compounds, the solvent has to penetrate the matrix, dissolve the compounds and break their interactions with the matrix, a process which in the most efficient way can be accomplished using a solvent of both high diffusivity and strong solvating power. Such a solvent can be a supercritical fluid or an organic solvent at elevated temperature. Supercritical fluid extraction (SFE) using supercritical carbon dioxide as a solvent has found applications in the recovery of value-added products such as lycopene in tomato processing byproducts *(10)* and tocopherols in soybean flakes and rice bran *(11)*. Güçlü-Üstündağ and Temelli *(12)* have in a review article studied solubility behavior of minor lipid components, among others squalene and α-tocopherol, from fats and oils in supercritical carbon dioxide. Recently Tabera et al. *(13)* have described a

procedure to extract value-added compounds in olive leaves by means of countercurrent supercritical fluid extraction in a pilot plant.

Pressurized fluid extraction (PFE) is a technique that uses organic solvents in pressurized cells at temperatures above their boiling point *(14)*. This technique has quickly become an attractive alternative to SFE and traditional extraction techniques in an analytical context. In general, PFE gives recoveries comparable to those obtained with Soxhlet extraction (using the same solvent) but with a shorter extraction time and a smaller amount of organic solvents *(15,16)*. Compared to SFE, method development of PFE is faster *(9)* and in some cases gives extracts with a higher recovery of target-analytes *(17)*. All these mentioned properties can be useful for the extraction of molecules from highly complex matrices, such as olive biomass, and could render good extraction yield in a short time.

The aim of this work was to develop a simple, environmentally sound and fast method for the extraction and determination of squalene in an olive biomass using the PFE technique, and investigate if this technique could be a viable processing technology for the extraction of squalene from olive oil pomace. The effects of different solvents and mixtures of solvent, extraction temperature and extraction time were also evaluated for their influence on yield of squalene. Squalene in the olive biomass extracts were quantified by liquid chromatography with UV absorbance and fluorescence detection. In addition, the proposed method was used to determine the content of α-tocopherol in the olive biomass.

Material and Methods

Chemicals and Samples

The solvents used, acetonitrile, ethanol, 2-propanol, 1-propanol, 2-butanol, ethyl acetate, toluene, acetone and hexane, were of pro-analysis grade. All the solvents were purchased from Merck (Darmstadt, Germany), except ethanol (99.5 %), which was obtained from Kemetyl (Haninge, Sweden). MilliQ-water was obtained from a purification system (Millipore). Nitrogen, (99.996 % by volume) was from AGA, (Stockholm, Sweden). Methanol of HPLC quality (Merck) was used as a mobile phase. Isolute sorbent (pelletized diatomaceous earth, International Sorbent Technology, Great Britain) was used as adsorbent in the initial studies.

The olive biomass samples (olive oil pomace) were obtained from an olive oil processing factory in Córdoba (Spain), which used a two phase centrifugation technique to extract the oil. The samples were kept frozen at –20°C. Two different samples (A and B) were used in all the experiments, which had been obtained using the same process, but at different time of the year. Sample A consisted of well grounded biomass, while sample B was crude and contained visual traces of kernels. The α–tocopherol and squalene standards were from Sigma Chemical (St. Louis, USA).

Instrumentation

In this study, an ASE™ 200 Accelerated Solvent Extractor, Dionex Corp. (Sunnyvale, CA, USA) was used. The extraction cells (Dionex) were of stainless steel with a volume of 11 ml and capped with PEEK seals and stainless steel frits.

Extraction Procedure

Approximately 2 g of olive biomass was weighed in an 11 ml stainless steel extraction cell equipped with a cellulose filter at the bottom of the cell (Dionex, 1.91 cm diameter). The following conditions were used for the extractions: Preheating for 5 minutes at 10 MPa pressure before static extraction at different temperatures and times, with 3 cycles of extraction. A flush volume of 60 % of the cell was used and finally the cell was purged for 60 seconds with nitrogen to collect the extract in the collection vial. Extraction solvents of different polarity with dielectric constants ranging from 37.5 for acetonitrile (most polar) and to 1.9 for hexane (less polar) were studied. The solvents tested were acetonitrile, acetone, ethanol, 2-propanol, 1-propanol, 2-butanol, ethyl acetate, toluene, and hexane . The range of extraction temperatures tested was 80 to 190°C. The system pressure was held constant at 10.0 MPa during all the extractions. The extraction vials were weighed before and after the extraction to calculate the volumes of the extracts.

Analysis of the Extracts

The extracts were analyzed using a liquid chromatography system, consisting of a Jasco (Tokyo, Japan) PU-980 pump, a Rheodyne (California, USA) injector (injection volume: 20 µl) and a Chrompack (the Netherlands) Chromsphere 100 mm x 3 mm I.D C18 column packed with 5 µm particles with

a pre-column of the same material. The extracts were filtered through a membrane filter, FGIP with pore size 0.2 µm (Millipore) before injection. Chromatography was conducted using a 100% methanol mobile phase (unless otherwise stated) in isocratic mode at a flow rate of 0.5 ml/min. Detection was accomplished using a UV detector (Jasco UV-970) at 210 nm, and a fluorescence detector (Jasco FP-920) at an excitation wavelength of 290 nm and an emission wavelength of 330 nm. Calibration plots were made from peak area of standards of squalene and α-tocopherol in 2-propanol and in ethanol. A PC computer system running Borwin software version 1.21 (JMBS Developements, France) was used for data acquisition and processing.

Factorial Design

An optimization study was performed to determine the interactions of the main variables and to find the optimum values of the factors expected to have the largest effect on the extraction yield of squalene from olive biomass. Statgraphic version 6.0 was used to design and evaluate the experiments. The studied factors were temperature, extraction time and concentration of acetone in a mixture of acetone in 2-propanol. The factorial design used was a central composite design with five central points. Nineteen experiments were performed in duplicates and in random order. The central composite design consisted of 8 experiments in the corners of a cube and 6 experiments in the star points of each side of the cube, expressing the limits of the design and 5 replicates in the center of the cube to estimate the variance (see Table I).

Results and Discussion

In a preliminary study nine different solvents were screened for the extraction of squalene from the olive oil pomace. The solvents recommended for Soxhlet extractions have generally been the starting point in choosing the solvents also in the PFE technique *(18)*. However, also more environmentally friendly solvents like ethanol and 2-propanol were investigated in this study. The solvents that were screened and the peak areas obtained for squalene with each solvent are shown in Figure 1. Acetone gave the highest yield of squalene under the present conditions (extraction temperature 100 °C, extraction time 5 min. x 3 cycles), while the second best solvent was 2-propanol. These two solvents have different functional groups and physical-chemical properties, and it therefore seemed logical to also consider a mixture of them (1:1, v:v) as extraction solvent. Since this solvent mixture also resulted in good yields, different combinations of acetone and 2-propanol were selected as solvents for the

subsequent optimization study together with pure acetone and 2-propanol, see Table I.

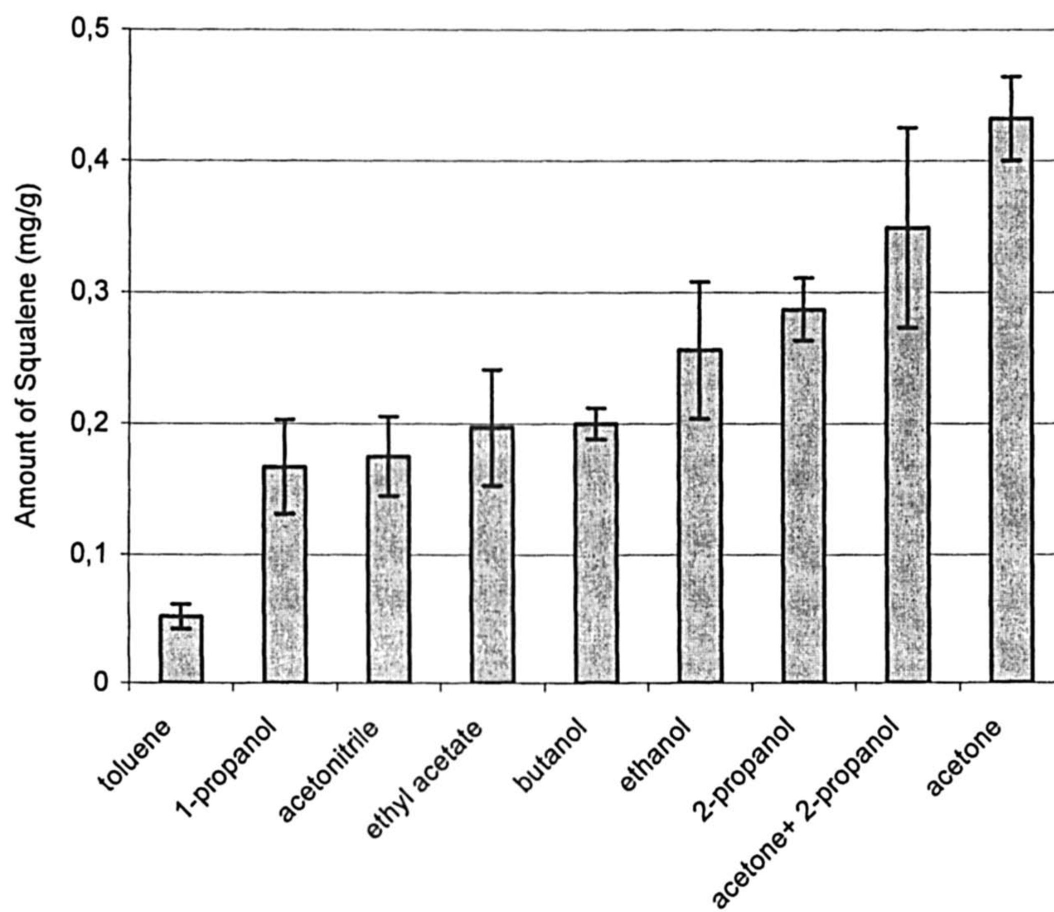

Figure 1. *Amount of squalene (mg/g) obtained from extraction of olive pomace using PFE with different solvents. Extraction conditions: temperature 100 °C, time 5 min and 3 cycles (n=2). Error bars show two standard deviations.*

Together with the extraction solvent, one of the main factors to optimize in PFE is the temperature *(14)*. Therefore, five temperatures between 80 and 190°C were tested. In order to study the possible synergetic effects between extraction time and temperature, a factorial design was performed, selecting these factors together with various solvent compositions of acetone and 2-propanol. The maximum temperature tested in this optimization study was 190°C, since the physical limitation of the instrument used was 200°C and extreme conditions wanted to be avoided. The extraction time was varied between two and 18 minutes. The obtained results for the nineteen experiments are shown in Table I.

Table I. Extraction yield in µg/g of squalene obtained with a central composite design from olive oil pomace, batch A.

Run	*Temp. (°C)*	*Conc. of Acetone in 2-Propanol (%)*	*Static extraction time (min)*	*Content of Squalene in olive biomass[1] (µg/g)*
17	80	50	10	230
9	102	80	15	403
12	102	80	5	207
14	102	20	5	163
18	102	20	15	341
1	135	100	10	456
2	135	50	10	420
4	135	50	10	450
6	135	50	10	413
7	135	0	10	424
11	135	50	2	265
13	135	50	10	321
16	135	50	18	404
19	135	50	10	463
3	167	80	5	478
8	167	20	15	518
10	167	20	5	439
15	167	80	15	520
5	190	50	10	575

[1] n = 2

Table II. Anova Table for the concentrations of squalene found in olive oil pomace. The factors investigated were; T (temperature), t (time) and c (concentration of acetone in 2-propanol). The significant effects have p-values < 0.05

Factor	*Sum of Squares*	*Df*	*Mean Square*	*F-ratio*	*P-Value*
T	148289	1	148289	95.05	0.0000
c	2961	1	2961	1.90	0.2015
t	39388	1	39388	25.25	0.0007
Tc	552	1	552	0.35	0.5727
Tt	7992	1	7992	5.12	0.0499
ct	40.5	1	40.5	0.03	0.8772
TT	300.8	1	300.8	0.19	0.6755
cc	1058	1	1058	0.68	0.4400
tt	11846	1	11846	7.59	0.0223
Total error	14040	9	1560		

The results illustrated in Figure 2 and 3 show that the highest temperature investigated (190°C) gave the highest yield of squalene. This study also shows that at temperatures above 100°C the yield of squalene decreases with extraction times longer than 12-15 min, which indicates that squalene either decomposes or reacts with other sample components or with the solvent (see Figure 3). For that reason, the optimum extraction time was found to be 10 minutes and this time was hence chosen for the following studies. To conclude, the optimized conditions were extraction with any mixture of acetone and 2-propanol at 190°C and 3 × 10 min extraction.

The factors and their interactions were evaluated from the estimated effects and the Anova Table, (Table II), with a significance level of $p<0.05$. The effects that were found to be significant were temperature, extraction time, the interaction between temperature and extraction time and the square of the extraction time. The factors were additive at low temperature which gave rise to the linear response clearly seen in Figure 3. The estimated effects of the three different factors and the interactions between them, show that the concentration of acetone in 2-propanol did not have a significant effect.

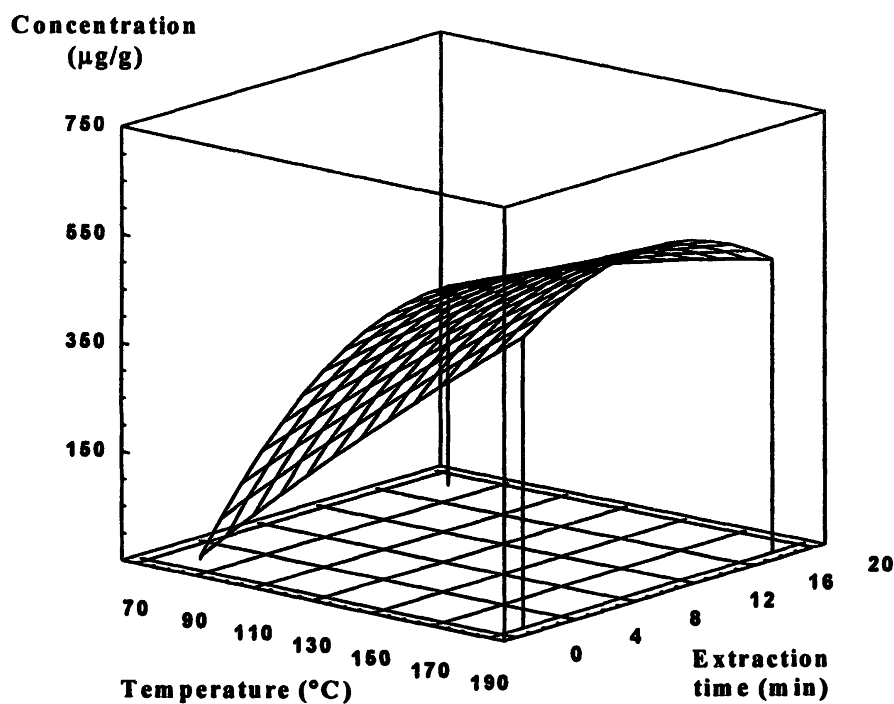

Figure 2. Response surface plot of the extraction yield of squalene from olive oil pomace using 2-propanol as solvent, as a function of extraction time and temperature.

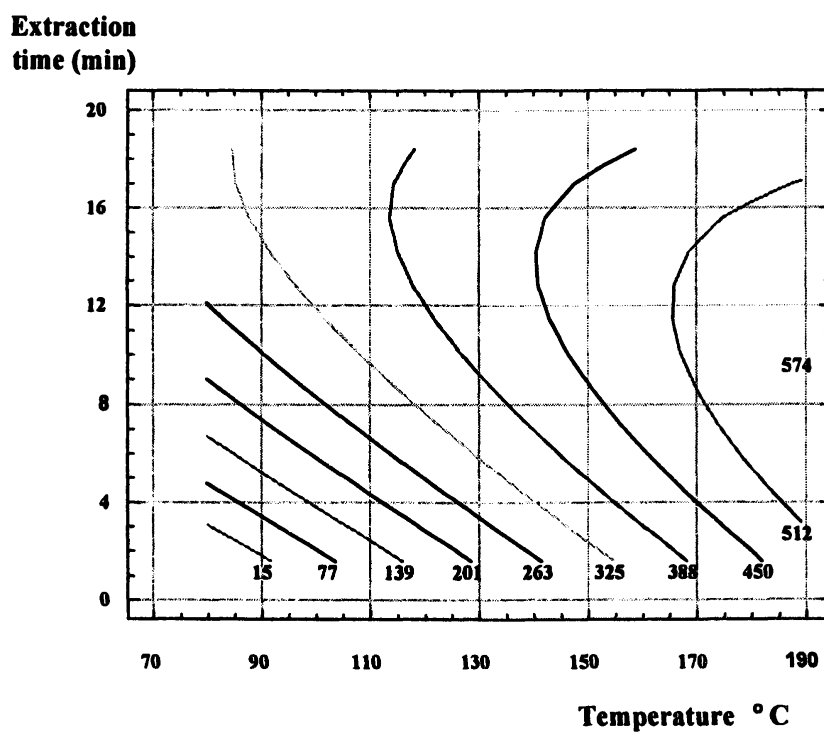

Figure 3. The same data as in Figure 2, but shown as a response contour plot.

A confirmation experiment was performed using the optimized parameters found in this study, where a new sample was extracted at 190 °C, 2-propanol as solvent, a static extraction time of 10 min. x 3 cycles with 60 % flush. The average yield of squalene from batch A was 509 μg/g (RSD value 16%, n=4), and from batch B 219 μg/g (RSD value 3.2%, n=5). The discrepancy in yield between sample A and B might be referred to the fact that the samples were taken at different times of the year, and might origin from different kind of olives.

For the extraction of α-tocopherol using the same extraction conditions (thus not optimized for α-tocopherol) the yield was 68 μg/g (n=3) from batch A, and 9.5 μg/g (n=2) from batch B.

An important finding in this study was that, due to the dominating effect of temperature, variations in the yield employing different composition of solvents are practically eliminated at higher temperatures. While in the pre-study acetone seemed to be the most appropriate solvent at 100°C, as shown in Figure 1, the difference between 2-propanol and acetone was practically eliminated at 190°C. In this study, 2-propanol was chosen for the optimized method instead of acetone, because of the slower rate of evaporation and due to health reasons. As the choice of extracting solvent seems more flexible at higher temperatures other aspects could be taken into account such as economical, environmental or health aspects as well as practical considerations, when choosing a solvent for a process. Therefore, ethanol was also tested in this study at optimized temperature (190°C) and extraction time (3 × 10 min). A t-test at 5% significant level showed no difference between the mean yield from extractions with 2-propanol and ethanol, thereby confirming that the choice of solvent has considerably less importance at higher temperatures. The average yield from batch B using ethanol as solvent was 209 μg/g (RSD value 9.9%, n=5) (compared to 219 μg/g with 2-propanol).

In conclusion, this study shows the versatility of the PFE technique and its fast method development, which makes it an effective tool to extract squalene and α-tocopherol from olive oil pomace. In an analytical context the described method gives fast and reproducible results. The samples were extracted without any sample pre-treatment, and the extracts were analyzed by liquid chromatography without the need of clean-up or concentration before injection. In addition, the PFE technique is fully automated and suits well for routine analysis requiring high sample throughput. In the context of "green" process-scale extraction, the results from this study indicate promising results. By using relatively small volumes of environmentally benign solvents, the PFE technique will provide improved sustainability of both the process and the final products For example, in the case of squalene, the exploitation of threatened shark species can be avoided by instead isolating squalene from renewable materials or plant biomass wastes.

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Chapter 8

Solutions for Online Coupling of Extraction and Chromatography in the Analysis of Food and Agricultural Samples

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This overview is focused on the on-line coupling of pressurized liquid extraction (PLE), microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) and sonication-assisted extraction (SAE) with liquid and gas chromatography for the analysis of solid agricultural and food samples. In addition, head-space techniques and direct thermal extraction are discussed.

Introduction

Even with the emergence of advanced techniques of separation and identification, it is rarely possible to analyze food and agricultural samples without manipulation. Particularly solid and semi-solid samples typically require complex, multistep sample preparation procedures. During recent years, much effort has been invested in the development of separation techniques, especially chromatography. However, the role of sample pretreatment is too often neglected in the method development. Often, the sample preparation is not at the same high

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level as the sophisticated separation methods. Many of the sample preparation methods currently in use have been around for decades, with essentially no modification over the years. The main problems with traditional sample preparation schemes are that they are too time-consuming, tedious and they even introduce serious errors to the analytical procedure.

In the sample preparation of semi- and nonvolatile compounds, solvent extraction is typically used for extracting the analytes of interest from a sample matrix. For volatile analytes, head-space or thermal extraction are good alternatives to solvent-based techniques. Several novel extraction systems that utilize elevated temperatures of pressures in the extraction have been developed particularly for solvent-based extraction methods. These new methods typically are much faster and often more selective than older methods and consume smaller amounts of organic solvents and reagents. Commercially available systems with the ability to heat and pressurize liquids include pressurized liquid extraction (PLE), microwave-assisted extraction (MAE) and supercritical fluid extraction (SFE). Also sonication-assisted extraction (SAE) has given promising results.

Most of the extraction methods are used off-line, meaning that extraction and analysis are done separately. Many of the systems could nevertheless be integrated as an on-line system, where the whole analytical procedure takes place in a closed, usually automated system. In this way, many of the problems associated with the traditional approaches could be avoided. Additional benefits are the increased sensitivity and reliability because sample clean-up in an on-line system tends to be more effective.

It should be noted, however, that on-line procedures are not always the best choice for the analytical task in question. For the preparation of an appropriate number of samples, the methods have to be selected not only on the basis of the expected performance of the analytical system, but also according to general requirements such as number and mass of samples, laboratory equipment and the experience of the analytical staff. An off-line procedure is a good choice, when the number of samples is small, because then there is usually no need for an automated method and no incentive for the time-consuming development of such a method, and conventional methods will suffice. Setting up an automated method, either at-line or on-line, can be worth the effort if the number of (similar) samples to be analyzed is large. In both off-line and at-line methods, optimization of individual steps of the sample pretreatment is quite flexible, in contrast to on-line methods, where compromises often have to be done in this aspect. Conversely, the sensitivity of off-line and at-line procedures is usually lower than in on-line methods because only a part of the sample is injected to the chromatographic system. On-line systems are beneficial when the amount of sample is limited, or very high sensitivity is required (*1-6*). In most cases, experienced personnel is required even though the use of an automated on-line

instrument is quite straightforward, for the method development and eventual trouble-shooting. In the case of labile or reactive sample components, on-line system can be clearly advantageous over the off-line and at-line methods. In an on-line system, where extraction and analysis take place in the same, closed system, the risk of decomposition is minimal.

Approaches For On-Line Coupling of Extraction with Chromatography

The most common extraction techniques for semivolatile and nonvolatile compounds from solid samples that can be coupled on-line with chromatography are liquid-solid extractions enhanced by microwaves, ultrasound sonication or with elevated temperature and pressures, and extraction with supercritical fluid. Elevated temperatures and the associated high mass-transfer rates are often essential when the goal is quantitative and reproducible extraction. In the case of volatile compounds, the sample pretreatment is typically easier, and solvent-free extraction methods, such as head-space extraction and thermal desorption/extraction can be applied. In on-line systems, the extraction can be performed in either static or dynamic mode, as long as the extraction system allows the on-line transfer of the extract to the chromatographic system. Most applications utilize dynamic extraction. However, dynamic extraction is advantageous in many respects, since the analytes are removed as soon as they are transferred from the sample to the extractant (solvent, fluid or gas) and the sample is continuously exposed to fresh solvent favouring further transfer of analytes from the sample matrix to the solvent.

In on-line analysis sample sizes are frequently small, often 4-1000 mg of the solid sample is sufficient for trace level analysis. The reason for this is that in on-line system, the whole sample extract reaches the analytical column, thereby increasing the sensitivity. As very small sample amounts are not always desirable with solid samples, careful homogenization of the sample is crucial in order to obtain representative sub-samples.

Solvent or Fluid Based Extraction

In the field of food and agricultural samples, relatively few on-line systems based on solvent extraction have been developed. SFE, PLE and SAE have been combined on-line with LC or GC (*7-20*). In theory, it would also be possible to use MAE in on-line combination, but so far, only one application for environmental analysis has been reported (*21*).

In solvent-based extraction methods, on-line coupling requires some modifications to the off-line extraction techniques. First of all, the volume of the extract must be kept small in on-line coupling. Typically, it should be less than 1-2 milliliters, or the extract must be concentrated before the transfer to chromatography. Moreover, the solvent (or fluid) must be compatible with the following chromatographic system. In coupling with GC this means that the solvent must be sufficiently volatile and preferably nonpolar. In coupling with LC the extracts should preferably be in a solvent of weak eluent strength in the LC (*1*). In on-line coupling of extraction with large-volume GC, the volumes of extracts transferred are typically up to milliliters, and special interfaces are required for the transfer. The most common techniques are based on large-volume GC and on-line LC-GC. Several interfaces have been implemented, including on-column, loop-type and vaporizer interfaces, which are almost exclusively used in on-line methods today. The situation is more straightforward in LC because rather large volumes can be transferred to an LC column without problems. If necessary, the coupling can be done via a short solid-phase extraction column, or the extract can be diluted with suitable solvent to reduce the solvent strength of the extract before the transfer.

Microwave-assisted extraction

Microwave-assisted extraction (MAE) is widely recognized as a versatile extraction technique especially for solid samples. It has also been used widely in digestion of food and agricultural samples, but it has also been utilized in the extraction of organic compounds (*22-26*). In several comparative studies, MAE has proven to be equal or superior to Soxhlet extraction, PLE and SFE (*22,23*). In MAE, organic solvent and the sample are subjected to radiation from a magnetron. The solvent (or the sample) must be dielectric in character. The main advantages of MAE are the fast rate of extraction due to the quick heating and elevated temperatures, but also the ease of instrument operation.

Almost all MAE applications involve off-line procedures, and on-line systems have not been utilized in food and agricultural analyses. Only a few approaches involving an on-line system have been published, for example in the determination of organophosphorus compounds in air particulates (*21*). In this MAE-GC system, an interface based on solid-phase trapping was used. Methanol was as the extraction solvent, and before the solid-phase trap the extract was diluted with water to enable efficient trapping to a polymeric sorbent. The sorbent was then dried with a nitrogen flow and the trapped analytes were eluted with organic solvent to the GC equipped with a PTV injector. A similar system should be applicable to the analysis of other types of solid samples as well.

Sonication-assisted extraction

In sonication assisted extraction (SAE), acoustic vibrations with frequencies above 20 kHz are applied to the sample. The vibrations cause cavitation in the liquid; that is, bubbles with a negative pressure are formed. When the cavitation bubbles collapse, shock waves are generated, which enhance the removal of analytes from the matrix surface. Moreover, implosion of cavities creates microenvironments of high temperatures and pressures. Traditionally, SAE is performed in static mode. In on-line combinations, the extraction is dynamic (*27,28*).

In principle, SAE can be coupled on-line with both LC and GC. However, no SAE-LC application has been reported and only one SAE-GC application for the analysis of organophosphates in air particulates (*27,28*). In this application, the extract was transferred directly to the PTV injector of the GC. Direct transfer was possible by using a miniaturized extraction vessel, a very short extraction time (3 min) and a moderate flow rate (200 μl/min) in the extraction.

Pressurized liquid and hot water extraction

In pressurized liquid extraction (PLE), rapid extraction is performed with small volumes of conventional solvents by using high temperatures (up to 200°C) and high pressures (up to 20 000 kPa) to maintain the solvent in a liquid state. The extraction can also be done with a purely aqueous phase, a technique often referred to as pressurized hot water extraction (PHWE) or subcritical water extraction. Temperatures of up to 325°C have been used in PHWE (*29-38*).

In principle, it is possible to combine PLE on-line with chromatography but so far, no applications have been published with organic solvents. On the other hand, several applications involving on-line coupling of PHWE with either LC or GC have been reported (*29-31,35-39*). The coupling of PHWE with LC is relatively simple because the solubility of the analytes in water decreases dramatically when the water is cooled to ambient temperature. Trapping of the extract in a solid-phase (SP) material is thus easy. In PLE, the extract is usually already in a relatively large volume of an organic solvent, and trapping is not easily achieved. Direct transfer is not possible either, because the volume of the extract tends to be too large. The connection of PLE to chromatography might be done in a similar manner to that described for on-line MAE-GC, i.e. by diluting the organic extract with water before trapping.

In on-line coupling, PHWE is performed in dynamic mode, i.e., the water is continuously flushed through the extraction vessel. After the extraction, the water is cooled and the extracted analytes are collected either on a solid-phase trap (for LC or GC) or a membrane extraction unit (for GC) (*35-39*). PHWE-LC

coupling with a SP-trap is more straightforward than PHWE-GC coupling, as the extract from the SP-trap can be directly eluted to the LC column by a suitable eluent (*7,38,39*). The apparatus closely resembles column switching LC systems. In PHWE-GC coupling using a SP-trap, the trap must first be dried with gas flow, after which the analytes are eluted with a suitable organic solvent (*35*). In PHWE-GC applying a membrane unit as interface, the extract can be directly transferred from the membrane extractor to the chromatograph (*36,37*). The membrane unit is based on microporous membrane liquid-liquid extraction (MMLLE).

In PHWE, degradation of the analytes can occur due to harsh conditions during the extraction, and this must be considered when choosing the extraction conditions. For relatively polar analytes, such as caffeine, chlorophenols and anilines, quantitative extraction can be obtained already at 100°C, whereas for less polar compounds, such as selected pesticides, PAHs or PCBs, 250 – 300 °C should be used (*16,29-32, 34-37*). Very high temperatures should be used with caution, however, because excessively high temperatures may cause degradation of analytes prone to hydrolytic attack (*33*). Moreover, the selectivity of the extraction decreases at high temperatures because of the larger amounts of unwanted low-polar matrix species that are coextracted.

PHWE-LC systems have been utilized, for example, to determine N-methylcarbamates in food. PHWE was done at 75 °C and a combination of static and dynamic extraction was used. In this system, the effluent from the chromatograph was coupled with a flow injection manifold where the N-methylcarbamates were hydrolyzed and then derivatizatized by reaction with a fluorogenic reagent (*o*-phthalaldehyde). The derivatized analytes were driven to a fluorimeter equipped with a flow-cell allowing monitoring (*31*).

For many pesticides, GC separation is more appropriate than LC in terms of separation efficiency and sensitivity. A system utilizing PHWE-MMLLE-GC has been developed for the determination of pesticides from grapes (Figure 1A) (*16*). A similar system has also been used for the determination of organic pollutants from soil and sediment samples (*35-37*). In this system, during the dynamic PHW extraction (120 °C, 40 minutes) the aqueous effluent flowed through a membrane extraction unit, and LLE took place through the organic liquid in the pores of the membrane. The MMLLE organic extract was obtained in a small volume and could easily be transferred directly to GC. In addition, the MMLLE was selective, as also size exclusion takes place due to the small pores of the membrane (*40*). Therefore, the extract was clean from high molar mass compounds that could disturb the GC analysis. Figure 1B shows PHWE-MMLLE-GC-MS determination of pesticides from grapes. The only sample pretreatment required prior to analysis was crushing and weighing of the samples. The LOQs of the method were in the range of 0.3-1.8 μg/kg, using total ion monitoring in the MS detection.

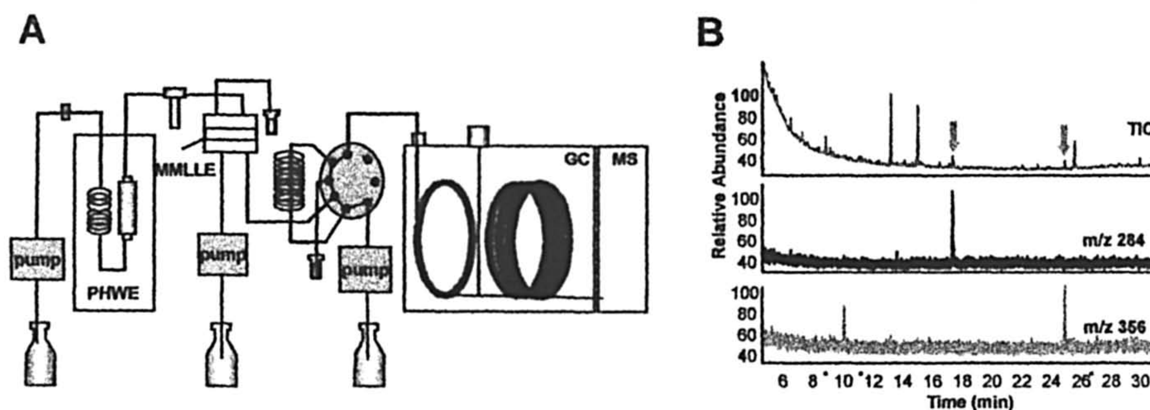

Figure 1. *(A) PHWE-MMLLE-GC-MS apparatus and (B) determination of pesticides in grapes by the system.*

Supercritical fluid extraction

Supercritical fluid extraction (SFE) has several distinct physical properties, and it is regarded as a promising alternative technique to conventional solvent extraction because it is fast, selective and environmentally friendly (*41-44*). SFE exploits the gas-like and liquid-like properties of a supercritical fluid, typically carbon dioxide. With addition of a suitable organic modifier to CO_2 it is possible to extract a range of compounds of different polarity. It should be noted that in SFE, water in samples can cause problems in the repeatability of the extraction. Therefore, samples containing large amounts of water, drying can be required before the extraction. In some cases it is also possible to add asuitable drying material directly to the extraction cell, if the analytes are not adsorbed onto it (*15*). Fat removal can often be combined with the extraction step, also by adding a suitable adsorbent material (e.g. Florisil) directly to the extraction cell (*41*).

SFE is well suited for on-line coupling with chromatography since CO_2 becomes gaseous upon depressurization and is easily eliminated from the analytical system. The solvating properties of the fluid are also decreased substantially during the depressurization. Typically, dynamic extraction combined with a sufficient static period is used in on-line coupling.

In the field of food and agricultural analyses, several SFE-GC (*11,12,17,19,20,44*) and SFE-LC applications have been published (*58-80*), and also a few SFE-SFC (*13,14*) applications have been reported.

In SFE-GC coupling, the main factor to be considered is the flow rate of the fluid, which increases upon depressurization by a factor of about 400. The interfaces used in SFE-GC are typically based on a split/splitless injector, an on-column injector or a programmable temperature vaporizer (*45-57*). In principle,

the interfacing is straightforward: the SFE restrictor is placed directly in the GC injector where the supercritical CO_2 is allowed to expand to gas. The expansion cools the injector and the analytes in the extract are trapped in the cold injector while the gaseous CO_2 is directed away, either via a split exit or through the GC column. The first approach is faster and more suitable. With pure CO_2 the interfacing is relatively easy, but the situation is more complex if modifiers are applied in the SFE. The effect of the modifier on the on-line system depends on the nature and amount of the modifier.

Examples of on-line coupled SFE-GC include determination of pesticides in honey (*12*), identification of volatile and thiolcarbamate herbicides in soil matrices (*17*), in the determination of free fatty acids and some other flavor compounds of Swiss cheese (*10*), in the analysis of aroma compounds sorbed by plastic packaging material (*11*), in the analysis of semivolatile compounds in wood and bark (*12*) and in the analysis of semivolatile compounds in plants (*44*). On-line SFE-GC allows also other interesting features of SFE, such as on-line fractionation, to be utilized in the analysis. For example, in SFE of *Thymus mastichina* L., two on-line separation vessels were used to obtain the less and the medium volatile fractions at two different temperatures and pressures (50°C, 150 bar and 25°C, 50 bar) and the extracts were collected directly to a glass liner of PTV packed with Tenax TA adsorbent to collect the volatile fraction (*18*). The first and second fractions were recovered by solvent elution, while the third fraction, which was retained in the glass liner, was introduced into a GC using thermal desorbtion. The on-line coupled SFE-GC is an especially useful for analyzing volatile compounds, and the trapping can be easily enhanced by using cryogenic focusing for retaining volatile constituents (*19*).

Also supercritical fluid chromatograph (SFC) has been combined with SFE (*13,42*). For example, SFE-SFC has been developed for the characterization of compounds in desert botanical species (*Dalea spinosa*) (13). A slightly more complex combination utilizing SFE-SFC-GC has been developed for the determination of volatile compounds in raw and baked Baltic herring (*Clupea harengus membras*) (*14*). In this study, the analytes were extracted with supercritical CO_2 (45°C, 10 MPa) and the volatiles and coeluted lipids were separated on-line using SFC, and the volatile fraction was introduced directly into a GC.

In SFE-LC, the most common interface is based on solid-phase trapping (*2,15,44,58-63,66-68,70-80*), although a few other types of interfaces such as impactor interface (*69*) open-tubular trapping (*64*) and the sample loop interface (*65*) have been developed as well. Direct trapping into a conventional packed LC column is not possible because of the high back pressure that analytical columns create. Because of the back pressure, the fluid cannot be efficiently decompressed and thus it will retain (partially) its solvation properties and efficient trapping will not be achieved, especially if modifiers are used in the

fluid. However, monolithic columns, which have a very low back-pressure, can be used for direct trapping of the extract (*15*). Both RPLC and NPLC have been used with SFE.

Solid-phase trapping is done using a multiport valve at the interface. During dynamic extraction, the analytes are trapped into the solid-phase, which is typically composed by C_{18}-modified silica or porous graphitic carbon (*60,74,79*). The analytes can be eluted from the trap directly to the analytical column, or a separate system can be used to remove gas from the trap before the transfer. The first approach may cause problems, because most liquid mobile phases are not miscible with gaseous CO_2, which will be present in the SP-trap after the trapping. For a conventional LC system, the mobile phase should be gas-free for optimal pump and detector performance. The depressurized CO_2 can be removed from the trap before elution by flushing the column with a suitable solvent of weak eluent strength before the transfer (*74,75*), or by using an extra, pressurized outlet (*79*). Also pressurization of the outlet of the LC detector has been tested (*60*).

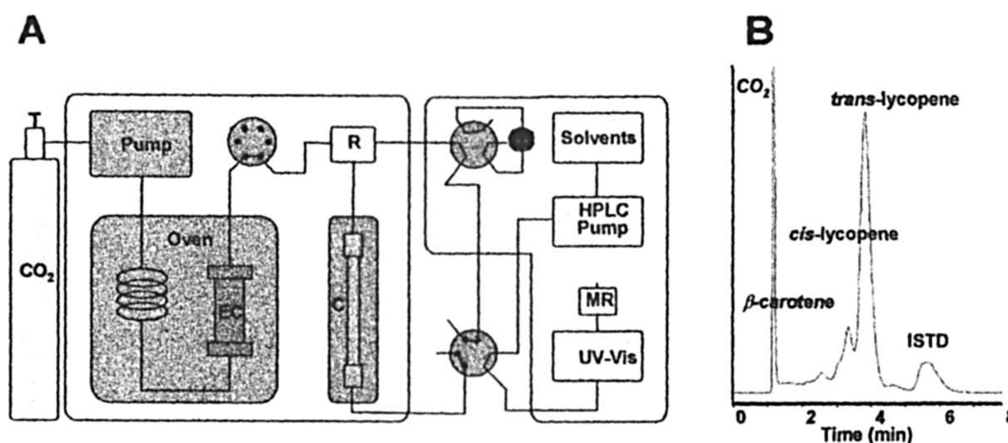

Figure 2. *(A) SFE-LC-UV system utilizing a monolithic column both for trapping and analysis and (B) determination of lycopene in tomatoes with the apparatus.*

When a monolithic column is used for SFE-LC (Figure 2 A), the coupling is much more simple (*15*). Monolithic columns have much lower low back-pressure than packed LC columns, and CO_2 is efficiently depressurized in them. In addition, monolithic columns can tolerate complete drying of the packing material without problems with the performance. In the on-line system, the extract is directly trapped in the beginning of the column, and after the extraction is completed, the LC analysis can be started.

SFE-LC applications used in the food and agricultural analyses include, for example, determination of lycopene in various fruits and foods and

determination of pesticides in food products (*15, 58-80*). An on-line SFE-LC system based on a monolithic column has been developed to determine lycopene in various food matrices, such as fruits and tomato products (*15*). Lycopene, which is an antioxidative compound, is challenging to determine with conventional methods, because of its reactivity towards atmospheric conditions. In the on-line system, lycopene could be determined reliably, as the extraction and analysis were carried out on-line in a closed system. Lycopene, which is an unonpolar analyte, was trapped efficiently in the beginning of the monolithic column during the extraction cycle, and the LC analysis started immediately after the extraction was completed. There was no signs of deteoration of the performance of the monolithic column. The whole analytical procedure took less than 25 minutes (Figure 2B).

Dynamic Head-Space Extraction and Thermal Desorption GC

If the analytes of interest are volatile or semivolatile, solvent extraction is not always necessary, and head-space techniques (HS) can be applied for the analysis, typically utilizing GC as the final analytical step. HS analysis can be defined as a vapor-phase extraction, involving first the partitioning of analytes between a non-volatile liquid or solid phase and the vapor phase above the liquid or solid. The vapor phase is then transferred further and either analysed as vapor or (ad)sorbed to an (ad)sorbent. The head-space techniques have been widely utilized in the analysis of volatiles, such as fragrances and aroma compounds, in various food and agricultural samples (*81-84*). The dynamic head-space (DHS), or purge-and-trap technique, is easily coupled on-line with GC. In an on-line system, desorption of trapped analytes for subsequent analysis is usually performed using on-line automated thermal desorption (ATD) devices.

DHS involves the passing of carrier gas through a liquid or a solid sample, followed by trapping of the volatile analytes on a sorbent or a cold-trap and desorption onto a GC. The sampling may be conducted at ambient or slightly elevated temperatures, but below the degradation temperature region for the material. Because the process requires several minutes to complete, the purged analytes need to be focused by cryofocusing on a column or trapping onto a solid phase adsorbent bed placed in-line with the GC. The trapped compounds are then subsequently desorbed to a GC column. In modern DHS instrumentation the two-stage thermal desorption process consists of tube desorption onto a Peltier-cooled trap, followed by trap desorption of the heated trap. It is essential that the thermal desorption is very rapid in order to get sharp initial bands to the GC.

Dynamic thermal desorption (DTD), which closely resembles the DHS technique, is also commonly used in combination with GC in the analysis of (semi)volatile compounds in both solid and liquid matrices (*85-88*). In DTD,

heat and a flow of inert gas are used to extract (semi)volatile organics retained in a sample matrix or on a sorbent bed. Similar adsorbent traps as in DHS are also used in DTD, and accordingly most samples require cryofocusing. It is also possible to perform the thermal desorption directly in the injector of a GC. In such a system, the solid sample is loaded into the cold injector, the carrier gas flow is temporarily halted, then the injector is rapidly heated to the desired temperature, the carrier gas is resumed and the thermally extracted components are swept onto the column. It should be noted that the DTD technique is appropriate only if the desired extraction takes place at a temperature below the decomposition point of other materials in the sample matrix, and the relatively small sample size that can be measured in a thermal desorption tube is representative of the sample as a whole.

DHS applications have been developed, for example, for the determination of aroma-active compounds in bamboo shoots (*83*), styrene in yoghurt (*84*) and volatile acids in tobacco, tea, and coffee (*88*), volatile compounds of strawberries (*89*) and odor-active compounds of hams (*90*). The applications of DTD-GC include, for example, in the determination of volatile components of Lavandula luisieri (*85*), in the analysis of volatile components of oak wood (*87*) and volatiles in various solid-food products such as spices and herbs (black pepper, oregano, basil, garlic), coffee, roasted peanuts, candy and mushrooms (*82*).

An example of a DHS application is the determination of aroma-active compounds in bambuu shoots. In this study, compounds such as *p*-cresol, methional, 2-heptanol, acetic acid, (*E*,*Z*)-2,6-nonadienal, linalool, phenyl acetaldehyde, were extracted from the bambuu shoot samples and analysed by GC. The required sample amount was 10 g, and the extraction temperature was 60°C, using a 30 min extraction time. The stripped analytes were first trapped into a cooled adsorbent tube (VOCARB 3000, at 0 °C), and then thermally desorbed to GC. In DTD, the sample amount required for the analysis is typically smaller than in solid head-space (SHS). In the determination volatile components such as camphor, 1,8-cineole and 2,3,5,5-tetramethyl-4-methylene-2-cyclopenten-1-one, from *Lavandula luisieri*, only 10-20 mg of (dry) plant sample was required for the analysis. The volatiles were desorbed from the sample under a helium flow and then cryofocused on a Tenax TA trap at −30 °C. The trap was then quickly heated and the desorbed volatiles were transferred directly to the GC column through a heated fused-silica line (*85*).

Conclusions

The number of on-line methods using solvent/fluid extraction developed for the analysis of solid food and agricultural samples is still small. The solventless

extraction methods (DHS and DTD) are, on the other hand, relatively widely utilized in the analysis of volatile analytes. However, their applicability is limited to relatively volatile, thermally stable analytes.

Most on-line methods utilizing solvent or fluid-based extraction methods provide quantitative recoveries for the analytes and samples investigated. It is impossible to state categorically, which method is best suited for a certain type of sample, as several factors should be taken into consideration in the comparison. Of the different extraction methods used for on-line combinations, SFE is the most selective method and the selectivity can be adjusted by changing the pressure and temperature of the fluid. It is best suited for relatively nonpolar or volatile analytes, and it can be easily coupled on-line with LC, GC and SFC. SFE generally produces clean extracts with so little residual organic solvent modifier that additional concentration may not be necessary before chromatographic analysis. On the other hand, SFE requires modifiers for efficient extraction of more polar analytes, which complicates the on-line coupling. In addition, since methods using SFE are heavily matrix-dependent, separate method development is required, which requires experienced operators.

For polar and medium polar compounds, PHWE, DMAE and DSAE suit better than SFE. On-line coupling of these techniques is possible with both LC and GC. PHWE is easily combined with (RP)LC, while DMAE and SAE can be easily coupled with RPLC if water is used in the extraction. With the use of organic solvents, coupling with GC is often more simple. The drawback of PHWE is the rather harsh conditions during extraction, which can lead to degradation of thermally labile analytes. DMAE and DSAE are less selective than SFE and PHWE and they suit to a large range of analytes. Of these techniques, DSAE seems to be faster and as the choice of solvent is less critical than in DMAE, it is more flexible in on-line coupling. On the other hand, DMAE offers a particularly intriguing feature for analysis of labile food flavor compounds, such as the extraction of plant or animal tissue in a microwave-transparent solvent.

SFE-GC system is the most widely applied on-line technique for the analysis of solid samples and it is well suited also for routine based applications. Also SFE-LC, PHWE-LC and DSAE-GC systems are relatively easily accomplished in an instrumental point of view even though more work will be required before these techniques are suitable for routine analyses. More complex systems, such as SFE-LC-GC, can be applied for research in special, very demanding applications, and due to their complexity, this type of systems are not suitable for routine laboratories.

The main benefit of the on-line coupling of an extraction to a chromatographic technique is that the whole analysis can be performed in a closed system. The main advantages are the improved sensitivity, minimal sample contamination and the possibility for a totally automated analytical

system. The main benefit of the on-line analysis of solid samples is that due to the high sensitivity, the amount of sample required is small, and the methods are consequently well suited for trace level determinations and to applications where the amount of sample available is small.

Unfortunately, the great potential of these on-line techniques is not reflected in present-day practice. One explanation for this may be that new techniques are not readily accepted, especially as the potential economic advantages of a new technique have to be balanced against the considerable costs of validation and standardization. The limited commercial availability of instruments also plays a role. Moreover, on-line coupling of an extraction system to a chromatographic instrument requires generally some adaptation and optimization as well as special knowledge of the underlying principles. The time invested in optimization of the conditions is rapidly repaid in more efficient sample throughput, better reproducibility and improved sensitivity.

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Chapter 9

Fast and Selective Analytical Procedures for Determination of Persistent Organic Pollutants in Food and Feed Using Recent Extraction Techniques

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This chapter describes the utilization of recent extraction techniques such as supercritical fluid extraction, pressurized liquid extraction and microwave-assisted extraction for the determination of persistent organic pollutants in food and feed samples. These techniques have been developed because the extraction step can severely limit the entire analytical procedure. Several food crises, for example dioxins entering the food chain, have stressed the requirement for high sample throughput. Increased sample throughput can be obtained by using automated clean-up systems following the extraction process; however, these techniques are often very costly. This text aims at describing how a substantial decrease in the time spent on sample handling can be achieved with for example integrated clean-up strategies.

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Introduction

Conventional extraction techniques for food and feed samples include liquid-solid extraction (shaking), Soxhlet extraction and sonication procedures. Shake-flask extraction procedures are simple and still in use today. Franz von Soxhlet introduced Soxhlet extraction 1879 and ultrasounds were started to be utilized in extraction procedures in the 1960's. Today there is an increasing demand for more efficient and reliable procedures leading to faster and simpler analyses with less labour demand. Therefore more sophisticated, automated, computerized analytical instruments have beed developed, which has minimized the time spent on the final analysis. However, the sample preparation step is still often based on old techniques, therefore making this step the most tedious and time-consuming of the analytical procedure. The demand for high sample throughput of the increasing number of substances that need to be analyzed, and concerns using hazardous solvents in the laboratories as well as new restrictions on solvent use, and costs of solvent waste disposal have pushed the development of modern extraction techniques forward. Recent extraction techniques named in this report include; supercritical fluid extraction (SFE), pressurized liquid extraction (PLE, also called accelerated solvent extraction, ASE™), and microwave-assisted extraction (MAE).

An important question to address is what demands we should have on alternative techniques. Below some desired requirements that would facilitate the extraction process have been listed.

- Shorter extraction times *(minutes)*
- Low consumption of organic solvents *(10-100 ml/sample)*
- Built-in clean-up step *(direct analysis of extracts)*
- Automated systems *(high sample throughput)*

Operating at elevated pressures provides a key path to shorter extraction times, since this allows the extraction to take place at increased temperatures above the normal boiling point of the solvent. As a result, extractions are frequently completed within 10-60 minutes instead of several hours or even days with conventional techniques performed at ambient pressure. The reason for this is that at elevated temperatures faster kinetics are achieved with better desorption of analytes from the matrix, higher mass transfer of the analytes from the core of the matrix to the surrounding solvent due to higher diffusion rates, and higher distribution constants due to enhanced solubility of the analytes in the extracting fluid. New techniques therefore require high pressure pumps and high pressure cells manufactured from thermostable materials to permit extractions at elevated temperatures and pressures.

This chapter mainly concerns solid food and feed samples. Today there are numerous papers published on the utilization of recent extraction techniques for the determination of persistent organic pollutants (POPs) in such matrices. However, there are still relatively few methods available for in-line or integrated clean-up in the extraction cell. In most cases, these integrated clean-up procedures aim at minimizing co-extraction of fat since these severely interfere with the chromatographic process. Depending on the concentration of the analyte, some extracts can be directly analyzed after integrated clean-up procedures, but obviously for more low-level analytes such as dioxins and furans these methods do not always provide a sufficient clean-up. Nonetheless, these extracts frequently require only minimal additional clean-up thereby significantly reducing the workload prior to final analysis. Often this can be achieved by simple means, while highly sophisticated external automated clean-up systems can be very complex and therefore costly.

Important aspects of the extraction step for POP analysis in food and other biological matrices have been reviewed recently (*1, 2*), but this discussion is further extended here because it is still of great importance to elucidate sources of food contaminants such as dioxins, furans and PCBs in food (*3*). This type of method development requires substantial time and investment, however, any technical improvement will ultimately lead to cost reduction in food monitoring laboratories.

Supercritical fluid extraction

Analytical scale SFE was introduced by German scientists at the end of the 1970's for the extraction of various compounds such as drugs, vitamins, and dyes using carbon dioxide as extraction media (*4-6*). A major increase in analytical scale SFE applications was however first seen in the late 1980's, when POPs were extracted from environmental samples (*7*), fats from food (*8*), and various components from polymers (*9*). Today SFE is a well-established extraction technique, adopted as a reference method by the U.S. EPA (*10-12*), and the theory and instrumentation is described in several review papers (*13-17*) and books (*18-21*).

In earlier years, integrated clean-up was given some attention, but the interest in SFE for POP applications has since decreased. This is not necessarily a consequence of inability of SFE to perform well but rather that many research groups have switched their attention to new fields of research and that alternative competing techniques have entered the market. People new to the field of SFE might therefore find useful information in historical research activities.

The concept of introducing fat retainer inside the extraction cell is nicely demonstrated in a work by Hopper who evaluated a C_1-silica based column for

in-line removal of fat from food extracts containing more than 60 pesticides (*25*). This procedure could extract and clean-up pesticides from samples containing 0.65 g animal fat or 1 g of vegetable oil, with lipid co-elution of 2.5 mg and 0.8 mg, respectively. The results were both reproducible and comparable to conventional tedious methodologies. Apart from C_1-silica based columns a number of other fat retainers have been studied as discussed below (*26-39*).

Alumina as fat retainer for chlorinated organic contaminants

Integrated fat removal from biological matrices using a fat adsorbing material inside the extraction cell was early investigated by Johansen et al. for the determination of PCBs in fish, crab, human milk and blood (*22-24*). These studies exploited basic alumina enabling on-line coupling of the SFE instrument to gas chromatography (GC). Lee et al. investigated the influence of the amount of basic alumina present in the extraction cell when extracting lipid rich fish (*26*). They found a strong correlation between the amount alumina and the fat retaining capability during the extraction as seen in Figure 1.

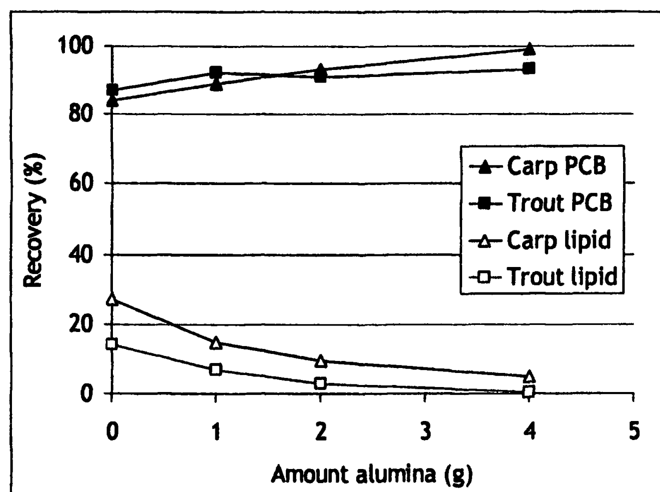

Figure 1. *Relation between lipid recovery, PCB recovery and the amount basic alumina added to the extraction cell when performing selective PCB extraction from fish. Data from ref (26).*

The extraction conditions were optimized to minimize fat co-extraction in the absence of fat retainer, but still strong enough to completely recover all PCBs from the sample. This lead to relatively harsh SFE conditions (100°C and 5000 psi) which still eluted less fat than Soxhlet. The most important finding however was to demonstrate that large amounts of fat retainer are needed to reduce the fat to acceptable levels. For the 4 g alumina data in Figure 1, a fat-to-fat retainer (FFR) value can be calculated as the amount fat added to the

extraction cell from the matrix, divided by the amount fat retainer in the cell. For trout a total of 0.27 g fat was added to 4 g alumina, while the corresponding value for carp was 0.38 g fat. This leads to FFR values of 0.0675 and 0.0950 for trout and carp, respectively. It is then revealed that for trout a FFR value of 0.0675 decreases the amount co-extracted fat to 6 mg, while for carp a FFR value of 0.0950 only coelutes 2 mg of fat. Despite these minute levels of fat, external clean-up had to be perform to make the chromatographic analysis work properly.

Later van Bavel and co-workers performed fat free extractions of a number of chlorinated organic contaminants such as dioxins from human adipose tissue by incorporating basic alumina in the extraction cell (*27, 28*). They used FFR values between 0.06 and 0.2 and did not experience problems with fat co-elution. Since POP concentrations sometimes are compared on a fat weight basis, Björklund et al. investigated the possibilities of performing a simultaneous determination of both PCBs and fat in a single run (*29*). This was accomplished by first extracting the PCBs with pure carbon dioxide, followed by elution of the fat fraction from the basic alumina using methanol-modified carbon dioxide, leading to 100% recovery of added triglycerides.

Neutral alumina has also been used for the extraction of organochlorine pesticides (OCPs) from chicken fat (*30*), OCPs and PCBs from human adipose tissue (*31*), and PCBs from fish (*32*). In only one of these studies was it possible to estimate FFR values (*32*), which were somewhere between 0.040 and 0.066. From this study, it is clear that neutral alumina also works properly for integrated clean-up procedures in SFE.

Comparison of fat retainers for chlorinated organic contaminants

Even though alumina is efficiently used as a fat retainer, there are other options. Silica was successfully utilized in the extraction of OCPs from chicken fat and lard (*30*). Later Alley and Lu investigated six different fat retainers and found that Florisil adequately separated spiked PCBs from bulk lipids in chicken egg and fish (*33*). More recently, Järemo et al. (*34*) did a systematic and comprehensive evaluation of basic alumina and Florisil as fat retainers. By sequential extractions, they could obtain extraction profiles, which revealed the length of the fat-free time window for the two fat retainers when using different FFRs. In order to obtain a fat-free time window of 60 min, during which other analytes could be extracted, Florisil required a FFR of 0.16, while corresponding value for basic alumina was 0.11. This fat-free time window was not dramatically changed by activating the retainers at 300°C for up to 58h, however deactivation of the retainer with 10% water had detrimental effects on the fat retaining capability of both retainers.

C_{18} and silica as fat retainers for polycyclic aromatic hydrocarbons

Ali and Cole (*35-37*) have performed lipid clean-up for the selective extraction of PAHs from various biological tissues in three different studies. In their first work, they demonstrated that by mixing fat with a C_{18} adsorbent the levels of fat in the extracts decreased with 83-91% (*35*). In the final method, 0.5 g of spiked pureed crab tissue was blended with 2g of C_{18} beads and the obtained extracts could be directly analyzed on GC/MS showing recoveries between 95 and 100% for ten investigated PAHs. An identical methodology was applied in two consecutive papers on smoked meat (*36*) and smoked fish (*37*). In no case was the fat completely removed, as was the case in most studies on chlorinated organic contaminants discussed above. In the three studies (*35-37*), between 5-20% of the fat was co-extracted, but even so the obtained extracts could be directly analyzed on GC-MS with quantitative recoveries. However, no attempts were made to optimize the fat retention using the C_{18} method, and no information was given on the possible decreased column lifetime when injecting the co-extracted fat on the chromatographic column.

Silica has also been used when extracting PAHs from birds of prey. Liver samples (0.5g) were mixed with 2 g of silica gel, and 1 g additional silica was added prior to extraction (*38*). This silica gel was partially deactivated with 15% water. The integrated clean-up procedure eliminated all other external purification steps, and in a recent publication, a modified version of this methodology was presented for the determination of PAHs in vegetable oil (*39*).

Pressurized liquid extraction

PLE is one of the most recently developed extraction techniques for solid samples. The first articles on PLE appeared in 1995 (*40-42*) and the year after that several applications for POPs in soil, sediment and fish (*43-46*) were published. PLE has today been thoroughly reviewed, with a focus on environmental samples (*47-49*).

Conventional PLE of pollutants in food and feed samples

PLE is used in many food and feed applications for the analysis of a great variety of POPs. One reason for this is the acceptance of this technique by the US EPA in Method 3545 (*50*). A classic PLE approach involves extraction of the contaminants together with the lipid fraction, which is removed by external clean-up. Already in 1997, Schantz and co-workers (*51*) evaluated this technique for the extraction of OCPs and PCBs in certified reference materials (CRMs)

(SRM 2974, mussle tissue) and (CARP I, II, carp tissue). It was shown that PLE is comparable to, or better than Soxhlet, as long as sufficient sodium sulphate is added to water-rich matrices. In later studies non-ortho PCBs were determined in fat-containing CRMs using PLE with methylene chloride (*52*). This was followed by a publication where fish tissue (SRM 1946) was thoroughly investigated for its PCB and pesticide content using both PLE with methylene chloride and Soxhlet (*53*). Methylene chloride mixed with acetonitrile (9:1, v/v) followed by fat removal with 9M sulphuric acid has also been used for the extraction of PAHs from commercial smoked meat (*54*).

Since there are a number of options in the development of new extraction methods, optimization of the extraction can be done with experimental designs. Chen and colleagues (*55*) extracted OCPs from animal feed and concluded the optimal conditions to be 100°C, with hexane-acetone (3:2, v/v) for 9 minutes using 2 cycles. However this is very close to the EPA method of 100°C, with hexane-acetone (1:1, v/v) for 2 minutes using 2 cycles, and from their own data set, this methodology would only give a couple of percent lower recoveries. When applying their optimized conditions to a CRM (BCR 115, animal feed), the recoveries were satisfactory as seen in Figure 2.

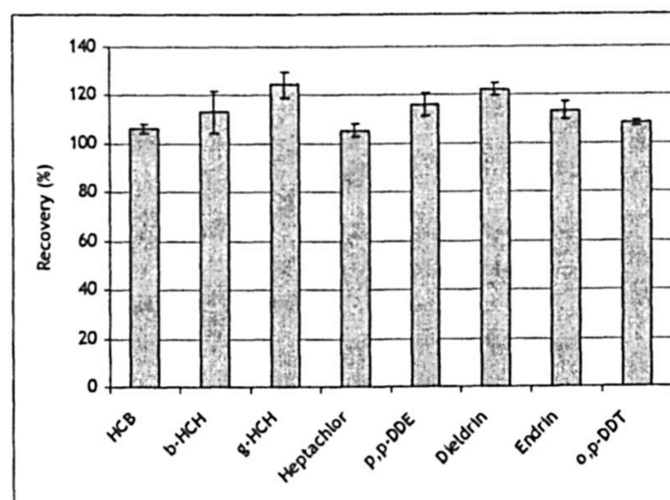

Figure 2. *Recovery of OCPs from animal feed (BCR 115). Data from ref. (55).*

In fact, the recoveries were consequently higher with PLE, which often is observed for this technique and is attributed to the higher extraction efficiency at elevated temperatures.

Conventional PLE combined with automated clean-up procedures

More recently, researches have been utilizing PLE in combination with automated clean-up techniques. Suchan and co-workers (*56*) extracted indicator-

PCBs and OCPs from fish samples and carefully optimized a PLE procedure. The extraction did not differ from conventional Soxhlet extraction. Clean-up of extracts was performed on an automated GPC system packed with Bio-Beads S-X3. Likewise, Saito and colleagues (*57*) developed a new analytical method suitable for determination of 59 different POPs, including PBDEs, PCNs, PCBs, and OCPs, in biological organ tissues. The obtained extracts were cleaned on Bio-Beads S-X3, and fractionated into two groups on SPE with silica gel. An improved clean-up strategy for a simultaneous analysis of PCDD/F, coplanar PCBs, mono-ortho PCBs and indicator PCBs in pork, beef, poultry and horse meat was also developed by Pirard and co-workers utilizing PLE combined with GPC on Bio-Beads S-X3 followed by fractionation on a Power-Prep automated clean-up system (*58*).

Apart from GPC with Bio-Beads, there are other automated clean-up approaches. Although GPC has several advantages, Focant and co-workers claimed that for the high-fat-content samples (4 g lipids) required in dioxin analysis, GPC suffered from several practical limitations and therefore could not be performed 100% automatically (*59*). Consequently, new high-capacity disposable silica (HCDS) columns were developed for the Power-Prep system. The combination of PLE and Power-Prep clean-up allowed 10 dioxin samples to be analyzed in 48 hrs.

PLE with integrated clean-up procedures

Draisci and co-workers performed interesting research for the extraction of relatively polar analytes such as musk compounds in Italian fresh water fish (*60*) and corticosteroids in bovine liver (*61*). For musk components, alumina was utilized in the extraction cell to hinder co-extraction of lipids. The FFR values for these investigations were in the range 0.002 and 0.05, and in no case did they report problems with the presence of fat during the chromatographic analysis. For corticosteroids, a different concept was used, where fat was selectively extracted with pure hexane, followed by elution of the analytes in a second step.

PCBs were first selectively extracted from fish using acidic alumina in the extraction cell, and the extracts could be directly analyzed on GC-ECD (*62*). Later Björklund and colleagues comprehensively investigated acidic, neutral and basic alumina as well as Florisil and sulphuric acid impregnated silica gel for the selective extraction of PCBs from fat containing matrices using hexane at 100°C as extraction solvent (*63*). In that study, sulphuric acid gave the cleanest extracts possible. Later, sulphuric acid impregnated silica was evaluated in more detail, and it was found that a FFR value of 0.025 was necessary in order to obtain completely fat free extracts for a number of matrices including feed for poultry, cod liver oil (CRM 349), and milk powder (CRM 450) (*64*). This resulted in less

than 1 mg co-extracted lipids. Recently an extended evaluation of sulphuric acid impregnated silica was conducted in order to reveal possible effects of the cell dimensions in PLE (*65*). This was due to the fact that most applications have been performed on a Dionex ASE 200 with 33 ml cells. However, another system is today available called ASE 300, with 34 ml cells, which are wider and flatter. The cross sectional area of the 33 ml cells is only 46% of the corresponding area in the 34 ml cells. However, it was demonstrated that the 34 ml cells perform similarly to 33 ml cells. The final methodology was performed with heptane at 100°C using 2 cycles at 5min with a FFR value of 0.025 and quantitative recoveries were obtained for vegetable feedstuff, feed for poultry, mackerel oil (BCR 350) and pork fat (IRMM 446). Furthermore, in this paper, the packing procedure was simplified and the neurotoxic hexane was exchanged with heptane. This methodology was recently tested in an international validation of bio- and chemical-analytical screening methods for dioxins and dioxin-like PCBs within the European DIFFERENCE project (*66*). It was shown that PLE is a valid alternative extraction and clean-up procedure for fish oil and vegetable oil since the results obtained with GC-HRMS and CALUX after PLE are equivalent to the results obtained with conventional extraction and clean-up procedures.

Florisil has also been used as fat retainer for the determination of PCBs in dried spoonbill eggs with a lipid content of 42% (*67*). They optimized their methodology with 15% dichloromethane in pentane at 175°C, but this caused severe fat breakthrough. Therefore, the temperature was lowered to 40°C in order to reduce fat extraction. Under the final optimized conditions, different fat retainers were tested, and the amount co-extracted fat are seen in Figure 3. Even though all fat retainers gave quantitative PCB recoveries, only Florisil delivered extracts that could be analyzed without further purification.

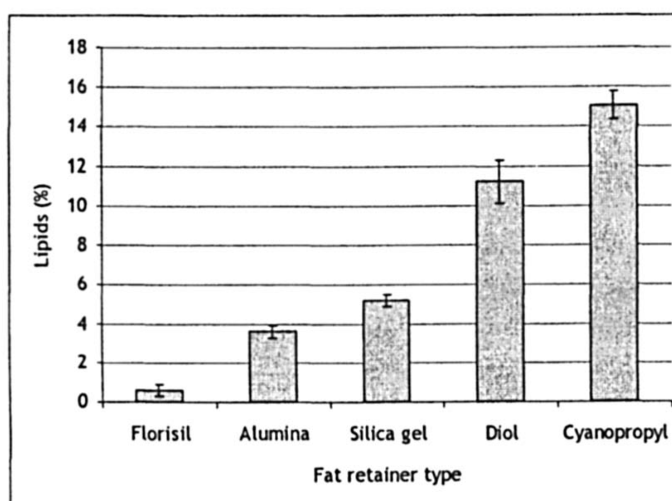

Figure 3. *Co-extraction of fat from dried spoonbill eggs with a lipid content of 42%, using different fat retainers in PLE. Data from ref (67).*

Microwave-assisted extraction

The fundamentals and practical use of MAE have been described in detail in several review articles (*68-70*) and books (*18, 71, 72*). The following text focuses on closed-vessel (pressurized) MAE, which permits extractions at elevated temperatures. A major difference of MAE compared to SFE and PLE, in addition to its unique heating performance, is that the commercially available MAE systems today operate in batch mode. The possibility of built-in clean-up is therefore difficult to perform and related to the design of the instrumentation. Both automated SFE and PLE are most commonly used in a dynamic or semi-dynamic mode, which simplifies the development of combined extraction/clean-up strategies.

Even though the interest in microwave-assisted extraction (MAE) has increased during the last 10 years, this technique has not been utilized much in food and feed applications. Only a few papers can be found with the combination of POPs and food/feed samples. This may be because MAE applications frequently require laborious and tedious clean-up of the extracts before final analysis. In some cases, only a simple filtration or centrifugation may be sufficient to separate the solid matrix from the extract but since MAE most often is more exhaustive than selective, extensive clean-up procedures based on for example solid-phase extraction is commonly needed for removal of interfering compounds (*73-75*). Other techniques that have been used for clean-up of MAE extracts are gel permeation chromatography (*76*), solid-phase micro extraction (*77, 78*), and liquid-liquid extraction (*79*).

Solvent type and volume

When selecting a solvent, considerations should be given to the microwave-absorbing properties of the solvent, the interaction of the solvent with the matrix, and the analyte solubility in the solvent. An important aspect is the compatibility of the extraction solvent with the analytical method used for the final analysis. A common mixtures is hexane/acetone (1:1), which has been used in environmental applications for the extraction of EPA listed semivolatile organics, PAHs, phenols, pesticides and phthalate esters (*80*), and was also the extracting solvent used recently by Barriada-Pereira and co-workers for the extraction of OCPs from plants (*81*). They extracted 21 OCPs from spiked grass samples and MAE recoveries were comparable to those obtained with Soxhlet. Gfrerer et al. optimized an extraction procedure for OCPs in spiked animal feed using acetone-hexane (1:1) and tested it on a CRM animal feed (BCR-115). The obtained recoveries were in the range of 96 to 116% with a precision of 0.4 to 3.8% (*82*).

The solvent volume needed for a single sample is often in the range of 10–30 ml. In some cases, solvent volume may be an important parameter for an efficient extraction. The solvent volume must be sufficient to ensure that the entire sample is immersed, especially for matrices that swell during the extraction process. Generally, a larger volume of solvent increases the recovery, but in some cases, a larger solvent volume may give lower recoveries (*83*). Carro et al. used a factorial design to develop an extraction method for OCPs in freeze-dried oyster samples (*84*). In this case solvent volume was shown to be important, with an optimum of 35 mL solvent / 1 g sample.

Temperature and time

The most investigated parameter in MAE is the extraction temperature, which is an important factor for achieving quantitative recoveries. For closed-vessel MAE, the temperature may reach well above the boiling point of the solvent. Extraction times in MAE are generally not exceeding 30 minutes. In many cases, 10 minutes is sufficient to obtain quantitative recoveries for extraction of organic pollutants (*80, 85*), but even 30 seconds has been demonstrated to give full recovery of pesticides from spiked fresh and cooked vegetables (*86*). A longer extraction time does not always lead to higher recoveries as often is the case using conventional techniques. During the extraction of pesticides from several crop matrices, it was demonstrated that increasing the extraction time did not affect the recovery (*79*). A method based on the combination of MAE and SPME was compared with a method based on sonication for the extraction of OCPs from Chinese teas. Tea leaves were mixed with water and irradiated. It was found that extraction time was not a critical parameter. Recoveries were in the range of 40 to 95% (*87*). Wittman and co-workers extracted spiked trichlorobenzenes from fish for only 15 min and compared it to a standard method based on LLE. The method gave higher values than those obtained with the standard method (*88*).

Effects of matrix characteristics

In many cases, matrix moisture improves extraction recoveries. The effect of this parameter is of course dependant on the extraction solvent used. Apart from increasing the polarity of the extraction solvent, added water (or that naturally occurring in a sample) affects the microwave absorbing ability of a sample and hence contributes to the heating process. Water may also swell the matrix and/or influence the analyte-matrix interactions, making the analytes more available to the extracting solvent. A comprehensive study was recently published by Bayen

et al. for the extraction of POPs in marine biological tissues (*89*). Spiked tissues were used for determining recoveries. Lipid content as well as tissue moisture content was determined for each sample type and the samples were not dried before extraction. Average analyte recoveries for the spiked samples were 89-97%. The method was used to quantify PBDEs in mussel tissue (SRM2978) and cod liver oil (SRM 1588a) and compared to results obtained with Soxhlet. As average recoveries for both OCPs and PCBs were in excess of 80%, analyte recoveries from both methods can be considered as acceptable and comparable. When extracting organic pollutants and pesticides, applying the same MAE method on different types of matrices such as soils, sediments and crops, the obtained recoveries may differ largely (*73, 79, 80*). This implies that the applicability of a MAE method to new types of samples is not straightforward, and that recoveries always need to be checked. This has also been observed in SFE, which is often claimed as being matrix-dependent but, obviously, the same holds true for MAE.

Dynamic MAE applications of non-food samples

In specific cases, MAE has been performed in a dynamic mode, with a subsequent on-line clean-up on a solid-phase extraction cartridge, for the extraction of PAHs from sediments and air samples using home-built systems (*90-92*). After completed extraction, the trap was dried with nitrogen and finally the analytes were eluted from the cartridge with an organic solvent before gas chromatographic analysis. A similar approach, developed as a screening system, was recently published by Criado and co-workers for extraction of PAHs in soils (*93*). Cresswell and Haswell (*94*) presented another set-up for dynamic MAE, also for the extraction of polyaromatic hydrocarbons, where the sediment sample was slurried in water or acetone before being introduced into a microwave field prior to filtration. The extracted components were trapped on an octadecylsilica cartridge and subsequently eluted and transferred directly onto a liquid chromatographic column. These kinds of systems have unfortunately not yet been tested in food or feed applications, although the determination of the pesticide dichlorvos from vegetables has lately been performed by on-line MAE coupled to headspace solid-phase micro extraction (*95*).

Comparative studies involving recent extraction techniques

Numerous publications have proven that recent techniques are viable alternatives and as efficient in terms of recoveries as conventional techniques but with additional benefits such as shorter extraction time, reduced solvent

consumption and increased sample throughput. Even though the investment in equipment for SFE, MAE, and PLE is high for a routine laboratory, the above benefits provide a cost-effective alternative to conventional methods in the long term. Several review papers comparing the different extraction techniques have been published lately (*70, 96-102*).

PLE versus SFE and MAE

The performances of PLE and SFE have been compared in applications such as for the extraction of oils and phenolic compounds from plant material (*103, 104*). In some cases, SFE has been the preferred technique due to a more selective extraction, exemplified by the fractionated extraction of different groups of polyaromatic hydrocarbons (*105*). The advantage of SFE to obtain extracts that can be directly analyzed without any additional clean-up is also stressed in environmental applications (*106-108*). In other cases, PLE has been preferred due to its matrix-independent properties, for example during the extraction of dioxins with toluene from high- and low carbon fly ash (*109*). The effect of water in the samples has shown to be much more pronounced in SFE than in PLE. When extracting pesticides from fortified tomatoes (*106*) or flavanones and xanthones from bark (*104*), decreased recoveries were observed for SFE. An evaluation study by Chuang et al. showed that PLE with external clean-up and with final analysis based on enzyme-linked immunosorbent assay (ELISA) or GC/MS was the preferable method for determination of pesticides in fatty baby food samples (*110*). An SFE method with carbon dioxide or 15% acetonitrile in carbon dioxide as extraction fluid with final analysis using ELISA was also tested. The SFE method did not provide quantitative recoveries (<50%) of the spiked pesticides, due to co-extraction of fat components. The best recovery results were obtained when baby foods were extracted using PLE with acetonitrile at 80°C. Finally, adult duplicate-diet samples (33 food samples provided by EPA) were analysed for target pesticides using the PLE-GC/MS method and concentrations ranged from <0.3 to 110 ppb.

Gfrerer et al. have recently published a study on the extraction of OCPs in animal feed comparing different methods, among them PLE and MAE (*82*). Both PLE and MAE provided significantly better extraction efficiency than Soxhlet extraction. The recoveries were 127 and 115%, respectively, of the values obtained with Soxhlet. The PLE method gave overall comparable or higher recoveries compared to MAE, but the extracts obtained were darker, indicating that more matrix compounds were co-extracted. In another work by Weichbrodt et al. MAE and PLE were compared for the extraction of OCPs in fish tissue (*111*). The results were quantitative for both techniques, but because of the water contents in fish tissue, the MAE procedure required two extraction

steps. During the first step, water was removed, while in the second step OCPs could be quantitatively extracted with pure solvent. This made MAE the more labour-intensive of the techniques investigated. However, addition of a drying agent such as sodium sulphate may have led to a simpler approach.

Choice of extraction technique

Although recent extraction techniques offer reduced extraction times and lower consumption of organic solvents, thereby making them superior to the traditional techniques in those regards, they differ in terms of selectivity, simplicity and sample throughput. An efficient method should provide high recoveries and high sample throughput. The recoveries obtained using SFE, MAE and PLE are most often comparable if optimization has been performed correctly for a given analyte-matrix couple. This conclusion can be found in numerous publications, mainly in environmental applications (*97, 112, 113*).

SFE appears to be the most selective technique, particularly when neat carbon dioxide is used as the extraction solvent, as the solvent strength can be continuously tuned by varying pressure and temperature (*i.e.* density). An exception is when non-polar analytes have to be extracted from a lipid-containing sample (e.g. food samples). When extracting polar compounds the selectivity of SFE is often reduced, since the addition of a modifier is then required, leading to a more exhaustive extraction and co-extraction of unwanted matrix components. Co-extraction is however present also in MAE and PLE, but it might be better to choose any of these organic solvent based techniques because they are easier to optimise using polar solvents.

Although careful method development may lead to good extraction selectivity, additional clean-up after completed extraction is still often needed for complicated samples, especially when using MAE or PLE. For some MAE applications, only a filtration step is needed, whereas for others, solid-phase extraction or liquid-liquid extraction steps are needed to prepare for the final analysis. Also in PLE further clean-up is normally needed, but here the possibilities are better for a simultaneous clean-up inside the extraction cell resulting in final extracts directly suited for analysis as discussed above.

In particular SFE has been claimed to be highly matrix-dependent (*i.e.* the extraction conditions should be optimized for each new matrix for the same type of analytes). This is however often not true and this opinion probably emanates from a wish to use SFE conditions that are as selective as possible in order to minimize co-extraction of matrix material. Since SFE based on carbon dioxide is unique with respect to the possibility to achieve high selectivity, this is understandable. For samples where analytes have a strong adsorption to the matrix, the matrix-dependence can often be eliminated by modifier addition

and/or increased temperature, which requires more care and experience than MAE or PLE.

In MAE and particularly PLE, few parameters have to be optimized. PLE instruments commercially available at present are more automated than MAE instruments, but also more expensive. Considering economical and practical aspects, the investment of MAE is lower but this technique requires manual operations, such as loading the solvent into the cell, and filtration or centrifugation to separate the extract from the matrix. The possibility to directly inject the extracts into chromatographic systems (on-line or off-line) makes SFE methods simple with less manual steps. On the other hand, SFE requires more experience to handle and is not that straightforward to operate as MAE and PLE.

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Chapter 10

Application of Membrane-Based Extraction Techniques to Food and Agricultural Samples

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The use of membrane-based extractions are increasingly seen as offering an alternative to conventional sample preparation procedures in analysis of chemical species in both food and agricultural samples. In this article, emphasis is placed on the attractiveness of membrane based extraction techniques to a variety of food and agricultural samples such as circulating nutrient solutions, fruit juices, fruits, beverages, vegetables, food oils and others. Examples of membrane based applications demonstrating how selective these techniques are even for food and agricultural samples are given.

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Introduction

The global food production has continued to increase in response to demand since the 1970s. The increase in food production can be attributed to modern practices of farming accompanied by widespread use of pesticides such as insecticides, fungicides and other related compounds. The concerns of adverse effects from these compounds found in food and other agricultural products to humans and other organisms are therefore a real problem. Persistent pesticides such as organochlorines have been banned and replaced by more easily degradable ones such as organophosphorus and pyrethroid compounds. However, because of the constant use of these pesticides, human health concerns is still there, especially in food products and agricultural crop residues. Regulatory agencies have emphasised more and more the need for the development and use of analytical methods able to determine these various chemicals in food and agricultural products. Diverse samples such as vegetables, fruits, frozen foodstuffs, juices etc require a sample preparation step before final determination on an analytical instrument such as gas chromatography or high performance liquid chromatography. Sample preparation is needed to isolate the analytes from complex matrices, remove interfering compounds and achieve a sufficient sensitivity. Most agricultural and foodstuff samples contain fat and/or dissolved solids. Isolating the analytes from such samples is therefore the most painstaking part of the analytical scheme.

The oldest sample preparation method that has been used for food and agricultural samples is liquid-liquid extraction (LLE) *(1,2)*. LLE extraction technique is still used today in many laboratories for determination of pollutants in agricultural and food samples *(3-6)*. Among its advantages, the technique is versatile as it can easily be applied to many types of food and agricultural samples. One of its main disadvantages is lack of selectivity for such complex samples. Often a clean-up step is needed before final analysis, therefore consuming a lot of time *(1)*. The basis of extraction is the partitioning of the dissolved analytes between the organic phase (extraction liquid) and the aqueous solution (sample solution) according to their partition coefficients. For solid samples, the analytes have to be partitioned between the homogenised solid sample and extraction organic liquid *(3-7)*. The technique is now being replaced by other techniques *(8)*.

Solid phase extraction (SPE) techniques are emerging as the perhaps most popular alternatives to liquid-liquid extraction for sample preparation of food and agricultural samples. The principle of SPE is now fully described *(9,10)*. Most sorbents are now available as disks, cartridges or precolumns *(11,12)*. Among the advantages of SPE, is the easiness for the method to be automated and the possibility for simultaneous combination of extraction and enrichment of the analytes *(12)*. This reduces the whole analytical scheme compared to liquid-

liquid extraction. For solid food and agricultural samples, liquid solid extraction is combined with SPE *(3)*. The major draw back of the SPE techniques using sorbents such as bonded phases, graphitised carbon blacks and functionalised polymers is the lack of selectivity especially when extracting complex samples such as food and agricultural products. This has led to the development of selective sorbents (molecularly imprinted polymers *(13)* and immunosorbents *(14,15)* suitable for complex matrices *(16)*. Another technique related to SPE, which is now becoming widespread for food and agricultural samples is solid phase microextraction (SPME) *(17,18)*. This technique is easily connected to gas chromatography in an automated way and uses little or no organic solvent. It has recently been reviewed for food, water and biological applications *(19)*. However, despite its simplicity, it lacks selectivity when extracting analytes in complex matrices like plant extracts and foodstuffs. In addition, the technique is not very sensitive and often it gives non-quantitative extraction. Related to SPME is the stir bar sorptive extraction (SBSE) technique *(20)*. Although SPME and SBSE techniques have different set-up and mechanisms, they are both solventless sample preparation techniques and use similar sorbents. SBSE has larger quantity of sorbent compared to SPME giving in general higher recovery and sensitivity *(20)*. Supercritical fluid extraction *(21,22)* and matrix solid phase dispersion *(23)* are other techniques but these are commonly used to extract solid food and agricultural samples.

Other sample preparation techniques currently emerging as alternatives in food and agricultural samples are based on membrane *(24-29)*. Membrane-based extraction techniques have general advantages in that they are versatile, selective, can easily be automated on-line to analytical system and simultaneously combine extraction and high enrichment in one step. Membrane techniques generally use small volume of organic solvents with few microlitres. Since most of them are miniaturised accompanied with high enrichment factors, membrane techniques also use small sample volumes. They are therefore suitable for application to food and agricultural samples although most previous efforts have been directed to applications for environmental and biological samples, as seen in the reviews *(30,31)* and book chapters *(32,33)*. One the disdavantages of membrane techniques is memory effects especially when extracting hydrophobic organic compounds.

Membrane Based Sample Preparation Techniques

The main membrane techniques that have been used for analytical applications can be classified based on whether the membrane is porous or nonporous *(34)*. A clear difference between these two is that selectivity for porous membrane processes is mainly based on pore size and pore size

distribution. A nonporous membrane can be either a porous membrane impregnated with a liquid or entirely a solid, such as silicone rubber. In both these cases, the chemistry of the membrane material can influence the selectivity and the flux of the process *(35)*. This review will focus on the membrane extraction techniques summarized in table 1. The general membrane process that drives the separation is the result of differences in the transport rates of the chemical species through the membrane (Figure 1). In most analytical applications of membrane-based extractions, the analytes are transported across the membrane under the influence of a concentration gradient (Figure 1). This is referred to as passive diffusion *(30,36)*. Various conditions can easily be fine-tuned in the different compartments of a membrane system depending on the type of membrane extraction technique *(37)*. This ensures that a concentration gradient across the membrane is maintained and in most cases is also the basis of the selectivity. Details of the principles of the membrane extraction techniques shown in table 1 are discussed briefly below.

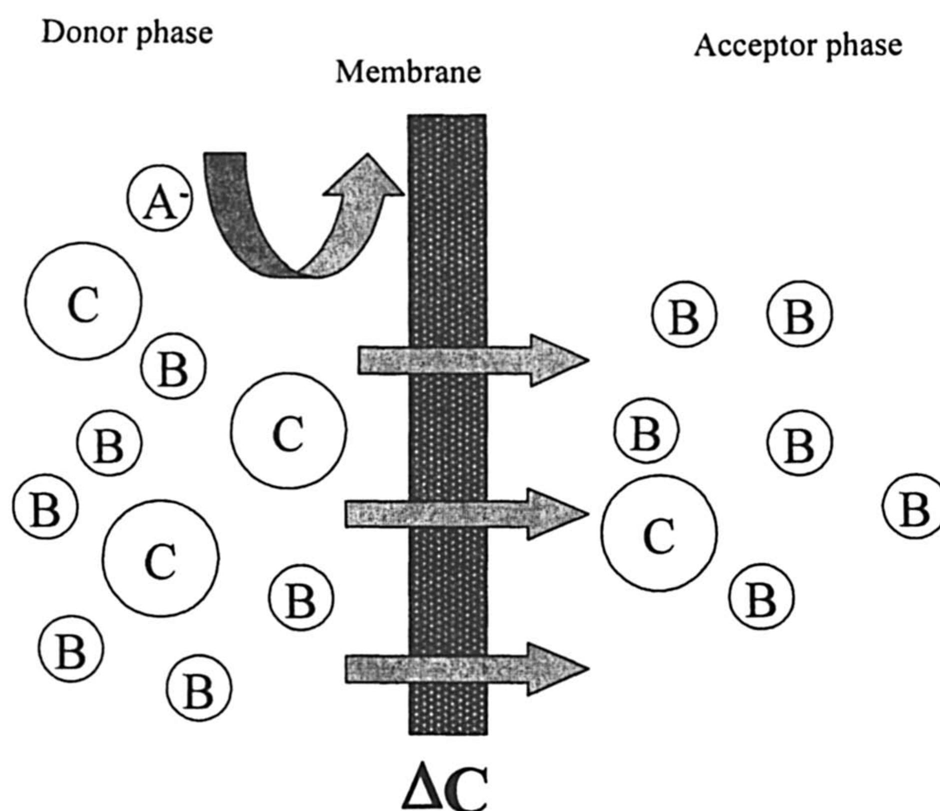

Figure 1. *General schematic representation of transport through hydrophobic nonporous membranes, where A represent charged small molecules, B small molecules able to diffuse through the membrane and C big molecules. ΔC is the difference in concentration gradient across the membrane.*

Table I. Major membrane-based extraction techniques used for food and agricultural samples

Name	*Abbrev.*	*Phase combinations (Donor/membrane/ acceptor)*	*Suitable type of organic compounds*
Supported-liquid membrane extraction	SLM	Aq/org/aq	Ionizable e.g. phenolic acids in nutrient solution (27,28), triazine herbicides in fruit juices (24).
Microporous membrane liquid- liquid extraction	MMLLE	Aq/org/org	Ionizable and neutral e.g. triazines in oil (38).
Polymeric membrane extraction	PME	Aq/polymer/aq, Org/polymer/aq, Aq/polymer/org	Ionizable and neutral e.g triazines, carbamates and organophosphorus pesticides in egg (29), vitamin E in butter (39), organophosphorus pesticides in wine and fruit juice (25).

Various modules or contactors have been used ranging from flat sheets to hollow fibres and with various acceptors volumes. Examples of such modules and different types of membranes have been reviewed recently *(34)*. Flat modules are made of two blocks of inert materials and grooves with depth 0.1-0.25 mm and width of 1-2 mm are machined in the inside of each block. Liquid connections are provided at both ends. A membrane is clamped between the blocks forming a flow-through channel at both sides of the membrane. Typical volumes for this type of modules have ranged from 1ml down to about $20\mu L$. For hollow fiber modules, the acceptor phase is the inside of the fiber lumen and the donor channel is the annular volume between the outside of the fiber and the inside of a surrounding tube *(34)*. This type of unit gives smaller acceptor volumes compared to flat modules *(34)* down to about $5\mu L$. Both of these module types can be used for any type of membrane based extraction techniques.

Supported-Liquid Membrane (SLM) Extraction

Principles of SLM Extraction

The principles of SLM extraction have been reviewed in several review papers *(34,36,37,40)* and book chapters *(32,33,41)*. Here, only brief details will be highlighted. Two important factors in an SLM extraction are pH conditions of the donor and acceptor solutions. The donor pH is adjusted such that the compound is uncharged and able to diffuse through an organic solvent immobilized in the pores of an inert support material. The acceptor pH is adjusted such that compounds are ionized and trapped. The analytes are thus partitioned from the aqueous sample stream into the organic membrane and are then re-extracted into the aqueous acceptor phase. The driving force is the difference of the concentration of the analytes in extractable form between the donor and acceptor phases. The different forms of analytes in the two compartments maintain the concentration gradient. SLM extraction is therefore particularly well suited for ionizable compounds such as medium to weak acids and bases. SLM extraction can provide very selective enrichment. Selectivity can be fine-tuned by proper choice of the conditions in the three phases (Aq-membrane-aq). Macromolecules are discriminated on the basis of their size while charged compounds are too polar to dissolve into the organic liquid. Neutral molecules merely distribute between the three phases without any enrichment.

Applications

The supported liquid membrane extraction is generally one of the most selective membrane based extraction techniques. Selectivity is tuned by adjusting the factors described above. However, like other membrane based extraction techniques, so far it has been applied to mostly environmental and biological samples as seen in review articles *(30,31,34)*.

Knutsson et. al., *(27)* was one of the first to developed a SLM extraction procedure for the determination of phenolic compounds in circulating nutrient solution, thus representing food and agricultural samples. The extraction was performed off-line. The sample pH was adjusted to pH 2 to make sure that the phenolic compounds were uncharged and extracted into dihexylether impregnated in the pores of hydrophobic filter. The stagnant acceptor solution was set to pH 9 with borate buffer thus making sure that the analytes were ionized and trapped. Jung et al. *(28)* extracted similar compounds in a

circulating nutrient solution using a modified SLM extraction procedure. Dihexylether containing 5% trioctylphosphine oxide (TOPO) and 0.1M Na_3PO_4 was used as acceptor solution. TOPO was added to increase the partitioning into the organic liquid, thus increasing the overall mass transfer and extraction efficiency. The extraction in this case was performed on site and extractions were performed over a three months period. In this case, three parallel membrane extraction cells were used to study similarly treated nutrient solutions. Extractions in each case were performed for three hours at donor flow rate of 0.3 mL/min. Figure 2 shows comparison of LC chromatograms from direct injection of standard mixture and after SLM extraction of a real sample. The real sample chromatogram is as clean as standard mixture, illustrating how selective SLM extraction can be if the conditions are properly chosen. A nutrient solution contains a lot of humic acids, which were not extracted. The detection limit ranged from 5 nM for phenazine-1-carboxylic acid to 300 nM for salicylic acid.

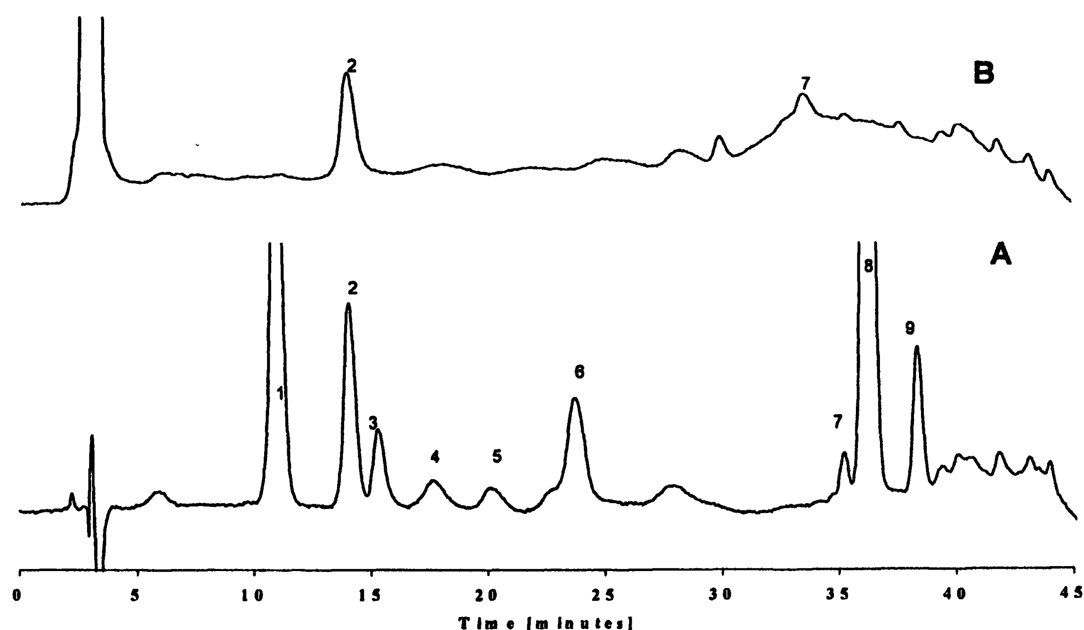

Figure 2. Chromatogram of a standard mixture of compounds (A) and corresponding circulating nutrient sample solution after SLM extraction (B). (1) p-Hydroxybenzoic acid, (2) vanillic acid, (3) salicylic acid, (4) benzoic acid, (5) ferulic acid, and (6) phenazine-1-carboxylic acid. The chromatogram additionally contains a number of other compounds. (Reproduced with permission from reference 28. Copyright 2002 Elsevier.)

Wiezorek's group recently combined SLM extraction and SPE for sample pre-treatment of triazine herbicides in juice prior to capillary electrophoresis determination *(24)*. In this method, triazines in orange juice were partitioned into dihexylether used as membrane and trapped into 0.5M H_2SO_4 acceptor solution. Ten ml of the acceptor solution was circulating during the extraction. The

acceptor solution was later neutralized with NaOH and extracted on a Bakerbond Speedisk C_{18} XF disk. The application of SPE alone for such samples could not provide sufficient clean-up *(24)*. However, by combining SLM extraction and SPE, much cleaner extracts were obtained and detection limits were lowered significantly from 1000 μgL^{-1} to 50 μgL^{-1}*(24)*. Valcárcel and co-workers have applied the SLM extraction principle to solid or semi-solid samples by designing the donor channel to accommodate such samples and permit a close contact between the sample and the membrane. This technique was used successfully to extract and quantify nicotine in snuff *(42)*, vanillin in chocolate *(43)* and caffeine in coffee and tea *(44)*.

Microporous Membrane Liquid-Liquid Extraction (MMLLE)

Principles of MMLLE Techniques

The detailed principles of MMLLE have been documented in reviews *(34,37)* and in a book chapter *(41)*. In this case, the organic acceptor solution fills the pores of the hydrophobic membrane forming a two-phase system (Table 1). The organic liquid is thus partly in the pores of the membrane and partly in the acceptor channel. The analytes partition from the aqueous donor stream into the pores of the membrane and diffuse to the bulk of the organic liquid in the acceptor channel due differences in concentration gradient. The concentration enrichment factor is therefore determined by the partition coefficient of the pollutant into the organic liquid. The highest enrichment factor that can be obtained is equal to the partition coefficient of the pollutant into the organic liquid *(37)*. The mass transfer can be improved if the acceptor solution is continuously or intermittently pumped, removing pollutant molecules successively from the acceptor *(37)*. In order for the pollutant to dissolve into the organic liquid, it must be uncharged or neutral (Table 1). Charged pollutants in the sample are therefore not extracted while macromolecules will not pass through the pores of the membrane. Polar compounds have too low partition coefficients and therefore are poorly extracted. The selectivity of MMLLE can thus be compared to that of PME where in both cases; an organic liquid is used as the acceptor solution with aqueous donor stream.

Applications

As in the case of SLM extraction, much of the reported applications have been to environmental and biological samples *(31,34)*. In MMLLE, the analytes are extracted from an aqueous sample stream into an organic acceptor solution, and has been easily connected to gas chromatography. Alternatively, the analytes can be extracted from the organic sample into an aqueous acceptor, and connected to liquid chromatography. Martínez et. al., *(38)* reported a membrane based extraction procedure for the determination of triazines in oils. Oil samples were diluted with hexane (1:4) to reduce the viscosity and then circulated over a porous Celgard 2500 membrane with a stagnant acceptor solution of 0.10 M $HClO_4$. The triazines were separated from the rest of the sample by diffusion to the acceptor solution due to concentration gradient, where they were ionised and trapped. In order to couple the extraction to a liquid chromatographic system, an acceptor solution of 0.005 M $HClO_4$ in methanol-water (90:10,v/v) was used instead. This was necessary to avoid working with strong acidic acceptor solution, which might damage the chromatographic system. In this application, very clean chromatograms were obtained and demonstrated the potential of MMLLE application to food and agricultural samples.

Polymeric Membrane Extraction (PME)

Principles of PME

In this case, an entirely solid membrane is used to separate the flowing donor (sample) and the stagnant acceptor solution. Silicone rubber is mostly used because it is hydrophobic and gives high permeability for small hydrophobic compounds such as pesticides and other related compounds *(45)*. Small compounds that are ionic at the sample pH conditions will not partition into the hydrophobic silicone rubber membrane. Larger molecules will have slow mass transfer through the silicone rubber compared to small molecules. The difference in the solubility and diffusion of various analytes into the polymer is therefore the basis of selectivity. The solid nature of silicone rubber makes it a versatile membrane extraction technique as it allows handling of various kinds of

complex samples such as those with high amounts of organic materials e.g. lipids (46). It also allows various phase combinations for extraction (table 1). Setting the pH of the aqueous acceptor solution such that ionisable organic compounds are trapped generally results in high enrichment factors. This also enhances the selectivity of the extraction just like in SLM extraction technique *(38)*. In a case where the acceptor phase is an organic liquid, the enrichment factor is based on the partition coefficient of the compound to the solvent which this condition is suitable for extracting neutral organic compounds.

Applications

Méndez and co-wokers have applied membrane extraction based on silicone rubber to a number of food samples with high organic content *(29,39)*. In all their applications, the membrane extraction was automatically connected to liquid chromatography thus reducing the analysis time. In one application *(39)*, vitamin E was extracted from butter after sample dissolution in a micellar medium (10% methanol-3% Triton X 100 - water) followed by alkaline hydrolysis on-line. The acceptor solution in this case consisted of acetonitrile, which was directly injected into a liquid chromatographic system with electrochemical detection. The coefficient of variation was studied to determine the day-to-day precision which after 10 days was found to be 6.7%. This means that the developed extraction procedure can be used for routine analysis of vitamin E in butter. Clean chromatograms were obtained showing the selectivity of the developed method. In another application from the same group *(29)*, a silicone rubber membrane extraction technique for pesticide multiresidues analysis in samples with high lipid content was described. In this case, egg samples were subjected to initial extraction in a Soxhlet system using hexane, after which the extract was introduced into the membrane separation device coupled to the HPLC system. The acceptor solution consisted of 0.01M H_3PO_4 in methanol-water (70:30,v/v). The acid helped to ionize basic pollutants (triazine herbicides) while methanol ensured that uncharged pollutants had sufficient partition coefficients to be enriched. This procedure offered clean chromatograms (Figure 3) as it was efficient in removing macromolecular compounds, considerably facilitating the determination. The detection limits varied from 0.018 $mgkg^{-1}$ for dichlorvos to 0.002 $mgkg^{-1}$ for atrazine.

Schellin et al., *(25)* reported a membrane-assisted solvent extraction combined with large volume injection GC-MS for determination of organophosphorus pesticides in wine samples. The method involved filling a

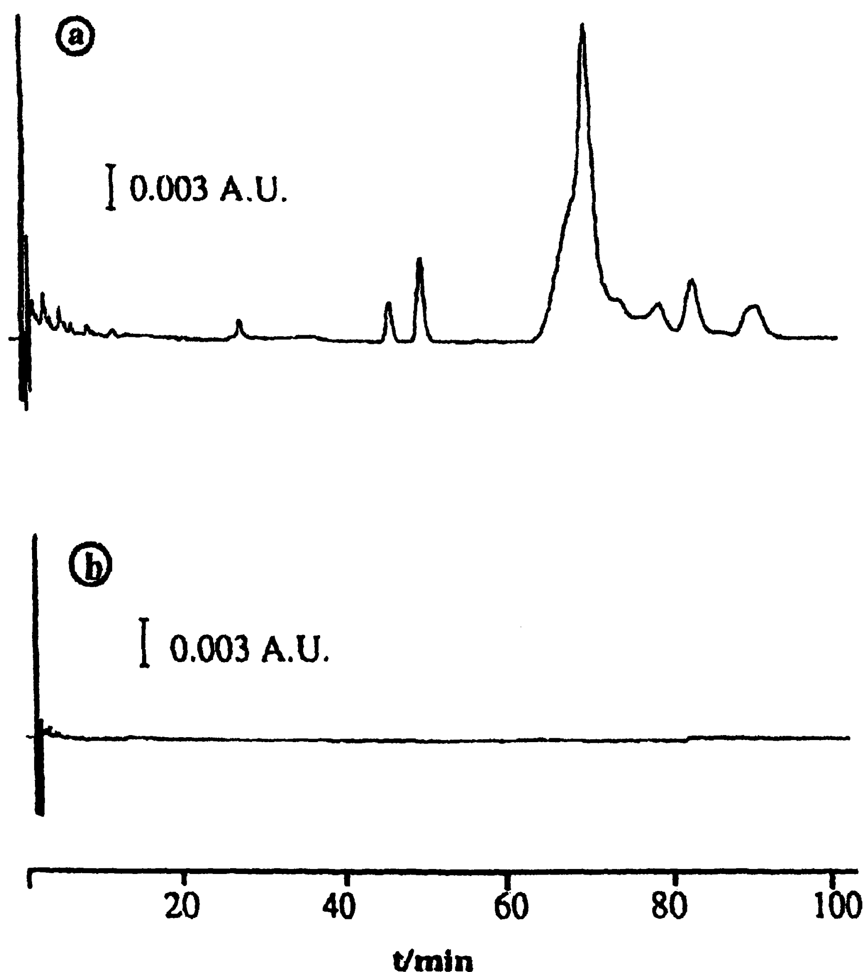

Figure 3. Chromatogram demonstrating the selectivity of PME of a non-spiked egg sample: (a) injected directly into the chromatograph without passing through the membrane; (b) after passing through the membrane. (Reproduced with permission from reference 29. Copyright 2002 Elsevier.)

20ml extraction vial with 15ml of aqueous sample. Then a membrane bag consisting of nonporous polypropylene was put into the vial and filled with 800μL of cyclohexane as acceptor solution. The analytes were separated from the rest of sample matrix by transporting them through the membrane material into the small amount of organic solvent. The technique was fully automated and successfully combined with large volume injection GC-MS. Figure 4 shows the selectivity of such a system by comparing red wine sample solution with that of the extract. The extract is very clear indicating the selectivity of the method. This was also seen in the resulting chromatogram that was identical to that spiked with water *(25)*. The detection limit for various organophosphorus pesticides in a variety of wines and apple juice was about 0.5 μgL^{-1}.

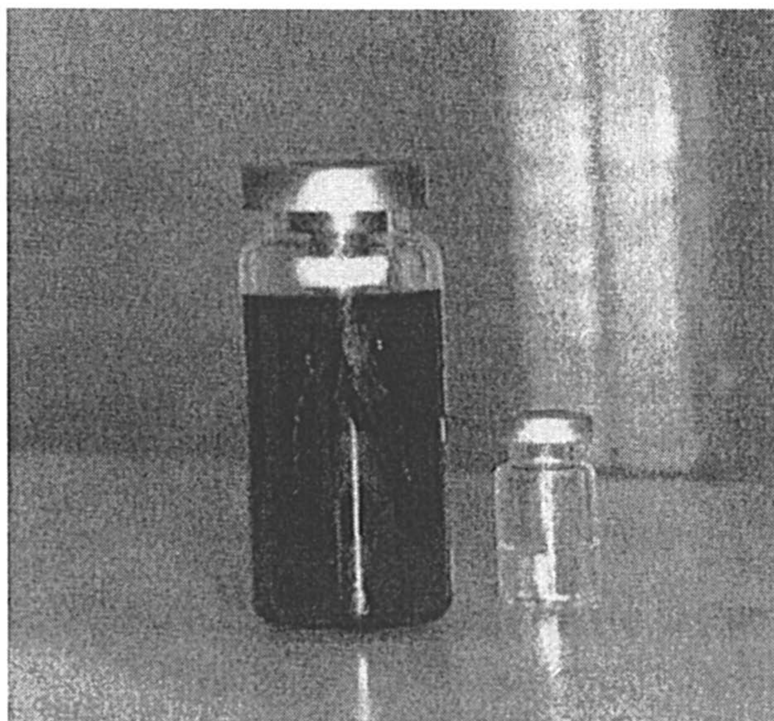

Figure 4. Red wine sample (left) and the obtained clear extract (right). (Reproduced with permission from reference 25. Copyright 2004 Elsevier.)

Conclusions

Membrane based extractions are capable of complementing conventional techniques (liquid-liquid and solid phase extractions) in food and agricultural sample preparation. Their attractiveness in sample preparation is based on their selectivity and ability to tolerate samples with high organic content and/or dissolved solid. They can also be easily automated and interfaced to other separation techniques. However, despite these advantages, membrane based extractions especially SLM extraction and MMLLE techniques have not been applied much to food and agricultural samples as compared to PME techniques.

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Chapter 11

Identification of Volatile Compounds in Shiitake Mushrooms Using Modern Extraction Techniques

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Dried Shiitake mushrooms (*Lentinus edodes*) were analyzed as part of the Generessence® program (*1*), with the aim of creating a new flavor incorporating only compounds identified in the starting material. Analytical data was obtained by three different methods: steam distillation-extraction (SDE), sorptive stirrer bar extraction (Twister™) and headspace trapping with Tenax™. A total of 391 compounds were found to be present in the three combined analyses, which is a significant increase on the recently reported 156 components in fresh shiitake mushrooms by Cho *et al* (2). Among the compounds identified, 79 compounds were sulfur containing, 82 were nitrogen containing and 18 contained both sulfur and nitrogen. This highly complex mixture contained a large variety of heterocyclic molecules: furans, thiazoles, thiazolines, pyrazines, pyrroles, pyridines and polysulfur cyclics. A comparison of the extraction techniques is presented with an emphasis on the positive and negative aspects of their use.

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Introduction

Shiitake mushrooms (*Lentinus edodes* Sing.) have long been a symbol of longevity in Asia because of their health promoting properties. They have been used medicinally by the Chinese for more than 6000 years. Their rich, smoky flavor has made them a delicacy in modern cuisine and they are cultivated on rotting hard wood. After harvesting, the mushrooms are heat treated for prolonged storage. Fresh mushrooms exhibit only a slight odor and the drying process is thought to be responsible for their unique "sulfurous" aroma (*3*). In addition several papers have been published on the aroma and composition of dried Shiitake mushrooms (*4,5,6*). They focused on the sulfur molecules present, especially the polysulfur cyclic class of compounds, of which examples are shown below. Compounds containing eight-carbons have been reported as major components of the aroma of fresh mushrooms and polysulfur cyclic compounds have been identified as characteristic in dried Shiitake mushrooms. The most significant polysulfur cyclic is Lenthionine in Figure 1, derived from Lentinic acid and discovered by Morita and Kobayashi in 1966 (7).

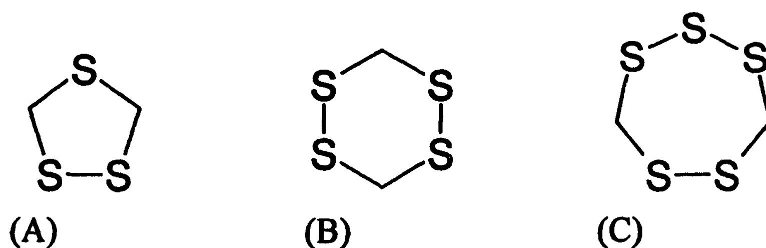

Figure 1. The main polysulfur cyclics in Shitake Mushroom: (A) 1,2,4-Trithiolane; (B) 1,2,4,5-Tetrathiane; and (C) 1,2,3,5,6-Pentathiepane (Lenthionine).

This analytical project was undertaken as part of the Generessence® program at International Flavors & Fragrances Inc. The program involves the in-depth analysis of a natural product to enable the creation of closer to nature flavors, using only the chemicals found in the analyses. An additional aim is the discovery of novel compounds in the natural product and occasionally in nature for the first time that may be synthesized and ultimately registered for use. Therefore, as much detail as possible is required for the analysis of volatiles, semi-volatiles and even non-volatiles present. Many extraction and isolation

techniques are available to the analyst and are well documented in the literature (*8,9*). In this particular case the extraction techniques used were simultaneous steam-distillation-extraction (Likens-Nickerson) (*10*), stir bar sorptive extraction (Twister™) (*11,12*) and headspace sampling (*13,14*). The steam distillation provided a liquid extract for GC-olfactory work. Sampling by stir bar sorptive extraction (Twister™) is particularly useful for the analysis of aqueous flavor solutions such as that of the rehydrated mushrooms. Headspace trapping of the aroma was conducted using pairs of Tenax™ traps to capture the highly volatile compounds. This in-depth analytical work was also recently reported at the 7th Symposium of Flavor Chemistry and Biology in Wartburg, Germany (*15*).

Experimental

Extraction techniques

Simultaneous steam distillation-extraction in Figure 2, was conducted using a modified Likens-Nickerson apparatus (*16*). The apparatus consisted of the sample flask (right) where approximately 150g of dried mushrooms from China were added and mechanically stirred with 2.5L of distilled water. Steam distillation involved collecting the mushroom volatiles in 150mL of dichloromethane in the extract collection flask (left) over 1.5 hours. The procedure was repeated with more dried mushrooms and dichloromethane; the combined extracts were dried over anhydrous sodium sulfate, filtered and reduced to a volume of 1mL using a Zymark™ TurboVap evaporation system. The extract was analyzed by a gas chromatograph fitted with a flame ionization detector (GC-FID), gas chromatograph with atomic emission detector (GC-AED), gas chromatography-olfactometry (GC-O) and gas chromatography-mass spectrometry (GC-MS).

For the stir bar sorptive extraction, 500ml of boiling water was added to approximately 50g of dried mushrooms and left to soak for 30 minutes to rehydrate. The mushrooms were removed by filtration and the resulting aqueous solution transferred to Twister™ (Gerstel Inc., Baltimore, MD) vessels on a stirrer plate. The Twister™ bars in Figure 3, were added and volatiles were collected with spinning for 4 hours and 24 hours respectively, at 1800 revs per minute. The stir bars were rinsed with distilled water, padded dry with tissue paper and thermally desorbed onto GC-FID and GC-MS systems.

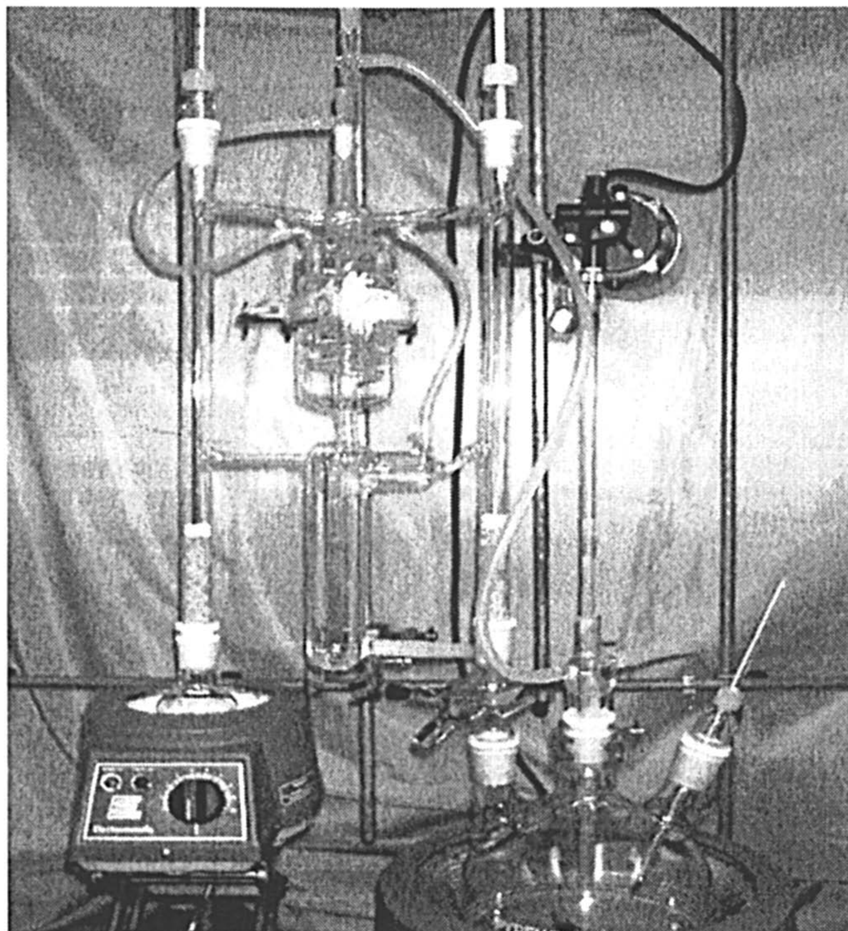

Figure 2. *Simultaneous steam distillation-extraction (Likens-Nickerson)*

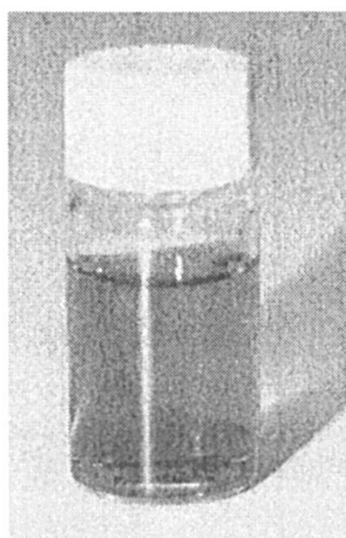

Figure 3. *Sorptive stir bar (Twister™) at the bottom of a glass vial in aqueous flavor solution.*

For the headspace analysis, approximately 100g of dried mushrooms were placed in a headspace sampling vessel (Figure 4) and the headspace collected onto pairs of Tenax™ TA traps (Supelco, Inc., Bellefonte, PA) using a vacuum pump (Fischer Scientific, Co., Springfield, NJ) with a flow rate of 25mL/min. The headspace was sampled over 4 hour and 24 hour periods, respectively. The traps were thermally desorbed onto GC-FID and GC-MS systems.

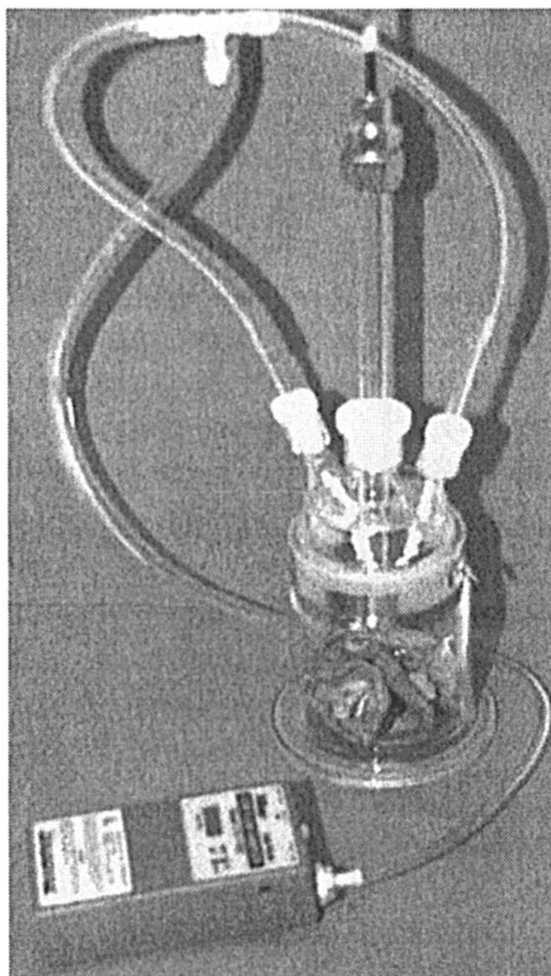

Figure 4. *Dynamic headspace trapping of using Tenax™ traps*

Gas Chromatography

The steam distillation extract was analyzed using a HP6890 gas chromatograph with a split/splitless injector and a flame ionization detector (FID) (Hewlett Packard, Wilmington, PA). The extract was injected onto a non-polar OV-1 capillary column (50m x 0.32mm i.d., 0.5μm film thickness, Restek,

Bellefonte, PA) in the split (split ratio 40:1) and splitless modes. The carrier gas was helium with a flow rate of 1.0 mL/min operated at constant flow. Injection port temperature was 250°C and the detector temperature 320°C. The column temperature was ramped from 40°C to 270°C at 2°C/min with a 10 minute hold at the final temperature.

To aid in the detection of sulfur and nitrogen-containing compounds, the extract was analyzed on a HP6890 gas chromatograph equipped with an atomic emission detector (AED) (Hewlett Packard, Wilmington, PA). The gas chromatograph conditions were the same as those described above.

A polar chromatogram of the extract was also obtained by injecting onto an HP5890 gas chromatograph with split/splitless injection and a flame ionization detector (FID) fitted with a Carbowax capillary column (50m x 0.32mm i.d., 0.3μm film thickness, Restek, Bellefonte, PA) using the previously described injection and detection techniques. The temperature program began with an initial temperature of 60°C held for 10 minutes, ramped at 2°C/min to a final temperature of 220°C and held for 20 minutes.

Tenax™ TA traps and Twister™ bars were thermally desorbed onto an HP6890 gas chromatograph equipped with a flame ionization detector (FID) using a Gerstel thermal desorber Model TDS 2 (Gerstel Inc., Baltimore, MD). Desorption time was 5 minutes at 250°C. The column was an OV-1 capillary column and the analysis was performed in splitless mode. The injector temperature was programmed from -150°C (held for 5 min during the thermal desorption) to 250°C. Detector temperature was 320°C.

All data was collected and stored by using HP ChemStation software (Hewlett Packard, Wilmington, PA).

Mass Spectrometry

Identification of components in the extracts was conducted by mass spectrometry. The sample was injected onto an HP5890 GC. The chromatographic conditions for the OV-1 column were the same as described for GC analysis. The end of the GC capillary column was inserted directly into the ion source of the mass spectrometer via a heated transfer line maintained at 280°C. The mass spectrometer was a Micromass Prospec high resolution, double-focusing, magnetic sector instrument. The mass spectrometer was operated in the electron ionization mode (EI), scanning from m/z 450 to m/z 33 at 0.3 seconds per decade.

Polar GC-MS analysis was on conducted on a Carbowax capillary column (50m x 0.32mm i.d., 0.3μm film thickness); the sample was introduced via an HP5890 GC into a Kratos Profile mass spectrometer (Manchester, UK). GC oven conditions were the same as outlined above. The mass spectrometer was

operated in EI mode scanning from m/z 450 to m/z 33 @ 0.3 seconds per decade.

Spectra obtained from both phases were interpreted on a MassLib data system (Max Planck Institute, Germany), using IFF in-house libraries and commercial Wiley 7, NIST 98 and other libraries. The identification of flavor components was confirmed by interpretation of MS data and by relative GC retention indices based on a calibration with ethyl esters.

Results and Discussion

As previously mentioned three hundred and ninety-one components were found to be present in the three combined analyses. This is also a significant increase compared to the eighty-one reported in the flavor database issued by TNO (TNO, 2000) (*17*). The analysis included nitrogen and sulfur containing compounds, which to the authors' best knowledge, were reported in Shiitake mushrooms for the first time.

Table 1 shows that the steam distillation-extraction of a large quantity of mushrooms yielded the most complete and complex sample for volatile analysis, when compared to the sorptive stirrer-bar and headspace trapping. The sorptive stirrer bar tends to extract fewer polar components and the headspace trapping is limited by volatility. A comparison of extraction techniques including supercritical fluid extraction as applied to Shiitake mushrooms is reported by Charpentier *et al* (*18*).

Table 1. Number of components detected with each technique.

Technique	*Total number of components*	*Sulfur containing*	*Nitrogen containing*	*Sulfur and Nitrogen containing*
Steam Distillation	260	67	57	21
Sorptive Stirrer-bar	156	23	42	3
Headspace	209	18	46	8
Total	391	79	82	18

The total ion chromatogram (TIC) profile (Figure 5) shows a wide spread of volatiles eventually tailing off as non-volatiles are excluded due to the boiling

point of water in the steam distillation. Analysis of the steam distillate yielded 260 components, of which 67 were sulfur containing, 57 nitrogen containing and 21 containing both sulfur and nitrogen. The major components were 1,2,4-trithiolane (43.8%), 1,2,3,5,6-pentathiepane (Lenthionine) (16.9%), 1,2,3,5-tetrathiane (5.2%), 1,2,4,5-tetrathiane (3.8%) and hexathiepane (3.2%).

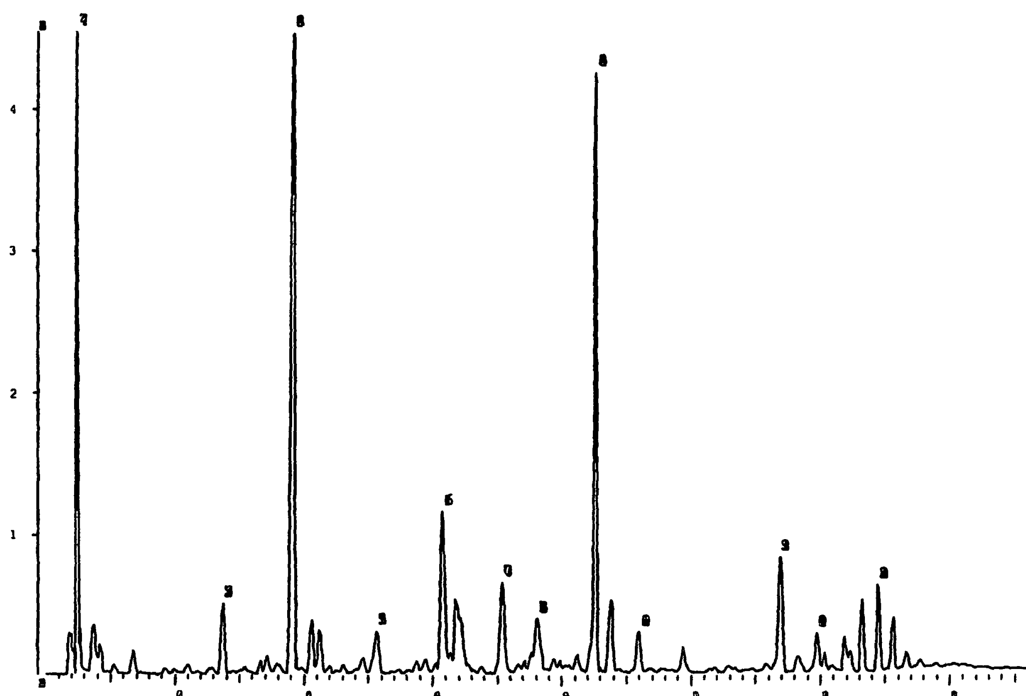

Figure 5. *Mass spectral TIC profile of the steam distillate.*

The Twister™ TIC (Figure 6) shows a wide spread of components affected by polar discrimination and with increased high boiling component content. Twister analysis yielded 156 components, of which 23 were sulfur containing, 42 nitrogen containing and 3 contained both sulfur and nitrogen. The major components were linoleic acid (63.8%), hexadecanoic acid (10.8%), 1,2,3,5,6-pentathiepane (Lenthionine) (7.6%), octasulfur (4.5%) and 1,2,4-trithiolane (0.9%).

The headspace TIC (Figure 7) shows the highly volatile components captured and then tails off quickly with higher molecular weight components. Analysis of the headspace yielded 209 compounds, of which 79 were sulfur containing, 82 were nitrogen containing and 18 contained both sulfur and nitrogen. The major components were 1,2,4-trithiolane (13.2%), isobutanol (8.7%), 2-methylbutanol (7.4%), isovaleric acid (5.1%) and 2-methylbutyric acid (4.5%).

Figure 6. Mass spectral TIC profile of theTwister™.

Figure 7. Mass spectral TIC profile of the dynamic headspace.

As seen from Figures 4, 5 and 6 each sample preparation method yielded a different flavor analysis. However, when combined, the results revealed a very complex and more complete profile of dried Shiitake mushrooms flavor.

Table 2 demonstrates the pros and cons of the various extraction and sampling techniques. The compounds listed above were selected based on different functional groups and increasing molecular weight. Even within this select group of compounds certain trends may be observed. Highly volatile, low molecular weight compounds tend to be higher in concentration in the headspace analysis, as expected. The low concentration of hexanoic acid and hexanal in the steam distillate is due to their hydrophilicity. These polar compounds tend to remain in the aqueous phase during distillation-extraction, whereas the relatively non-polar undecanone extracts well.

Table 2. *Comparison of three sampling techniques in the extraction of Shiitake Mushrooms.*

Component	*Steam distillate (ppt)*	*Twister (ppt)*	*Headspace (ppt)*
Hexanal	0.08	1.52	15.35
2,5-Dimethylpyrazine	2.77	0.27	19.48
1-Octen-3-ol	0.40	0.15	1.32
Hexanoic acid	0.00	0.09	0.71
1,2,4-Trithiolane	437.70	8.48	132.45
2-Formyl-N-methylpyrrole	0.59	0.02	0.23
N-isoAmylacetamide	0.23	0.14	1.23
Undecan-2-one	16.20	0.22	1.55
Lenthionine	168.59	75.89	4.85

(ppt = parts per thousand in the extract)

The concentration of Lenthionine versus that of trithiolane is greatest in the Twister™ analysis. This ratio appears to be reversed in the headspace and steam distillation. In the headspace analysis this is explained by the differences in molecular weight and volatility of these two compounds. In the steam distillation it is thought that thermal decomposition of Lenthionine leads to a higher concentration of trithiolane and other polysulfur cyclics.

Comparison of the different techniques

Below are summarized the many advantages and disadvantages of the particular extraction and sampling techniques used in this study. They include observations learned through frequent use on various types of samples.

Simultaneous Steam Distillation-Extraction

Advantages:

- Extracts do not contain high-boiling or non-volatile material, kind to GC liners and columns.
- Usually is the greatest source of compositional detail and novel components.
- Can concentrate extracts further for trace component detection.
- Works best for samples that have previously been thermally treated.

Disadvantages:

- Highly polar or hydrophilic components extract poorly or not at all.
- Fresh fruits give "cooked" extracts.
- Possible artifact formation and thermal degradation.
- Less quantitative than liquid/liquid extraction.
- Complicated apparatus for set-up and time consuming cleaning.

Sorptive Stirrer-Bar (Twister™)

Advantages:

- Quick and simple to use.
- Solventless technique.
- Requires little sample to perform sampling.
- Can be used to identify trace components obscured by higher concentration polar components such as acids and alcohols.

Disadvantages:

- Polar component discrimination.
- Need to desorb Twister™ relatively quickly due to potential loss of volatiles with time.
- Possible artifacts from adsorbent.
- Less quantitative than a liquid extract.
- Difficult to predict optimum sampling/stirring time from one sample to another; several sampling times recommended.

Dynamic headspace

Advantages:
- Quick and simple to use.
- Solventless technique.
- Requires little sample to perform sampling.
- Truly analyzing what is smelled.

Disadvantages:
- Relative concentrations of components do not reflect the true concentrations; can be corrected by vapor pressure adjustment.
- Aroma profile is dependant upon sampling temperature and time.
- Difficult to predict optimum sampling time from one sample to another; several sampling times recommended.
- Possible artifacts from adsorbent.
- Does not detect non-volatiles.

Conclusions

There is as yet no perfect extraction method for all types of samples. A minimum of a liquid extraction and a headspace sampling should lead to a fairly well rounded flavor or fragrance profile and analysis. The more techniques used to study a particular subject, the better the quality of the analytical results. However with experience the choice of extraction techniques will ensure more detailed analyses from fewer extracts.

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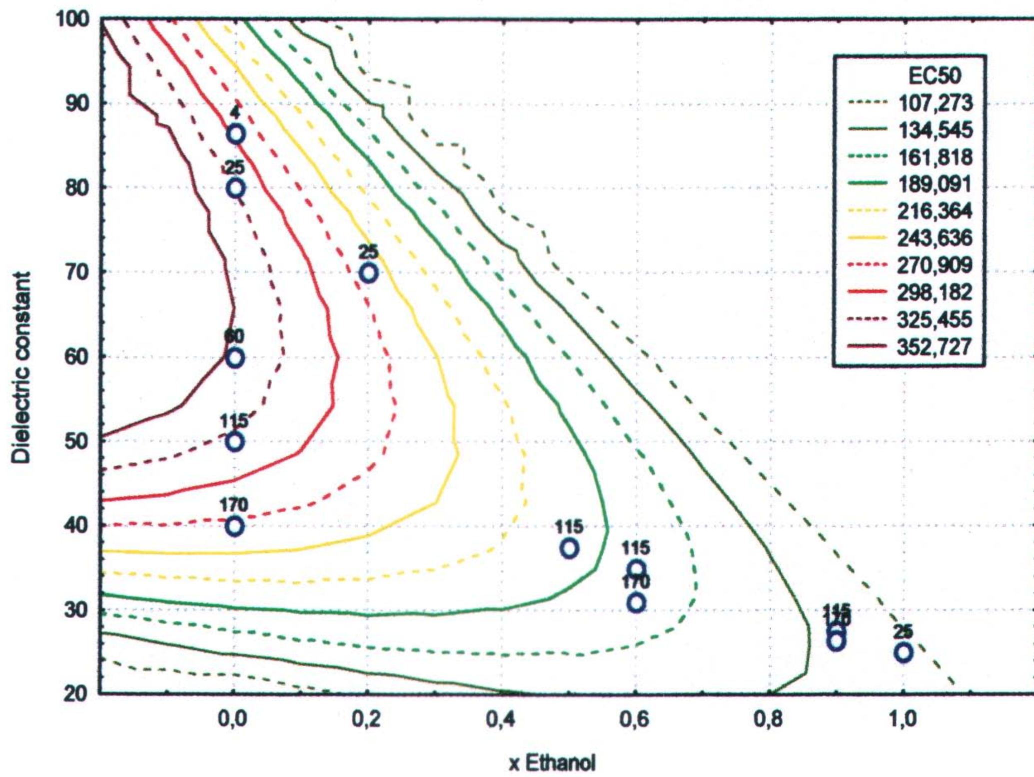

Plate 5.3. *Surface plot of EC_{50} vs. molar fraction of ethanol and dielectric constant.*