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Solid Phase Extraction: Applications to the Chromatographic Analysis of Vegetable Oils and Fats

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CONTENTS

RESUMEN

Extracción en fase sólida: Aplicaciones al análisis cromatográfico de aceites y grasas vegetales.

Se analizan las aplicaciones de la extracción en fase sólida para el aislamiento de ciertos lípidos previamente al análisis cromatográfico. La mayor parte de la información se refiere a los esteroles y compuestos relacionados, fenoles polares y contaminantes como los hidrocarburos aromáticos policíclicos. Se muestran y discuten numerosos protocolos analíticos en detalle.

PALABRAS-CLAVE: Aceites vegetales - Análisis cromatográfico - Extracción en fase sólida - Grasas vegetales - Lípido.

SUMMARY

Solid phase extraction: Applications to the chromatographic analysis of vegetable oils and fats.

Applications of solid-phase extraction for the isolation of certain lipid classes prior to chromatographic analysis are given. More information was found for sterols and related compounds, polar phenols and contaminants such as polycyclic aromatic hydrocarbons. Detailed analytical protocols are presented and discussed in many cases.

KEY-WORDS: Chromatographic analysis - Lipid - Solid-phase extraction - Vegetable fats - Vegetable oils.

1. INTRODUCTION

Adequate sample preparation techniques help to ensure an accurate and precise estimation of the true value of the food components. Solid phase extraction (SPE) is a convenient approach for sample preparation in the chromatographic analysis of edible fats as it requires a small amount of the test sample, low volumes of organic solvents, whereas the treatment is accomplished in a greatly shorter period of time than in other techniques.

SPE typically involves commercially available pre-packed polymer or glass mini disposable columns (cartridges) or disks that can be used to remove contaminants or to further fractionate the analytes prior to analysis. The technique is highly selective and versatile due to the large number of phases introduced in the last ten years. New formats (96-well plates, microfibers for solid phase microextraction), automation and on-line use are the important advantages for the current and future applications of SPE. Many recent review articles and other special editions cover in details the needs of a new comer both in the theoretical background and practical aspects of SPE (Thurman and Mills 1998; Simpson1997; Fritz 1999; Horack and Majors 1993; Hennion 1999).

Gas Chromatography (GC) and, primarily, High Performance Liquid Chromatography (HPLC) are the two techniques widely applied for the analysis of the major (triacylglycerols) and minor constituents of edible oils and fats (Christie 1987; Hammond 1993; Cert *et al.* 2000). The second class consists of subgroups of chemical compounds (diacylglycerols, monoglycerols, phospholipids, free fatty acids, sterols, vitamins, hydrocarbons, pigments, waxes, polar phenols, etc), the presence and the levels of which are characteristic features of each oil. Bioavailability of these compounds is associated with health benefits and problems. The same techniques are also used to monitor the total quality and shelf life of edible fats. Therefore, accurate determination of mandatory compounds is of great importance for both the industry and the official quality control.

Since the first steps of chromatography, the extraction of the examined compounds from the lipid matrix has been performed using liquid-liquid extraction. Solid phase extraction is progressively replacing traditional procedures reducing, thus, the cost of analysis and augmenting the sensitivity of existing analytical protocols. More use of SPE in the isolation of lipid classes has been carried out for biological fluids and tissues. Adsorption (silica or

bonded phases, e.g. amino), reversed-phase (C_8 and C_{18}) and ion-exchange (quaternary amino, aminopropyl) packing materials have been used by a great number of investigators over the years.

Recent reviews on the chromatographic techniques applied to lipid classes or devoted to the analysis of specific classes of lipids give also information on many of the applications of SPE in oils and fats (e.g.: Ebeler and Ebeler 1996; Aparicio and Aparicio-Ruíz 2000; Cert *et al.* 2000; Lercker and Rodriguez-Estrada 2000; Ruiz-Gutiérrez and Pérez-Camino 2000). In the present review emphasis was paid to update the information and present in details some of the applications concerning the vegetable fats. Information on other edible fats or fatty foods associated with the above objectives is given in certain cases. The classes of compounds are presented in descending order of frequency of SPE applications found in the literature.

2. STEROLS (4-demethylsterols) AND THEIR OXIDATION PRODUCTS

Sterols, free or esterified with fatty acids, are found in small concentrations (0.1-5.6%) in edible fats. Cholesterol is found in animal fats and at relatively low amounts in certain plant lipids (e.g. palm oil). Phytosterols, the plant sterols, are rather simple mixtures of closely related organic compounds. Some of them always accompany each other (e.g. ß-sitosterol and campesterol). The analysis of sterols is important for detecting oil adulteration, e.g. of butter fat with vegetable oils or of virgin olive oil with cheaper oils. More recent is the interest in the nutritional value of sterols and the monitoring and accurate quantification of oxysterols. The latter, especially cholesterol oxidation products, have been examined thoroughly due to their potential health risk (Bosinger *et al.* 1993).

Capillary gas chromatography (cGC) is mainly used for the analysis of sterols. The sterol fraction is obtained from the unsaponifiable matter by thin layer chromatography (TLC) prior to analysis. This tedious procedure, part of the official ones adopted for the control of edible oils (EC 1991; IUPAC 1992; AOAC 1999) has a little potential for automation and limits the number of samples that can be handled daily. Alternative sample preparation procedures involve derivatization of the unsaponifiable (Slover *et al.* 1983), automatic removal of the interfering triacylglycerols by continuous on-line transesterification with potassium methylate in methanol (Ballesteros *et al.* 1996), fractionation on column chromatography (CC) (Homberg 1987) or on-line LC-GC (Grob *et al.* 1989 and 1990; Artho *et al.* 1993; Biedermann *et al.* 1993). Using these alternative procedures tocopherols, hydrocarbons and related compounds are also determined in the subsequent analysis. The merits of HPLC procedures have also been investigated in the literature for their potential either for sample treatment, as direct analytical means or in conjunction with other chromatographic techniques (Holen 1985; Cert *et al.*, 2000). Thus, there is an increasing interest in developing rapid methods for sterol analysis (Alonso *et al.* 1997).

Step	Action
A	Saponification of sample [0.25-0.50 g oil + 5ml 0.5 mM ethanolic KOH, 20 min reflux, 80°C]
B	Addition of betulin as internal standard [1 mg betulin in 5ml $CHCl_3$]
C	pH adjustment, filtration [few drops 5M HCl, 0.45 mm nylon membrane filter]
D	Application to C_{18} cartridge

Scheme 1
Extraction of sterol fraction according to Toivo *et al.* (1998).

SPE procedures in the analysis of sterols in edible fats are rather limited though more information can be found in the case of biological fluids. More emphasis is paid to isolate the oxidation products, mainly those of cholesterol (Ruiz-Gutiérrez and Pérez-Camino, 2000).

Horstmann and Montag (1986) obtained sterols from fats or unsaponifiable matter using silica gel cartridges and eluents of increasing polarity and then performed normal phase HPLC. The less polar compounds (triacylglycerols, waxes and hydrocarbons) were removed with hexane. Silica cartridges were also used by Bortolomeazzi *et al.* (1990), Bello (1992) and Nota *et al.*(1995) to isolate the sterol fraction from various oils. Bortolomeazzi *et al.* (1990) isolated free and total sterols from egg yolk, corn and olive oil on both unused and recycled silica columns (600 mg SepPak) with fairly good results. Amelio *et al.* (1992) used quaternary amine columns conditioned with diethyl ether to simply purify the unsaponifiable matter, which was already separated from soap on a Chem Elut 2050 column. The SPE eluate was then fractionated on silica-HPLC, the collected material was derivatised and, finally, sterols, as well as uvaol and erythrodiol were determined on capillary GC. The procedure was applied to crude, extra virgin, refined and crude pomace olive oils. Toivo *et al.* (1998) extended the efforts made by Tsui (1989) on C_{18} packing material to clean up the unsaponifiables according to the scheme presented in Scheme 1. They validated the method for rapeseed (ten samples) and butter (ten samples). The cartridges (Mega BondElut, 1.0g/6ml, Varian) were first activated with 5ml methanol and 5 ml deionized water, respectively. The sterol fraction was then

eluted (98.5% recovery) with 5% methanol in chloroform (15 ml). Concentration of the eluate (0.5ml) was necessary prior to silylation and subsequent cGC analysis. The authors claim for a significant reduction in the overall analysis time and report that preparation of about 20 samples is conveniently carried out in a working day by one technician. The latter is a worth mentioning improvement of the existing official procedure. Lechner *et al.* (1999) introduced a procedure for the simultaneous determination of tocopherols and sterols in vegetable oils. Prior to SPE, the free hydroxylgroups of the compounds were silylated to reduce polarity. Bakerbond silica columns (1000mg/6ml) from Baker were conditioned with about 3 ml n-hexane:methyl *tert*-butyl ether (99:2, v/v) under vacuum. Then sample was applied and the analytes were eluted with 4.5 ml of the same solvent system. The eluate was subjected to cGC without further treatment. Recovery checked for compounds differing in polarity (squalene, cholesteryl stearate, 5,7-dimethyltocol, stigmasterol and betulin) was excellent (~100%). The method was applied to rapeseed, sunflower, soybean, castor, poppy and cuphea oils to check robustness and reliability.

The complex mixtures of sterol oxidation products are usually found at low concentrations in food. Among them cholesterol oxides (COPs) are mainly found in processed products of animal origin. Prior to chromatographic analysis precautions are necessary to avoid decomposition of these products. Therefore, many efforts have been made to simplify the sample pretreatment and solid phase extraction on silica, aminopropyl, reversed phase or combinations of these have been applied. Noorooz-Zadeh and Appelqvist (1992) separated free sterols from oxides on a Lipidex-5000 column (Packard Int., Downers, Grove, IL) and then using an NH_2 cartridge (100mg, Analytichem Int.) achieved to concentrate the sterol oxide fraction. The recovery of the combined sample preparation process was 85-88%. The method was applied to isolate oxysterols from soyabean oils (fresh and stored for one year) and also from wheat flours. Schmarr *et al.* (1996) developed a routine screening method involving aminopropyl cartridges suitable to give an estimation of the distribution of oxysterols in fatty foods of animal origin. Mild transesterification of the lipids extracted on a Soxhlet apparatus using methyl tert-butyl ether (MTBE) was first carried out. To ensure reproducible retention pattern a small amount of anhydrous sodium sulfate was placed on the top of the cartridges (500 mg, amino-phase, BondElut) and the conditioning was made with hexane (5 ml). The transesterified lipid from 150-220 mg of fat dissolved in chloroform (250 μl). The sequence of solvents for the removal of the lipid matrix was: hexane (10 ml), hexane:MTBE, 5:1,v/v (5ml), hexane/MTBE, 3:1,v/v (5ml). Acetone was then used (7ml) to elute the fraction containing the oxides. It is interesting that the authors as many others preferred elution under gravity (flow rate 0.3-0.5 ml/min) to get the most of the retaining power of the sorbent. Recoveries were checked using coconut oil (free of cholesterol) which was subjected to the entire procedure. The recoveries for 7α-and 7ß-hydroxycholesterol, 19-hydroxycholesterol, 20α-hydroxycholesterol, 25-hydroxycholesterol, cholesterol -α and-ß epoxides, 7-ketocholesterol, 3ß,5-dihydroxy-5a-cholestan-6-one, cholestane-3ß,5α,6ß-triol were 86-107% as quantified after GC analysis on a SOP-50 column. Contamination of cholesterol was minimal (recovery 1.2%). Ulberth and Rössler (1998) compared several solid-phase extraction methods as the sole sample pretreatment for cholesterol oxidation products from milk fat prior to chromatographic analysis. In the same paper one can find tabulated a useful summary of previous relevant papers (e.g. Nourooz-Zadeh 1990; Chen and Chen 1994; Lai *et al.* 1995; Penazzi *et al.* 1995; Johnson 1996). The literature information was comparatively examined using Supelclean LC-NH_2 SPE (500 mg, Supelco), Sep-Pak classic silica (690 mg) and C_{18} (360 mg) cartridges (Waters). In all methods 500 mg of milk fat spiked with 30 mg of COPs was applied to the columns. A combination of silica SPE and NH_2 cartridges given in details in Scheme 2 allowed the optimum removal of interfering components and the reliable separation (free of spurious peaks) and quantification of COPs.

3. OTHER ALCOHOLS AND RELATED DERIVATIVES

Waxes: Waxes are esters of long chain alcohols with fatty acids. They are found in vegetable oils and

Step	Action
A	LC-Si SPE column (300mg/3ml); Conditioning with 3 ml hexane
B	Sample application
C	Elution with 10 ml *n*-hexane/ethyl ether (95:5,v/v); 25 ml *n*-hexane/ethyl ether [90:10,15ml *n*-hexane/ethyl ether (80:20, v/v), vacuum 29 kPa, solvent flow rate 0.6 ml/min v/v];
D	Elution of COPs with 5 ml acetone
E	Concentration of the eluate to 1ml of *n*-hexane/ethyl ether (90:10, v/v)
F	Application to NH_2-SPE column (500mg)
G	Elution with 15 ml of *n*-hexane/ethyl ether (90:10, v/v)
H	Elution of purified COPs with 10 ml acetone

Scheme 2
Combined elution of COPs using silica and NH_2 SPE columns (Lai *et al.* 1995; Ulberth and Rössler 1998).

their levels depend on the technology of extraction from fruits and seeds. The analysis of waxes is important in the quality control of dewaxing and winterization processes (Mariani *et al.* 1987). The composition of waxes varies in the various oils so that information can be obtained for the presence of inferior quality oils as it is established in the case of virgin olive oil (EC 1993). The wax esters are obtained from the oil after saponification and then analysed by capillary GC. Simplification of the sample preparation step has been attempted by several authors (Grob *et al.* 1989 and 1990; Amelio *et al.* 1993). Reiter *et al.* (1999), who applied for the first time SPE to isolate waxes from fennel and caraway seed oils, claimed that the procedure is applicable to other vegetable oils. The oil (30 mg/500 μl *n*-hexane) containing the internal standards was applied to silica cartridges (500mg/3 ml, Bakerbond, J.T. Baker) preconditioned with the same solvent. The sample was drawn through the column at a flow rate of 2.5 ml/min (controlled by a vacuum manifold processor) and the waxes were then eluted with n-hexane:diethyl ether (99:1, v/v) (10 ml). Recovery studies were very satisfactory. Yields from the capillary gas chromatographic analysis for stearic acid palmityl ester and behenic acid behenyl ester (40 mg/ml, each) ranged between 95-98% (SD%<5). Nota *et al.*, (1999) simplified the method of isolation of waxes of olive oil in an attempt to improve the existing official method for their determination. The separation was carried out on SPE cartridges (1g Chromabond, Macherey-Nagel) rinsed first with carbon tetrachloride (6ml). Oil (20 mg) plus the internal standard was transferred onto the column with a minimum amount of carbon tetrachloride (3x100μl) and the waxes were eluted with the same solvent (6ml). The speed of elution ranged between 0.5-1.5 ml/min. High levels of free acidity (up to 10%) did not lower the accuracy of the method.

Steryl esters: Sterols can also be found esterified with fatty acids. Steryl ester composition depends on the type of oil (e.g. Worthington and Hitchcock 1984; Boskou and Vlahopoulou 1986) and their content does not change significantly during refining as it happens with the free sterols. Thus, the determination of steryl esters is an aid to the identification of questionable oil samples (Kioseoglou *et al.* 1987; Gordon and Griffith 1992; Gordon and Miller 1997). This piece of information is lost during conventional sterol analysis, which involves saponification as the means for sample preparation. Many attempts are found in the literature concerning the determination of intact esterified sterols in oils and fatty foods. SPE has been used in some of them for the sample pretreatment prior to chromatographic analysis. Hopia *et al.* (1992) used SPE to separate the different lipid classes of edible oils prior to high performance size-exclusion chromatography. The authors preferred amino to silica columns to avoid water effects to cartridge efficiency. The recoveries for standard compounds of the nonpolar and polar fraction were >95%. Steryl esters were found in the nonpolar fraction. Mono- and diglycerides and free fatty acids occurred in the polar one. The sample size was 20-60 mg/500 mg SPE column. The nonpolar fraction was eluted with hexane:diethyl ether (9:1, v/v) (20ml).

4,4-dimethylsterols (triterpene alcohols): The triterpene erythrodiol and uvaol is commonly used to detect residue oil in virgin olive oil (Blanch *et al.* 1998). Amelio *et al.*(1992) used quaternary amino SPE columns to elute the two dialcohols during sterol determination. Bello (1992) was able to separate them on silica cartridges in the same run with sterols and squalene. The recovery for both alcohols was >85%.

4. POLYCYCLIC AROMATIC HYDROCARBONS

Polycyclic aromatic hydrocarbons (PAHs), one of the most contaminating groups of environmental pollutants may be found in oils and fats through various ways related to the seed drying, extraction technology, packaging materials or any other activity taken place from the field to the processing area (Moret *et al.*, 2000). Oils and fats represent one of the major sources of (PAHs) in the diet because of their lipophilic nature. Processed fatty foods, e.g. dried, roasted and smoked products, contain higher levels of PAHs compared with the untreated ones (Kayali-Sayadi *et al.* 1999; Moret *et al.* 1999; Roda *et al.* 1999). Thus, there is a trend to establish legal limits and implement regulations to control them because of the long-term adverse effects (mutagenic and carcinogenic) these compounds may induce to humans (Moret *et al.*, 2000). Certified materials help the analysts to validate their methods or even use standardised procedures (ISO, EN, etc.) (Win *et al.* 1998). All separation techniques, liquid and gas chromatography, supercritical fluid chromatography and capillary electrophoresis have been used for their analysis. HPLC coupled with a fluorescence detector is the most widely applied technique. Extraction, purification and pre-concentration of the sample prior to analysis is, therefore, a critical step considering the low amounts that are expected to occur in an oil.

In a recent review (Marcé and Borrull, 2000), a lot of attention was paid on technical details concerning the employment of SPE procedures for the isolation of PAHs. Indeed, though the elution of PAHs from C_{18} sorbents, used in most, should be straightforward, there are inherent drawbacks that should be taken into account (El Harrak *et al.* 1998; Kayali,-Sayadi, *et al.* 1999). Moret and Conte (2000) described in retrospect the analytical methods for the

determination of PAHs in edible oils and fats. Solid-phase extraction is basically used for the purification of extracts obtained using liquid-liquid extraction, caffeine complexation or saponification. All sample preparation methods aim at removing the triacylglycerols or other interfering compounds prior to analysis. The potential of solid-phase extraction in this field is not so promising and other emerging techniques are powerful means for sample preparation and determination to address the needs of organic trace analysis (Moret and Conte 1998; Dadoo and Zare 1998).

5. PHENOLIC COMPOUNDS

Phenolic compounds are transferred from fruits and seeds to raw oils during processing. Refining eliminates the content of phenolic compounds, therefore, their presence is mainly of interest in virgin olive oil and to a certain extent in cold-pressed seed oils, e.g. cold -pressed canola oil. In recent reviews and papers the importance of phenolic compounds in the sensory quality and the stability of virgin olive oil and their effect to human health has been discussed in details (Tsimidou 1998; Morales and Tsimidou, 2000; Owen *et al.*, 2000). The major phenolics found in virgin olive oil are hydroxytyrosol, tyrosol, aldehydic secoiridoids, flavonoids and lignans (acetoxypinoresinol, pinoresinol) (Pirisi *et al.*, 2000; Brenes *et al.*, 2000). The content of these natural antioxidants depends on the olive oil processing (two-phase extractors yield in general oils with more phenols) and storage conditions. Except from the colorimetric procedures using the Folin-Ciocalteu reagent for the determination of total phenols, gas chromatographic methods and, basically, HPLC procedures have been used for the analysis of the oil fraction containing the phenolic compounds (Ryan and Robards 1998).

Liquid-liquid extraction and more recently solid-phase extraction have been used for the isolation of the so-called “polar fraction”. The solvent system usually applied is aqueous methanol in various proportions. Tsimidou (1999) has recently reviewed the advances in extraction and analysis of virgin olive oil polyphenols. Ruiz-Gutiérrez and Pérez-Camino (2000) presented also applications of solid -phase extraction of phenolic compounds of virgin olive oil. A thorough list of applications found in literature either for the extraction or for the clean-up of the polar fraction is given in Table I. C_{18} phases appear to be used in most applications. The first systematic study was carried out by Papadopoulos and Tsimidou (1992), who, in a comparative study of two SPE systems and a liquid-liquid one, found, using HPLC, that polyvinylopyrrolidone (PVP) traps selectively the phenolic compunds. C_{18} cartridges of the same bed volume and with the same solvent system did not retain the bound forms of phenolic compounds. Recovery studies using hydroxytyrosol and tyrosol on an oil matrix (40 mg/kg each) gave yields of ~50% and 90%, respectively. Recoveries

Table I
Application of SPE to virgin olive oil phenolics

Reference	SPE column; bed volume	Sample application	Elution system, v/v/v	Comments
Gutiérrez et al. (1989)	C_{18} (Baker Chem. Co.); 6 ml	1g oil/4ml hexane	Conditioning: 10ml hexane; Elution: water:methanol (30:70)	
Papadopoulos and Tsimidou (1992)	a)PVP (Rigas Labs); 100mg/ml b) C_{18} (Analytichem Int.) 100mg/ml	1g oil/2.5 ml hexane	Conditioning: 10ml hexane; Elution: (3x2ml) hexane; (2x2 ml) ethylacetate; (15x2 ml) methanol; (2x2 ml) methanol:water (60:40); (1x2ml) water	Comparison with liquid-liquid extraction; vacuum 15-25 inHg (BondElut/VacElut system)
Mannino et al. (1993)	C_{18} (BondElut),6 ml	0.5 g oil	Conditioning: 2ml hexane:ethylether (98:2); Elution: 10 ml hexane:ethylether (98:2); 8ml methanol	
Andreoni and Fiorentini (1995)	NH_2(BondElut SAX), 50 mg	0.4-1 g oil	Conditioning: methanol, $CHCl_3$, n-hexane and buffer. Elution: (5x2.5ml) methanol:water(1:1); (5x2.5ml) 1mM ammonium acetate in methanol:water(1:1)	Colorimetric determination of phenols
Favati, F. et al. (1995)	C_{18} (J.T. Baker), 1g/6ml	1g oil/10ml hexane	Conditioning: (2x5ml) methanol and (2x5 ml) hexane Elution: (3x5 ml) hexane; (2x5ml) methanol.	
	CN or Florisil (Varian), 1g/6ml	5g oil/50 ml hexane	Conditioning: (4x5ml) methanol and (2x5 ml) hexane. Elution: 25 ml hexane, (4x5 ml) methanol.	
Pirisi et al., (1997; 2000)	C_8 (Alltech), 500mg/3ml	1g oil/10 ml hexane	Conditioning: 10 ml n-hexane, 10 ml acetonitrile. Elution: 10 ml n-hexane: cyclohexane (1:1); 2.5 ml acetonitrile	Vacuum 30 mm Hg, flow rate 1.0 ml/min
Litridou et al. (1997)	C_{18} (Millipore Waters),	Polar fraction from 50 g oil	See text for details on the collection of 40 different fractions	Colorimetric and HPLC analysis.
Servili et al.(1999)	ExtractClean highload C_{18} (Alltech), (5g/20ml)	5g oil	Conditioning: 20 ml hexane or hexane;ethyl ether (98:2). Elution: a) 100ml of conditioning solvent; 80 ml methanol b) methanol:Tween 20(98:2,v/w) c) methanol:Tween 80(98:2, v/w) d) methanol:Triton X-100 (98:2,v/w)	Comparison with liquid-liquid extraction systems
Liberatore et al.(2001)	C_{18} (2g/6ml) and C_{18} end capped (2g/6ml) (Int.Sorb. Tech. Hengoed, U.K.)	3g oil/15 ml hexane	Conditioning: (2x10 ml) methanol and (2x10 ml) hexane Elution: (4x10ml) hexane; (4x10 ml) methanol	Gas chromatographic analysis of phenolic compounds

with the liquid-liquid system were better. It is worth mentioning that a selective elution of polyphenols on a PVP: celite glass column had been carried out by Solinas and Cichelli (1981) some years ago. The authors had claimed that the uptake of phenols was inversely proportional to the dielectric constant of the organic eluent. Mannino *et al.* (1993) reported higher recoveries (tyrosol, 98%, vanillic acid 96% and gallic acid 101%) but they did not refer the level of addition of the standards detected using an electrochemical system. Andreoni and Fiorentini (1995) reported their first attempts to fractionate further the polar fraction with methanol: water (1:1) and 1M ammonium acetate in the same mixture as eluents. Three different packing materials, cyano, C_{18} and Florisil (Mg_2SiO_3) were tested for their efficiency in retaining the phenolics of virgin olive oil by Favati *et al.* (1995). The results were then compared with those obtained by two common liquid-liquid methods. The reduction in overall analysis time as well as the almost 100% recovery for three levels of tyrosol addition (10, 330 and 550 mg/kg) are the two main findings when the CN column was used. Cortesi *et al.* (1995) studying in parallel the merits of phenol extraction with aqueous methanol or aqueous tetrahydrofuran used C_{18} cartridges to purify the polar fraction obtained with methanol prior to HPLC analysis. In papers on the quantitative determination of complex phenols Pirisi *et al.* (1997 and 2000) describe the employment of C_8 (500mg/3ml, Alltech) cartridges for the isolation of phenolic compounds from olive oil (recovery: 70-105 %).

Aiming at the assessment of the antioxidant activity of the two main subgroups (A and B) of the polar fraction of any virgin olive oil (Figure 1), Litridou *et al.* (1997) collected forty different fractions using different proportions of methanol:water. After RP-HPLC examination of the fractions they concluded that simple phenols could be eluted using methanol:water (20:80, v/v) whereas the combined

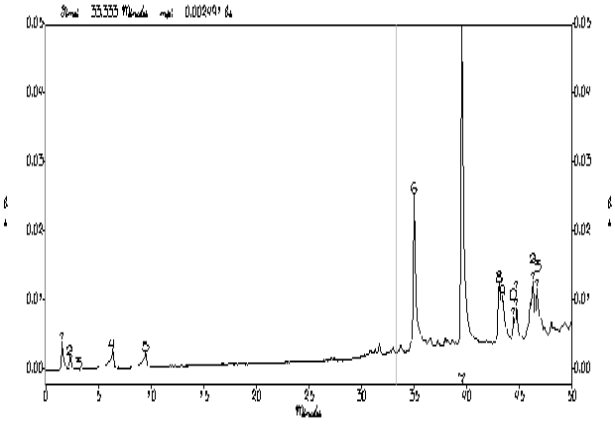

Figure 1
Olive oil phenolic components. Peaks with retention time greater than 30 min correspond to compounds of part A (simple phenols and acids); Peaks with retention time lower than 30 min correspond to compounds of part B (bound forms) [M. Tsimidou, unpublished data].

forms required a more hydrophobic system consisting of methanol:water (80:20, v/v), pure methanol and finally chloroform.

In a thorough study published in 1999, Montedoro and co-workers added to knowledge of the phenols present in olive fruit, virgin olive oil, vegetation waters and pomace (Servili *et al.* 1999). They used ExtractClean highload C_{18} (Alltech) columns (5g/20ml) in the case of the oil and compared the results with those derived from liquid-liquid extraction using various nonionic surfactans (Montedoro *et al.* 1992). A detailed table gives information for the recoveries of ten simple or complex constituents. Methanol led to the highest recovery in total phenols whereas no significant differences were found between the yields of phenols and phenolic acids. The four elution systems used are given in Table I.

In 2001 (Liberatore *et al.*) observed the non-homogeneous and even contradictory results concerning the recovery of phenolics using different methods. They reported recovery data for eight representative olive oil phenols (tyrosol, p-hydroxybenzoic, vanillic, protocatechuic, syringic, p-coumaric, ferulic and caffeic acids) at two levels of addition (50 and 500 mg/kg) using three different extraction procedures. The efficiency of two SPE procedures (C_{18} and C_{18} end capped) was compared with that of a liquid-liquid extraction previously reported by Tsimidou *et al.* (1992). End capped reversed-phase was proved less efficient than C_{18}. Suppression of the residual polar groups of silica had an adverse effect on the release mechanism of the analytes. Still, the recovery data were not consistent at the two levels of addition to permit a better understanding of retention of components of similar molecular weight and polarity.

6. PHOSPHOLIPIDS

Phospholipids contribute to the stability and quality of edible oils, fats and fatty foods through their antioxidative activity or contribution to the texture (Singleton and Stikeleather 1995; Carelli *et al.* 1997). On the other hand, they are responsible for oil discoloration during deodorization and steam distillation so that their determination is necessary to evaluate the efficiency of degumming (Mounts and Nash 1990; Mounts *et al.* 1992; Nzai and Proctor 1998). Some oils contain substantial amounts of phospholipids, e.g. soyabean oil, sunflower oil and canola oil or peanut oil (Przybyski and Eskin 1991; Smiles, *et al.* 1988). The most important members of this class of lipids found in edible fats are phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and phosphatidyl choline (Singleton and Stikeleather 1995; Boskou 1996; Carelli *et al.* 1997; Mounts and Nash 1990). Egg yolk and milk fat contain significant amount of

sphingomyelin, too (Ramesh et.al. 1978; Gunstone, *et al.* 1994). The fatty acid composition of the various phospholipids depends on the fat origin (Lendrath *et al.* 1991). Fish oils contain similar to mammalian oil phospholipids. After refining oils and fats of vegetable origin have rather low amounts so that a concentration step is needed prior to HPLC, which is the preferred method that replaced thin layer chromatography (Singleton 1993; Nzai and Proctor 1998).

SPE has been employed in certain cases for the pre-concentration of phospholipids. Nash and Frankel (1986) fractionated degummed, crude soybean oils using silicic acid columns (Sep-Pak, Waters Assoc.) prior to HPLC analysis. Oil, 1g of a 10% solution in petroleum ether:diethyl ether (95:5, v/v), was applied, the nonpolar lipids were eluted with the same system (10 ml), the intermediate polarity lipids were eluted with diethyl ether (20 ml) and finally the phospholipids were obtained with methanol (10 ml). The applicability of the system was checked by thin layer chromatography of the various fractions. Caboni *et al.* (1996) separated phospholipids from total lipids that had been extracted from food samples (egg powder, chicken meat, ripened cheese and salami) by the Folch method. The lipid extract was purified by using normal phase (silica) ion exchange (aminopropyl) and reversed–phase (C_{18} and C_8) SPE cartridges (500 mg, BondElut, Varian). The purified fractions were analyzed by HPLC coupled with an evaporative light-scatttering detector. Details for the four SPE elution systems are given in Table II.

Based on phosphorous determination in the various fractions, the authors reported that phospholipids eluted in the third fraction from polar phases (silica and amino) and in the first fraction derived from the reversed-phase bonded materials. Though precision data were satisfactory in all four procedures, accuracy data varied significantly. C_8 packing was proved more efficient for individual and total phospholipid recoveries.

Carelli *et al.* (1997) enriched sunflower oil samples with phospholipids using SPE cartridges and subsequently performed HPLC analysis based on the IUPAC standard method for soybean lecithin and ultraviolet detection. The bonded diol cartridges (500 mg, J.T. Baker) were preconditioned with methanol (2ml), chloroform (2ml) and hexane (4ml). From the oil dissolved in chloroform (50-150 mg) triglycerides were removed with chloroform (2.5 ml). Phospholipids were eluted with 7ml methanol that contained 0.5 ml/100 ml of a 25% ammonia solution. Accuracy and precision data reported were quite satisfactory. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol were sequentially eluted from an aminopropyl bonded silica with acetonitrile/n-propanol (2:1, v/v), methanol, isopropanol/methanolic HCl (4:1, v/v) and methanol/methanolic HCl (9:1, v/v). The recoveries were excellent (Pietsch and Lorenz 1993).

7. VITAMINS

The analysis of lipophilic vitamins is a well investigated area of analytical chemistry. In a recently published book, Eitenmiller and Landen (1999) carefully reviewed analytical methods for all vitamins and related compounds. They incorporated long tables with detailed information not only for compedium, regulatory and handbook procedures but also complete lists for methods reported in scientific papers. Sample matrix, analytes, sample preparation (extraction and clean up) and further details (columns, elution and detection systems, validation data) for the chromatographic analysis of lipophilic vitamins were given for each reference. The demand for precise determination of vitamins is steadily increasing the last 20 years. For example, products as infant formula, medical and nutraceutical products containing mixtures of vitamins should be in compliance with the requirements for nutrient labeling analyses in line with the evolved legislation in the USA, Canada and the European Union (Waysek 1993). Developments in the packing

Table II
SPE Separation of phospholipids from fatty foods according to Caboni et al. (1996)

SPE mode *	Lipid sample	Conditioning solvent system	Elution system (v/v/v)
Silica	200 mg / 0.5ml $CHCl_3$	3 ml *n*-hexane	4 ml *n*-hexane:diethyl ether (8:2); 4 ml *n*-hexane:diethyl ether (1:1); 4 ml methanol or 4ml 1-5% CH_3COOH in methanol
Amino-propyl	200 mg / 0.5ml $CHCl_3$	3 ml *n*-hexane	2x2.5 ml $CHCl_3$:isopropanol (2:1); 2x2.5 ml 2% CH_3COOH in diethyl ether; 4x1ml methanol
C_{18}	200 mg / 0.5ml $CHCl_3$:methanol (2:1, v/v)	3 ml methanol	4ml methanol/water (4:1); 4ml methanol; 4ml methanol:$CHCl_3$ (4:1)
C_8	200 mg / 0.5ml $CHCl_3$:methanol (2:1, v/v)	3 ml methanol	4ml methanol; 5ml $CHCl_3$: methanol (3:2) 5ml $CHCl_3$

* Column regeneration with 3ml $CHCl_3$; 3 ml $CHCl_3$: methanol (2:1); 5 ml methanol.

materials (polymeric C_{18} or C_{30} columns) facilitated resolution of the many isomeric compounds of retinoids, carotenoids and tocopherols (Epler *et al.* 1992; Sander *et al.*, 2000). Vegetable oils and fats are good sources of carotenoids having provitamin A activity (in particular ß-carotene) and tocopherols whereas vitamin K_1 is found at very low levels.

ß-Carotene: The multiple functions of carotenoids have drawn the interest of investigators from many scientific fields. Their content in vegetable fats is eliminated during refining. Chromatographic analysis on normal or reversed-phase columns is well-documented (Britton 1985; Tee and Lim 1990; Oliver and Palou, 2000). However, no application was found for the use of SPE prior to analysis of oils and fats.

Tocopherols: Natural vitamin E is a complex of eight vitamers having different biological activity. Tocopherols are commonly determined separately from the other lipid classes. The preferred HPLC mode is normal phase (silica, amino or diol packings) (Kamal-Eldin *et al.*, 2000). Separation on normal phase columns does not require any sample pretreatment so that SPE applications are limited (Hopia *et al.* 1992; Lechner *et al.* 1999).

Vitamin K: The role of vitamin K in hemostasis and its contribution to the synthesis of certain proteins (Gla) is an area of concern for nutritionists and epidemiologists. On the other hand, the control of K_1 (phylloquinone) and menaquinones (formed by the intestinal flora in the colon) in foods is important for those undergoing anticoagulant therapy, who should avoid foods rich in vitamin K. The richest sources of dietary vitamin K_1 are green and leafy vegetables (Ferland and Sadowski 1992). Human milk is low in vitamin K content a fact that explains the fortification of infant formula (Huang 1985). Oils contain extremely low amounts of K_1, the responsible for the antihemorrhagic activity of the vitamin, (average< 2mg/kg; soyabean>rapeseed>olive>sesame>walnut >safflower>almond>corn>peanut oils) (Jakob and Elmadfa 1996). The level of vitamin K_1 in any oil seems to be closely affected by the origin of the oil, the technology and the overall handling conditions (Gao and Ackman 1995). Menaquinones are predominantly present though at low levels in cheese.

Methods to determine the very low levels of K vitamers (ppb range) in oils and fats are mainly liquid chromatographic. Extraction with an organic solvent is preceded taking into account that vitamin K is degraded under alkaline conditions. The extract is subsequently purified to avoid interference during analysis. Ferland and Sadowski (1992) reported the use of SPE on silica (3.0 ml, J. T. Baker Inc.) for the purification of oil extracts with hexane prior to RP-HPLC using post-column chemical reduction of the vitamin to its fluorescent hydriquinone. Oil (0.25 –1.0 g) together with an appropriate amount of dihydrovitamin K_1 as internal standard was subjected to extraction with hexane (3 min, vigorous shaking). An aliquot of the extract was applied to the preconditioned with hexane SPE column, a further washing with hexane (8.0 ml) followed and then vitamin K_1 was eluted with an equal volume of hexane-diethyl ether (97:3, v/v). A similar purification system performed on semi-preparative silica column (µPorasil, 5µ, 300x3.9 mm) by Piironen *et al.* (1997) could be with some changes potentially adopted as an SPE procedure for on-line SPE-HPLC analysis. The group of Sadowski (Booth *et al.* 1994) extended their work in fatty and non-fatty matrices (vegetable juice, whole milk, raw spinach leaves, plain bagel and lean raw ground beef). SPE silica columns were employed in all cases to purify the hexane extracts according to the above-mentioned protocol. The extract from beef was cleaned up on a C_{18} SPE column to separate phylloquinone from the large amounts of saturated fats. Canola oil is an important source for dietary lipids for Canada and many of the Nordic countries. Evaluating the K_1 content of this oil Gao and Ackman (1995) enzymatically hydrolysed the lipids and then purified the extract on silica SPE columns. In this way residual non-polar or fluorescent closely eluted to the internal standard interfering compounds were eliminated.

8. PARTIAL GLYCERIDES AND FREE FATTY ACIDS

The analysis of partial glycerides, mono-and diglycerides is usually carried out by capillary gas chromatography. HPLC may only give information on the total amounts of different classes of partial glycerides (e.g. 1,2- and 1,3- diglycerides) (Cortesi *et al.* 1992). These compounds, intermediates in the biosynthesis of triglycerides, are also markers of oil freshness or of employed technological treatments. Pérez-Camino *et al.* (1996) reported on alterations on diglyceride composition using different types of SPE columns (diol, silica, amino). They found that only the diol phase did not cause isomerization during the extraction. In their recent review (Ruiz-Gutiérrez and Pérez -Camino, 2000), the authors commending on these and other literature data stated that" a lot of care must be taken before choosing a support until more explanation and importance is given to the absorbent employed". Similar observations were also made by Conte *et al.* (1997).

Free fatty acid content is not a problem in the analysis of vegetable fats that are consumed refined with the exception of virgin olive oil. Hopia *et al.* (1992), describing the analysis of lipid classes by solid-phase extraction gave information for the elution of free fatty acids from edible oils and fats. Procedures related to this group of lipids is found for

other categories of food products, where the level and composition of free fatty acids, in particular short chain ones, affect the organoleptic characteristics of the finished products (e.g. cheese) (Innocente *et al.*, 2000).

9. CHLOROPHYLL PIGMENTS

Among all the vegetable oils, VOO is consumed containing high amounts of chlorophyll pigments. Chlorophyll pigments are usually removed during refining. Analysis of chlorophylls in oils presupposes careful extraction or direct injection onto the chromatograph to avoid the formation of artifacts (Psomiadou and Tsimidou, 2001). Mínguez-Mosquera *et al.* (1992) reported a first attempt of the separation of individual chlorophyll pigments using C_{18} SPE cartridges preconditioned with methanol and then with hexane. The sample (1g oil/4ml hexane) applied onto the cartridge was first washed off from glycerides with hexane (3 ml) and then the pigments were eluted with acetone (5ml). Still, ß-carotene and part of pheophytin α eluted in the hexane fraction. In the acetone fraction many other carotenoids, and chlorophyll pigments were identified through HPLC. No recovery data were reported.

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