Recent trends in the advanced analysis of bioactive fatty acids

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Abstract

The consumption of dietary fats have been long associated to chronic diseases such as obesity, diabetes, cancer, arthritis, asthma, and cardiovascular disease; although some controversy still exists in the role of dietary fats in human health, certain fats have demonstrated their positive effect in the modulation of abnormal fatty acid and eicosanoid metabolism, both of them associated to chronic diseases. Among the different fats, some fatty acids can be used as functional ingredients such as alpha-linolenic acid (ALA), arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), γ-linolenic acid (GLA), stearidonic acid (STA) and conjugated linoleic acid (CLA), among others. The present review is focused on recent developments in FAs analysis, covering sample preparation methods such as extraction, fractionation and derivatization as well as new advances in chromatographic methods such as GC and HPLC. Special attention is paid to trans fatty acids due its increasing interest for the food industry.

Key Words: fatty acids, CLA, extraction, derivatization, fractionation, chromatography, trans fatty acids, bioactivity

Abbreviations: AA, Arachidonic acid; ADHD, Attention-deficit hyperactivity disorder; Ag^+-HPLC, Silver-ion HPLC; AHE, Automated acid hydrolysis-extraction; ALA, Alpha-linolenic acid; APF, 6-oxy-(acetyl piperazine) fluorescein; ASE, Accelerated solvent extraction; BDETS, 1,2-benzo-3,4-dihidrocarbazole-9-ethyl-*p*-toluenesulfonate; BDEBS; 1,2-benzo-3,4-dihydrocarbazole-9-ethyl-benzenesulfonate; BSTFA, *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide; CLA, Conjugated linoleic acid; CME, Capillary microextraction; CMTS, Chlorotrimethylsilane; CN-PDMS, Cyano- polydimethylsiloxane; CW-DVB, Carbowax-divinylbenzene; DHA, Docosahexaenoic acid; DMC, Dimethyl carbonate; DMF, Dimethyl formamide; DMOX, Dimethyloxazolyne; EPA, Eicosapentaenoic acid; EPMF, 6-oxy-(ethylpiperazine)-9-(2´-methoxycarbonyl)fluorescein; FAs, Fatty acids; FAMEs, Fatty acid methyl esters; FFAs, Free fatty acids; FID, Flame ionization detector; FTIR, Fourier transform infrared spectroscopy; GC, Gas chromatography; GL, Glycolipids; GLA, Gamma-linolenic acid; HDL, High- density lipoproteins; HMDS, Hexamethyldisilazane; HPLC, High performance chromatography; LA, Linoleic acid; LCFA, Long chain fatty acids; LDL, Low-density lipoprotein; MCFA, Medium chain fatty acids; SBSE, Stir bar sorptive extraction; MIS, Microwave-integrated soxhlet; MS, Mass spectrometry; MSD, Mass selective detector; NL, Neutral lipids; PA, Polyacrylate; PDMS-CAR, Polydimethylsiloxane-Carboxen; PDMS-CAR-DVB, Polydimethylsiloxane-Carboxen- divinylbenzene; PDMS, Polydimethylsiloxane; PDMS-DVB, Polydimethylsiloxane-divinylbenzene; PL, Phospholipids; PLE, Pressurized liquid extraction; PUFAs, Polyunsaturated fatty acids; SDE, Simultaneous distillation-extraction; SPE, Solid phase extraction; SPME, Solid-phase micro-extraction; STA, Stearidonic acid; THM, thermally assisted hydrolysis and methylation; TLC, Thin-layer chromatography; TMAE, trimethylaminoethyl ester; TMAH, Tetramethylammonium hydroxide; TMCS, trimethylchlorosilane; TMS-DM, Trimethylsilyldiazomethane; TMSH, Trimethylsulfonium hydroxide; TSPP, 1-[2-(*p*-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f]-9,10-phenanthrene; UFAs, Unsaturated fatty acids; USE, Ultrasound-assisted extraction; UV, ultraviolet-visible; VFAs, Volatile fatty acids; VLCFA, Very long-chain fatty acids.

Contents

1. Introduction

Dietary fats are the most targeted nutrients related to chronic diseases such as obesity, diabetes, cancer, arthritis, asthma, and cardiovascular disease. The public perception of fats, in general, is not favorable. However, while there is some truth in this perception, a wide range of nutrients and non-nutrients can be used as functional ingredients in fats and spreads, providing specific health benefits [1]. The benefits and functional roles of dietary fat in providing calorie density to the diet, as a source of essential fatty acids, and as a vehicle for fat-soluble vitamins, nutrients and antioxidants for the human body have been long established. Consumption of certain foods or fatty acid supplements can modulate abnormal fatty acid and eicosanoid metabolism, both of them associated with the above mentioned chronic diseases. Among all the fats, certain fatty acids have the potential to be used as functional ingredients since their intake has been found positively related to health [2 - 8, to name a few].

Fatty acids are aliphatic monocarboxylic acids which act as the building blocks of lipids. These can either be saturated, monounsaturated or polyunsaturated depending on the number of double bonds. They differ in length as well, having a chain of 4 to 28 carbons. Long chain fatty acids (LCFA) are fatty acids with aliphatic tails of 16 or more carbons [9]. This group of fatty acids includes the polyunsaturated fatty acids (PUFAs), which are fatty acids containing two or more double bonds. There are two principal families of PUFAs - the omega-3 and the omega-6 families. Although there are several systems of nomenclature, fatty acids are most commonly expressed by their trivial names e.g. "linoleic acid (LA)". In *delta-x* nomenclature each double bond is indicated by Δ^x, where the double bond is located on the xth carbon-carbon bond counting from the carboxylic acid end. Each double bond is preceded by a *cis*- or *trans*- prefix, indicating the conformation of the molecule around the bond. For linoleic acid, a polyunsaturated fatty acid of 18 carbon atoms with two double bonds, the exact structure is then given by the systematic name *cis*-9, *cis*-12-octadecadienoic acid, and by the abbreviation 18:2 *cis*,*cis* Δ^9,Δ^{12} [10, 11]. The *n-x* nomenclature is shorthand to categorize fatty acids by their physiological properties. A double bond is located on the xth carbon-carbon bond, counting from the terminal methyl carbon (designated as *n* or ω) toward the carbonyl carbon. For example, α-Linolenic acid is classified as n-3 or omega-3 fatty acid, and so

it shares properties with other compounds of this type. Since humans cannot synthesize double bonds at position 6 or lower, omega-3 (n-3) and omega-6 (n-6) PUFAs are essential fatty acids necessary for human health and must be obtained from the diet. The parent compound of the n-6 family, linoleic acid (LA) (C18:2, cis,cis Δ^{9},Δ^{12}, n-6) is plentiful in nature, and it is found in the seeds of most plants being its content high in oils commonly used in cooking, such as corn and sunflower oil [12]. Alpha-linolenic acid (ALA) (C18:3, *cis,cis,cis* Δ^{9},Δ^{12},Δ^{15}, n-3), the parent compound of the omega-3 family, is far less common and is found primarily in soya bean, rapeseed and flaxseed oil. Both α-linolenic acid and linoleic acid can be elongated and desaturated to long chain PUFAs: linoleic acid to arachidonic acid (AA) (C20:4, *cis,cis,cis,cis* $\Delta^{5}\Delta^{8}$,Δ^{11},Δ^{14}, n-6), and α-linolenic acid to eicosapentaenoic acid (EPA) (C20:5, *cis,cis,cis,cis,cis*-Δ^{5},Δ^{8},Δ^{11},Δ^{14},Δ^{17}, n-3) and docosahexaenoic acid (DHA) (C22:6, *cis,cis,cis,cis,cis,cis*-Δ^{4},Δ^{7},Δ^{10},Δ^{13},Δ^{16},Δ19, n-3).

PUFAs, along with γ-linolenic acid (GLA) (C18:3, *cis,cis,cis* Δ^{6},Δ^{9},Δ^{12}, n-6) and stearidonic acid (STA) (C18:4, *cis,cis,cis,cis*-$\Delta^{6}\Delta^{9}$,Δ^{12},Δ^{15}, n-3) have shown biologically beneficial effects. An increased intake of ALA has been associated with a decrease in blood cholesterol levels, a reduction in the risk of heart attacks, and a growth reduction in breast, colon and prostate cancer [13-16]. ALA is the precursor of EPA and DHA and it is also stated to have an effect in the production of different eicosanoids, and in lowering the blood pressure as well as blood triglyceride levels [17]. LA has become increasingly popular in cosmetics because of its beneficial properties on the skin. Research points to linoleic acid´s effective properties when applied topically on the skin, i.e. anti-inflammatory, acne reduction, moisture retention properties [18, 19]. Linoleic acid has also been found to actively lower the serum cholesterol [20, 21]. On the basis of the inverse relationships between linoleic acid intake and plasma cholesterol concentration, and the link between plasma cholesterol concentration and the incidence of coronary heart diseases, advisory agencies in Western countries have long recommended increasing linoleic acid intake to 4-10% of energy intake [22, 23]. Gamma linolenic acid is reported to feature anti-inflammatory effects as well as anti-cancer actions [24, 25], as well as STA, which is reported to show anti-inflammatory properties in the treatment of diseases such as cystic fibrosis due to the inhibition of leukotriene B4 synthesis [26 - 28]. The long-chain n-3 PUFAs, such as EPA and DHA have the ability to prevent and treat hypertension, arthritis, inflammatory and

autoimmune disorders, as well as cancer [29-38]. However, the hallmark effect of these two PUFAs is the decrease in plasma triglyceride concentration and therefore to reduce the risk of fatal coronary heart diseases [39 – 42] mediated by several mechanisms, including reduction of triglyceride synthesis and chylomicron secretion from intestinal cells in addition to suppression of fatty acid synthesis and triglyceride production in the liver [43]. In addition to all the beneficial properties described above, it has been reported that low levels of DHA result in reduction of brain serotonin levels [44] and have been associated with ADHD, Alzheimer´s disease, and depression, among other diseases, and there is mounting evidence that DHA supplementation may be effective in combating such diseases [45, 46]. Arachidonic acid is also one of the essential fatty acids required by most mammals and is accumulated rapidly in the developing brain during the last trimester of gestation [47]. A deficit in AA results in reduced growth in infants [48] and it has also been stated that infant formulas supplemented with AA from fungal oil enhanced the growth of prematural infants [49]. Arachidonic acid supplementation has also shown beneficial effects in human amnesic patients, improving the cognitive dysfunction due to organic brain damages or aging [50]. Arachidonic acid is also involved in the growth suppression of human lung tumor A549 cells [51].

Other long-chain fatty acids which feature beneficial effects are the conjugated linoleic acid (CLA) and the pinolenic acid. Conjugated linoleic acid consists of a series of positional and geometric isomers of conjugated derivatives of linoleic acid and they are mainly *trans* fatty acids. Among all the isomers, only 18:2 *cis,trans* Δ^{9},Δ^{11} and 18:2 *trans,cis* Δ^{10},Δ^{12} are considered biologically significant and active. Surprisingly, even if *trans* fatty acids have been generally regarded as detrimental to health [52], in recent years CLA has attracted a great deal of attention because of its potential biologically beneficial effects in attenuating many chronic diseases. Different CLAs are reported to be anti-cancer, anti-atherogenic, anti-adipogenic, anti-diabetogenic and/or anti-inflammatory [53], and the use of dietary CLA has been suggested to have a chemopreventive effect in animal prostate [54, 55], gastrointestinal [56] and skin [57] cancer, although the anti-carcinogen effects are exerted irrespective of differences in position or geometric distribution among different isomers [58]. CLAs have been reported to elicit beneficial effects on a variety of other important biomarkers of disease related to diabetes prevention [59] and reduction of cholesterol-induced atherosclerotic

lesions in aortic arch and thoracic aorta in a number of animal models by plaque regression [60-62]. Mixtures of CLA isomers have also been found to decrease body fat in humans by 3% through a modification of eicosanoid synthesis [63] and to increase the level of antioxidant enzymes in cells [64]. On the other hand, studies on the pinolenic acid (18:3, *cis,cis,cis* $\Delta^5,\Delta^9,\Delta^{12}$) have proven beneficial functions in the human body: decreasing lipids, increasing HDL and prostacyclin, decreasing blood pressure, inhibiting aggregation of platelets as well as anti-inflammatory properties [65, 66]. It has also stated that pinolenic acid may have LDL-lowering properties due to enhancing of hepatic LDL uptake [67].

The above mentioned fatty acids may be found in free form, but in general they are combined in more complex molecules through ester or amide bonds. The most common procedures used to measure free fatty acids (FFA) concentration and fatty acid enrichment consist in multiple-steps methods involving (a) an extraction procedure to extract lipids from the sample, generally based on Folch or a modified Folch method; (b) the isolation of FFA from the rest of the lipids by thin-layer chromatography (TLC) and/or solid phase extraction (SPE); (c) derivatization of FFA to fatty acid methyl esters (FAMEs), and (d) the FAMEs extraction for the final chromatographic determination. The chromatographic determination of FAMEs and the separation into individuals is by far mostly done using capillary gas chromatography (GC), and less frequently by high performance liquid chromatography (HPLC) [68-71].

This review is focused on recent developments in FAs analysis, covering sample preparation methods such as extraction, fractionation and derivatization as well as new advances in chromatographic methods such as GC and HPLC. Special attention is paid to *trans* fatty acids due its increasing interest for the food industry.

2. Sample preparation

2.1 Fatty acid extraction techniques

Accurate determination of FA compositions of different matrix-bound FAs is a predominant problem in total fat extraction. A broad range of extraction techniques (Folch, Blight & Dyer, Soxhlet, percolation, maceration, digestion, steam distillation,

etc.) are currently used for this purpose. Traditional or modified Folch procedure, which employs chloroform/methanol (2:1 v/v), or the commonly called Bligh and Dyer method, in which a chloroform/methanol/water mixture is used to separate the lipids from all the non-lipids in samples containing 80% water, are generally employed in laboratories to quantitatively extract lipids. Exhaustive Soxhlet extraction is probably the most commonly used technique for the extraction of fats and oils from food matrices [72, 73]. Petroleum ether is the solvent recommended by AOAC for fat determination using the Soxhlet method; however, "n-hexane" has also been used for defatting and for analysis of fat, lipids and fatty acids in cereals [74], oil seeds [75], egg [76], milk [77] and peanuts [78], among others. Almazan and Adeyeye performed a comparative study uing hexane and petroleum ether in the fat extraction of different food commodities and reported that the results were different not only in terms of extraction yield but also in terms of fatty acid composition [79]. Besides the simplicity of Soxhlet methodology, it presents some disadvantages such as long extraction time, relatively high solvent consumption and poor reproducibility [80], and in this sense, much work has been done to improve the Soxhlet extraction in terms of time and solvent use [81]. An important improvement of the Soxhlet extraction device was developed by Randall, who proposed a three-step extraction namely: boiling, rinsing and solvent removal [82]. More recently, Virot et al. proposed an improved process of Soxhlet extraction assisted by microwave, called microwave-integrated Soxhlet (MIS) for the extraction of fats from different food matrixes such as oleaginous seeds, meat and bakery products. Few articles have been reported in the literature using MIS; the advantages of using microwave energy as a non contact heat source include: more effective heating, faster energy transfer, reduced thermal gradients, selective heating, reduced equipment size, faster response to process heating control, faster start-up, increased production, and elimination of process steps [83]. MIS process involves the use of polytetrafluoroethylene/graphite (Weflon) stir bar. Weflon allows diffusion of heat created by the microwaves to the surroundings and is particularly useful for solvents transparent to microwave radiations such as hexane. MIS was compared with conventional Soxhlet extraction in the extraction of fats from peanuts, sunflower seeds, beef steaks and croissants. The authors concluded that the MIS process provides a valuable alternative for fats and oils extraction. After 32 min of MIS extraction, it is possible to extract and concentrate the total amount of fat from foodstuffs, whereas conventional Soxhlet method requires 8 h with an additional step to

concentrate fats and oils by evaporation of the solvent using a vacuum rotatory evaporator [84].

Other solvent extraction methods have been evaluated for the extraction of fat from different food products. Alabalá-Hurtado et al. made a comparison between the Pont and the pentane fat-extraction methods from powdered infant milk. The pentane fat-extraction method [85] uses pentane to extract the fat after shaking in the absence of heating, whereas in the Pont method a de-emulsification reagent is used to release the fat from the food matrix, followed by heating at 70 ^{o}C and centrifugation [86]. Pont method gave better recoveries than the pentane method, however, the peroxide values, calculated to measure the oxidative damage that occurred during the extraction, were lower when using pentane, probably due the absence of heating [87].

Simultaneous distillation-extraction (SDE) technique was applied for the extraction of FFA of the Parmigiano-Reggiano cheese. This method allows the extraction of the less volatile aroma compounds, like FFA, and their simultaneous concentration in a rather small volume of organic solvent. The authors used a face centered cube experimental design to study the effects of extraction time, solvent and sample temperature on the SDE extraction yields of 13 FAs. According to the results obtained, the authors proposed a 3 h. run extraction time, and 125 and 55 ^{o}C as heating temperature for sample and solvent, respectively [88]. Alewijn et al. developed a fast and simple method for the quantitative determination of fat-derived medium and low-volatile compounds in cheese. The extraction procedure use acetonitrile as extraction solvent, and an extraction temperature of 45 ^{o}C for 10 min. This procedure led to a concentrated solution of potential flavor compounds, virtually free from TG, protein and salt from the cheese matrix. Twenty-three FAs were found in the samples of the three cheeses analyzed, most of them consisted of medium-chain FAs [89].

More recently, Abdulkadir and Tsuchiya have developed a one-step method combining extraction and esterification processes in one tube for the quantitative and qualitative analysis of FAs in marine animal samples. In this method, the sample is mixed with hexane and BF_3 (14% in MeOH) in a capped tube that is heated at 100 ^{o}C for 120 minutes under continuous stirring. With this method, 36 FAs were identified in oyster samples, 29 in clam samples and 16 in squid samples; when compared with the Bligh

and Dyer conventional extraction method, the total FA concentrations were higher when using this one-step method for all the samples tested [90].

The AOAC 996.01 method is the universally accepted method for the determination of total, saturated, polyunsaturated and monounsaturated fat in cereals. However, this method involves several steps in the extraction of the FAs such as the hydrolysis of the ground sample, extraction of fat into diethyl and petroleum ether solvents and the evaporation of the solvents. Robinson et al. have recently developed an automated acid hydrolysis-extraction (AHE) system for fat extraction and quantification of total, saturated, polyunsaturated, monounsaturated, and *trans* fat in cereal products [91]. The method involves a combination of automated acid hydrolysis and rinsing of the sample in a closed system followed by reflux boiling with solvent and automated Soxhlet extraction of the lipid, also in a closed system [92]. The percentage of total fat is measured gravimetrically. In addition, the extracted fat can be recovered and total fat and lipid classes measured by capillary GC as in AOAC Method 996.01. Because the AHE system is automated and closed the operator has less contact and exposure to fumes. Furthermore, six samples can be analyzed simultaneously with one unit, less solvent is consumed per sample and 80% of the solvent can be recovered and reused [93].

Low sample throughput, time, and solvent-consuming associated to classical extraction such as Folch and Bligh & Dyer methods, have been outperformed by a relatively new extraction technique called pressurized liquid extraction (PLE, Dionex trade name ASE for accelerated solvent extraction). In this technique, a solid sample is packed into an extraction vessel, and the material is extracted using an organic solvent at high pressure (3.5 to 20 MPa) and temperature above its boiling point (usually from 60 to 200 ^{o}C) [94, 95]. PLE has become one of the most powerful extraction approaches in routine analysis of lipids/fatty acids in biological matrices such as foods. The high performance of PLE can be attributed to the following factors: (1) higher solubility of the analytes in the solvent at higher temperature, (2) higher diffusion rate as a result of higher temperature and (3) disruption of the strong solute-matrix interactions [96]. Currently, PLE is attracting interest due to its short extraction times, high extraction yields and total automation, besides, PLE is a technology that reduces human contact with solvents, and reduces the volume of solvents used [97, 98]. In PLE, the selection of a suitable

solvent is a key factor for the optimization of the extraction process. Several factors can dramatically affect the extraction efficiency, kinetics and selectivity such as the physicochemical properties of the analytes, the chemical composition of the sample matrix, the sample particle size, the temperature and pressure and the choice of the solvent [99, 100].

A modified Folch procedure was compared to PLE in their ability to extract lipids from cereal, egg yolk and chicken breast muscle samples. The effects of different binary solvent mixtures, such as chloroform/methanol (2:1, v/v) and isopropanol/hexane (2:3, v/v), and the extraction conditions on FA composition were tested. Results showed that the FA contents of cereal and yolk lipids extracted by PLE were highest when using isopropanol/hexane, however, the extraction of muscle lipids resulted in higher FA contents when chloroform/methanol was used. Also, the FA contents of lipids extracted by PLE were similar or improved compared to conventional extraction [101]. Toschi et al. compared the recovery of total lipids from poultry meat obtained by ASE extraction to those obtained by Folch and to acid hydrolysis followed by Soxhlet conventional methods. Two different solvent mixtures (chloroform/methanol and n-hexane/2-propanol) were tested at various temperatures (60, 80, 100 and 120 ^{o}C) and pressures (15 and 20 MPa) and with different sample preparations. Results showed that consumption of solvents were significantly reduced using ASE from a half to one third, relative to those required by the traditional Folch method. Authors found a high correlation between the Folch method and the ASE experiments performed at 120 ^{o}C and 20 MPa using chloroform/methanol as solvent mixture with a total extraction time of 10 min. However, the FA compositions of the poultry lipid, extracted by the three extraction methods were found to be equivalent [102]. Poerschmann and Carlson proposed a new preparation scheme to extract and fractionate neutral lipids (which included LCFA) from polar phospholipids in biological matrices. Basically, a two-step PLE scheme was combined with an “in-cell-fractionation” using silica-based sorbents placed at the outlet of extraction cell. The two-step PLE scheme consisted on a first extraction step, which included the utilization of either solvent n-hexane or n-hexane/acetone solvent mixtures followed by a second extraction step, with solvent mixtures of chloroform/methanol. The optimal extraction temperatures were of 50 and 110 ^{o}C for the first and second extraction steps, respectively. The pressure was of 12 MPa and two static cycles, each 10 min, were conducted across all experiments. This

proposed protocol was found to be superior to commonly used approaches consisting of an exhaustive lipid extraction followed by off-line lipid fractionation using SPE regarding fractionation efficiency, time and solvent consumption [103]. The extraction efficiency of PLE has also been compared to other extraction techniques such as ultrasound-assisted (USE) as well as to classical Soxhlet, in the extraction of fatty acids from leaves of *Piper gaudichaudianum* Kunth. The solvents employed in the PLE extractions were petroleum ether and ethanol, and the extraction pressure was of 10 MPa. The extraction temperatures were optimized to 85 ^{o}C for ethanol and 150 ^{o}C for petroleum ether, and the extraction time was 10 min for both solvents. Results showed that PLE decreased significantly the total extraction time, the amount of solvent and the manipulation of the sample and solvents in comparison with Soxhlet and USE. In addition, PLE was more effective in the extraction of FAs [104].

More recently Sheibani and Ghaziaskar used a modified supercritical fluid extraction apparatus, to develop a PLE method for the extraction of pistachio oil (called PFE, pressurized fluid extraction). The apparatus was able to pump liquid solvent and CO_2 into the extraction vessel alternatively. The effect of different variables on the extraction yield such as the extraction temperature (4 - 80 ^{o}C) and pressure (10 - 150 bar), among others, were tested. Two different solvents, n-hexane and ethanol, were used to investigate the influence of solvent polarity on the yield of oil extraction from pistachio. An increase in the extraction temperature led to higher extraction yields due to a decrease of the viscosity of the solvents tested. On the contrary, small change was observed when operating at extraction pressures of 1 or 15 MPa. Therefore, a pressure of 1 MPa was applied only to keep the solvent as liquid at the high temperatures and drive the solvent through the sample in order to improve extraction yield. When compared to conventional Sohxlet extraction, the yields and FAs composition were found similar although PFE extraction required lower solvent consumptions and extraction times [105]. Also, Assis Jacques et al. extracted different saturated and unsaturated FAs from mate leaves using PLE extraction. Authors studied the influence of the extraction time, polarity of the solvent, amount of sample, number of PLE cycles, flushing volume and extraction temperature in the extraction yield. Results obtained indicated that the solvent polarity was the most significant variable in the study, being the medium yield obtained with methanol about eight times higher to those obtained when using n-hexane as extraction solvent [106]. PLE has also been applied for the

quantitative extraction of different samples of environmental organic compounds from soils [107]. In this sense, Jeannotte et al. made a comparison of solvent mixtures for PLE of soil FA biomarkers which included saturated, branched and mono- and poly-unsaturated FAs. In the study, four solvent mixtures were selected for testing: chloroform/methanol/phosphate buffer (1:2:0.8, v/v/v), chloroform/methanol (1:2, v/v), hexane/2-propanol (3:2, v/v) and acetone. Results showed that the solvent mixtures containing chloroform and methanol were the most efficient for extracting lipids from agricultural soils [108]. Table 1 shows a summary of the new extraction techniques employed to extract fatty acids from different food matrices.

2.2 Fatty acid fractionation techniques

Traditionally, FA profiling of lipids by GC does not allow correlating FA identification with their lipid class of origin (e.g., acylglicerols, phospholipids, cholestryl esters, etc) except when previous separation of the lipid classes is accomplished usually by column chromatography or thin layer chromatography (TLC). As for column chromatography, a method for the fractionation of lipids and purification of GLA from the microalgae *Spirulina platensis* ARM 740 has been recently developed by Sajilata et al. [109]. In this method, lipids from freeze-dried biomass were extracted according to the Bligh and Dyer method and the separation of the lipids into the individual classes, namely neutral lipids (NL), glycolipids (GL) and phospholipids (PL) was achieved by silica gel column chromatography and preparative TLC. The individual classes were eluted with chloroform acetone and methanol. For elution of glycolipids, mixtures of varying proportions of chloroform:acetone to pure acetone were used. *S. platensis* ARM 740 was found to contain 94% of the total GLA in the glycolipid fraction and the GLA methyl ester was purified by fractionation of FAMEs by argentated silica gel column chromatography according to the procedure described by Guerrero et al. [110].

However, TLC on silica gel plates is probably the most commonly used chromatographic procedure for the fractionation of lipid classes. Although HPLC provides a more efficient separation of lipid classes, the TLC method enjoys several advantages including the lower cost, less rigorous sample preparation, the ability to analyze multiple samples simultaneously, and the ease to visualization. Moreover, most laboratories now use commercially available plates because its uniformity in thickness,

stability of the silica gel layer, and greater reproducibility of separations. Examples of TLC fractionation of lipid classes followed by FA analysis by GC are given elsewhere [111 – 114]. These strategies gave FA information on small amounts of sample but care had to be taken to control the weight ratio of silica gel versus "scrapped-off" lipid, where significant losses of PUFAs and unsaturated fatty acids (UFAs) have been reported [115]. The group of Sowa et al. has been successful using TLC for the separation and quantification of phospholipids and subsequent analysis of their FA composition by GC [116], however, they noticed that the recovery of UFAs was dramatically reduced following the separation of lipid classes by TLC, direct transmethylation of FAs in presence of the silica gel, and subsequent GC analyses. The source of the error came from the oxidation of UFAs during methylation, in the presence of certain brands of silica gel. These results show that some brands of TLC plates may be unsuitable for lipid analysis if the aim is to determine the FA composition by GC using direct methylation [117]. Advances towards the coupling of TLC to GC/MS analysis of the FA profile from different lipid samples such as marine lipid samples, have recently been accomplished by Estévez and Helleur [118]. The authors developed a method by introducing an intact TLC silica-coated rod (15 cm) into a vertical furnace pyrolyzer coupled to a GC/MS instrument. Using this technique, a fatty acid profile corresponding to the neutral and polar fraction of a lipid extract previously separated by TLC was obtained. The FAs were analyzed directly off the TLC rod, as their methyl derivatives were obtained following thermally assisted hydrolysis methylation in the presence of trimethylsulfonium hydroxide (TMSH). Compared to conventional TMSH/lipid solution, in which previous studies have demonstrated that the mole ratio of TMSH/lipid constituted a critical parameter for the control of the degree of isomerization of PUFAs [119], there was a significant decrease in the degree of isomerization for PUFAs using the TMSH/silica-supported reaction, when a relatively high TMSH/lipid molar ratio (>2500) was used. TLC was also recently employed as a separation method in the characterization of FFAs from crude wax from sunflower oil refineries. The wax was extracted with chloroform, concentrated, precipitated and then subjected to TLC to separate and purify it into various fractions, such as fatty esters, free fatty alcohols, and FFAs. Separations were done in 20 x 20 cm glass plates coated with a silica gel layer of 0.25 mm thickness, and the plates were developed using the solvent system toluene:chloroform (7:3). Each fraction was identified by GC-MS indicating the presence of C_{18}-C_{30} fatty acids in the free form

[120]. On the same basis, FFAs were separated by TLC from the total lipids extracted from beans [121].

When it comes to separate FAs according to both the number and the configuration of their double bonds, TLC on silica gel impregnated with silver nitrate has been of enormous value to the lipid analysts. This kind of TLC named silver-ion TLC or "argentation" chromatography is based on the ability of the double bonds in the alkyl chains of FAs to form polar complexes reversibly with silver compounds. Fully saturated lipids do not form complexes and migrate to the top of the plate, those containing one monoenoic fatty acyl residue come next and components of increasing unsaturation then follow. Silver-ion TLC was first introduced by Morris [122] and has been employed in the milk fat analysis [123] and determination of coffee triacylglycerol molecular species according to their degree of unsaturation [124], to name a few. Silver-ion TLC has been more recently employed in the development of a method for the separation of geometrical isomers of EPA and DHA formed during fish oil deodorization, according to their number of *trans* double bonds [125]. The TLC plates (silica gel, 20 cm x 20 cm) were impregnated by immersion in a silver nitrate solution (10%, w/v in acetonitrile) for 30 min and then eluted with toluene:methanol (85:15, v/v). The fractions containing mono-, di-, tri-, tetra-, penta-*trans* for EPA and DHA and hexa-*trans* for DHA were scraped off the plate and the FAMEs recovered with 1% NaCl in methanol:water 90:10 (v/v) solution and then extracted twice with hexane. These results were confirmed by the same authors by using Ag-HPLC fractionation to separate the geometrical isomers of EPA and DHA.

More modern methods are based on solid-phase extraction (SPE) typically using amino-bonded phase and C18 bonded-phase columns [126]. These procedures are quite fast, minimizing volumes of organic solvents and lead to a good recovery and a high reproducibility. The methodology has been extensively reviewed in the past by Christie [127] and Ruiz-Gutiérrez et al. [128]. Related to FA, some SPE methods have been suggested: Wilson et al. proposed a simple and reliable method for preparing the concentrates of methyl or ethyl esters of *n-3* PUFAs by SPE using NH_2 columns preconditioned with dichloromethane and hexane [129]. As PUFAs have strong dipoles at methylene-interrupted carbon atoms, strong interactions between the hydrogen atoms of these carbons and the NH_2 phase takes place. The amino-bonded phase columns were

also used for estimating the amount of FFA in fermented dairy products [130] and to isolate a fraction containing the FFA from stomach samples from cows [131]. Prasad et al. analyzed arachidonic acid (C20:4, ω-6) from tissue lipids and compared the recovery of the acid isolated from NH_2 column and TLC concluding that the amino bonded-phase column method yielded a higher recovery of arachidonic acid as compared to that of TLC [132]. Ruiz et al. checked the feasibility of two different SPE methods for separating major lipid classes from the masseter muscle of Iberian pigs [133]. One method (OM) was based in that developed by Kaluzny et al. [134] in which the fat dissolved in chloroform is added to an aminopropyl minicolumn, which was previously activated with chloroform. The FFA eluted as the second fraction, after washing the column with a mixture of diethyl ether:acetic acid (98:2). The other method (MM) tested was based on that developed by Pinkart et al. [135] and in this case, the intramuscular fat was dissolved in a mixture of hexane:chloroform:methanol (95:3:2) and then added to an aminopropyl minicolumn which was previously activated with hexane. The FFA eluted also as a second fraction after the neutral lipids, by washing the column with diethyl ether:acetic acid (98:2). The fractions obtained with the two methods were separated by TLC (silica gel plates 60 F_{254} plates) and after examination of the TLC it was concluded that, using the first method, the second fraction was mainly constituted by FFA, with a small contamination of phospholipids, whereas using the second method, the fraction was exclusively constituted by FFA and showed no evident presence of phospholipids nor neutral lipids.

More recently, Lacaze et al. have developed a novel analytical protocol for the determination of FFA (saturated, monounsaturated and polyunsaturated) from shellfish which also involved a SPE step of the lipidic extracts based on the method above described by Kaluzny et al. [136].

Many analytical procedures have been used to isolate, identify and quantify volatile fatty acids (VFAs) including liquid-liquid extraction [137], distillation [138] or purge-and-trap techniques [139] combined with GC. Among these extraction techniques, solid-phase microextraction (SPME) is a solvent-free sample preparation technique which has become very popular due to its easy use, high sensitivity and reproducibility and low cost. It requires neither solvents nor previous sample preparation and it is easy to automate. This method, developed by Arthur and Pawliszyn [140] has recently been

used by Ábalos et al. in the determination of free VFAs in waste waters [141]. In that work, a variety of SPME fibers of different polarity (polyacrylate, PA, Carbowax-divinylbenzene, CW-DVB, polydimethylsiloxane-divinylbenzene, PDMS-DVB, polydimethylsiloxane-Carboxen, PDMS-CAR, polydimethylsiloxane-Carboxen-divinylbenzene, PDMS-CAR-DVB) were evaluated for the extraction of underivatised C_2 – C_7 fatty acids. PDMS-CAR fiber was selected for the extraction of VFAs, and temperatures and extraction times of 25 ^{o}C and 20 min yielded the best recoveries. The same method was used for the determination of free VFAs in aqueous samples using GC coupled to chemical ionization mass spectrometry [142]. PDMS-CAR fiber gave also the best effectiveness and repeatability in the extraction of VFAs ethyl esters from raw spirits when compared to other fibers such as PDMS, PDMS-DVB and PA (polyacrilate) [143]. However, SPME of polar analytes such as carboxylic acids from aqueous medium is often difficult because of the high affinity towards water. For efficient extraction of such analytes from aqueous samples, the SPME coating must have a high enough polarity to compete with water for the analyte molecules. When using polar stationary phases for the extraction of polar analytes from aqueous media, desorption step becomes problematic, often leading to undesired effects such as incomplete desorption and sample carryover. In this sense, sol-gel coatings [144, 145] have been developed to provide an effective solution to these problems inherent in conventional SPME coatings. The sol-gel coatings offer several advantages. First, sol-gel coatings are chemically anchored to the fused silica substrate ensuring thermal stability of the coatings, and thereby facilitate application of higher temperatures for effective desorption of high-boiling analytes. Second, the sol-gel coatings usually give a porous structure enhancing the surface area of the extraction phase which allows the use of thinner coatings to achieve faster extraction and desired level of sample capacity. Third, the selectivity of a sol-gel coating can be easily fine tuned by changing the composition of the used sol solution. These coatings have been designed and used by Kulkarni et al., who have recently developed a sol-gel inmobilized cyano-polydimethylsiloxane (CN-PDMS) coating for capillary microextraction (CME also called in-tube SPME) of aqueous FFAs. The sol-gel CN-PDMS microextraction capillaries provided efficient extraction of FAs without using any derivatization, pH adjustment or salting-out procedures [146]. Other novel SPME fiber prepared by sol-gel technology such as TMSPMA-OH-TSO [3-(trimethoxysilyl)propyl methacrylate hydroxyl-terminated silicone oil], a medium polarity coating, was found to be very

effective in carrying out simultaneous extraction of VFAs in beer [147]. Other new improvements in the SPME technology were made by Horák et al., who have developed a method for the determination of medium-chain free fatty acids (MCFA) –caproic, caprylic, capric and lauric acids- from beer using stir bar sorptive extraction (SBSE). SBSE uses magnetic stirring rods incorporated in a glass tube and coated with a layer of polydimethylsiloxane (PDMS) phase. The extraction theory of SBSE is the same as for solid phase micro-extraction (SPME) with PDMS fibre coating, in which the efficiency of partitioning of the analytes into the PDMS phase on the stir bar at equilibrium can roughly be predicted by the octanol-water partition coefficients [148]. The procedure devised for practical extraction utilized 10 mL of sample stirring with "Twister" (length 10 mm, film thickness 0.5 mm) at 1000 rpm for 60 min at room temperature, combined with solvent back extraction with a mixture of dichloromethane:hexane (50:50 v/v) at 1000 rpm for 40 min [149]. SBSE had been shown to have a much higher sensitivity than SPME by a factor within 50 and 250 due to the higher volume of the PDMS phase (ranges 25 – 125 µL), in which the amount of analyte extracted is proportional to the coating thickness, increasing the limit of detection of ultratrace compounds during sampling [150].

3. Sample analysis

In general, GC has become widely adopted as a highly applicable tool in micro-scale analysis of fatty acids in different research areas. For instance, in biomedical research, GC data on human fatty acids had already been published by the end of the 1950s [151]. The basic features of PUFAs, and especially of essential fatty acid metabolism could be verified in high detail by GC analysis [152 – 154]. Authentication methods for genetic varieties of olive oils have been most frequently established using GC [155 - 157] and many laboratories are currently using GC to analyze fatty acid composition in various matrices such as cell membranes and cultures [158], microorganisms [159], plasma [160], tissues [161], etc.

3.1 Gas chromatography of fatty acids

3.1.1 Derivatization of fatty acids by gas chromatography (GC)

Gas chromatography is the routine procedure for the analysis of LCFAs (>C_{12}) in many branches of biological sciences. However, separating carboxylated compounds by GC is complicated by their relatively high polarity and therefore it is necessary to prepare non-polar derivatives of fatty acids which are also more volatile than the free acid components. In this sense, fatty acid methyl esters (FAMEs) are used almost universally for GC analysis of fatty acids [162, 163]. The generation of FAMEs can be done in acidic or in alkaline conditions on isolated lipids or fatty acids but also directly by one step procedure combining lipid extraction and transesterification of vegetal oils with sodium methylate, NaOH or KOH in dry medium [164]. Under acidic conditions, the most common derivatives of fatty acids are the methyl esters obtained by heating FFAs with a large excess of anhydrous methanol in the presence of a catalyst, boron trifluoride (BF_3) [165], although acidic conditions generated by HCl in dry methanol or methanol sulphuric acid have been also described [166]. BF_3 has revealed a high methylation power requiring a short time period to react, however some reports have claimed that BF_3 leads to irreversible damage of GC column [167]. Methanol-HCl has been considered a mild and useful derivatization reagent due to its almost quantitative yield but its transmethylation capacity is low, thus requiring reaction times higher than 30 min. [168]. On the other hand, Christie has reported that the use of H_2SO_4 led to the decomposition of PUFAs under certain conditions [169].

In the derivatization under the presence of basic catalysts, the fatty esters form an anionic intermediate which is transformed in the presence of a large excess of the alcohol into a new ester. Free fatty acids are not sensitive to nucleophilic attack by alcohols or bases and thus are not esterified in these conditions [164]. Potassium hydroxide, sodium hydroxide and sodium methoxide are the most common catalyst used in basic catalysis. Basic catalysis is faster than acid catalysis, however alkali catalyst reactions require strict anhydrous conditions, which may be difficult to fulfill in case of biological samples. Potassium hydroxide and BF_3, both in methanol can be used in combined catalysis, which involves a two step process. Whereas basic catalysis is faster than acid catalysis, the former will transform free acids into their carboxylic salt and prevent their determination by GC analysis. A second acid catalysis avoids this problem but increases sample preparation time [170].

Recent improvements in derivatization protocols reported in literature differ widely in terms of catalyst reagent, derivatization time, and temperature. Eras et al. have used chlorotrimethylsilane (CTMS) as an acid catalyst for the transesterification of some fatty esters in 1-propanol [171], and more recently they have developed a one-step method to transesterify acylglycerides and esterify FFAs at the same time using CTMS as reagent. This methodology provides some advantages compared to traditional derivatization protocols: CTMS is cheaper than, for example, BF_3-MeOH and is likely to permit the use of different alcohols; the possibility of using 1-propanol instead of methanol ensured a major solubility of fat, which resulted in a short reaction period without a considerable increase in GC analysis time; CTMS is easier to handle than either HCl gas or concentrated H_2SO_4, and its solubility in organic solvents ensured one reaction phase in which the esterification of FFAs and the transesterification of acylglicerides occurred at the same time [170].

Thermally assisted hydrolysis and methylation (THM) provides an interesting alternative to other derivatizing sample preparation techniques because of simplicity and rapidness. The method was first introduced by Challinor [172] in the analysis of polyesters and, in the field of lipid analysis, has recently been applied for profiling fatty acids in PUFAs rich oil [173, 174]. THM procedures show a series of advantages over conventional fatty acid analysis protocols that include: smaller sample size, minimum sample treatment and handling, and faster analysis time. THM uses organic bases, such as tetramethylammonium hydroxide (TMAH) in a one-step hydrolysis/methylation reaction that releases the FAMEs of the free or esterified fatty acids as thermal decomposition products of the tetramethylammonium salts [175]. THM coupled to GC in the presence of TMSH has recently applied by Nakanishi et al. to the analysis of the lipid contents and their fatty acid compositions in individual zooplankters, especially focusing on PUFAs (DHA and EPA) components [176]. In the procedure for one-step THM-GC, in which a vertical microfurnace pyrolyzer was directly attached to a gas chromatograph, 2 µL of a methanol solution of TMSH was added as a derivatization reagent to the sample and heated to 400 ^{o}C under a flow of Helium as a carrier gas. Results obtained by THM-GC were in good agreement with those achieved by conventional Bligh-Dyer solvent extraction for every fatty acid component including EPA and DHA PUFAs. Also, as previously stated, Estévez and Helleur combined silica rod TLC with THM and analysis by GC/MS for the fatty acid profiling of neutral and

polar fractions of marine lipid samples [118]. Results showed that silica-supported THM using TMSH substantially decreased the degree of isomerization for PUFAs, unlike what happens when using TMAH solutions, which seems to cause isomerization of double bonds due the elevated temperatures required for the TMA-salts decomposition [177]. Isomers of the methyl ester of linoleic acid have been reported as TMAH thermochemolytic products of soybean oil and linoleic acid itself, attributed to base-catalysed Z to E conversion and double bond migration [178]. Moreover, pyrolysis with TMAH may cause overloading and column deterioration when on-line Pyrolysis-GC-MS is adopted. The use of TMSH prevents these side-reactions but the methylating cation which forms part of its chemical nature may produce a disturbing smell for its thermal degradation compounds (trimethylamine, organic sulfides). In this sense, dimethyl carbonate (DMC) has also recently been investigated as a mild, harmless and odorless reagent for pyrolytic methylation of fatty acids. Fabbri et al. developed a transmethylation procedure in analytical pyrolysis for profiling fatty acids using DMC as methylating reagent. Since PUFAs are partially difficult to analyze under pyrolitic conditions for the aforementioned reasons, the authors selected soybean oil as test due its PUFAs high content. Methyl esters of palmitic, linoleic, oleic and stearic acid were formed as prominent products from off-line pyrolysis of soybean oil in the presence of DMC and zeolite 13X. Isomerisation of linoleic acid was found out to be less important with DMC than with TMAH, but the employed zeolite had the inconvenience of promoting the formation of thermal degradation products [179]. When using DMC in the presence of nanopowder titanium silicon oxide resulted in the production of FAMEs as unique products. In this case, underivatized fatty acids and hydrocarbons, which are typically formed upon pyrolysis without any added reagent, were not detected. Titanium silicate also showed a stronger efficiency than zeolite 13X in trapping DMC and thus promoting its methylating activity responsible for the rapid formation of the FAMEs [180]. Figure 1 shows the chromatogram obtained from DMC/titanium silicatePY-GC-MS of olive, linseed and walnut oil.

More recently, the same group compared the activity of nanopowder and mesoporous titanium catalysts towards pyrolysis transmethylation of soybean oil (with a high content of unsaturated fatty acids) with DMC. The manipulation of dry nanoparticles in routine operations can be cumbersome with respect to coarser materials hence, mesoporous materials containing titanium ions (Ti-MCM-41) were selected as a

potential alternative to nanopowder titanium silicate. Results showed that, in the case of silicates, the mesoporous structure and the content of titanium were determinant factors to obtain FAME yields comparable to those of nanopowders. When applied to on-line pyrolysis-GC-MS, Ti-MCM-41 with 10% molar Ti content provided a better analytical performance than titanium silicate nanopowder. The additional benefit was the coarse nature of Ti-MCM-41 particles (> 1 μm), that are easier to handle and pose less health hazard than dry oxide nanoparticles [181].

Basic or acid catalysts can be used for the preparation of FAMEs, however, the use of an homogeneous catalyst has the disadvantage of the miscibility of the catalyst in the reaction medium, which causes separation problems. Hence, the use of heterogeneous catalyst such as zeolites and ion-exchanged resins has clear advantages since they are non corrosive and are easy to separate from the reaction mixture; furthermore, no washing of the ester is required, presenting fewer disposal problems [182, 183]. Bondioli has reported that the cationic resins are active at low temperatures and owing to their molecular sieve action, produce few by products and may be highly selective [184]. More recently, Simone et al. have described transesterification of brazilian vegetable oils with methanol over ion-exchange resins [185]. Talpur et al. have developed a simple, rapid and fairly selective method for the preparation of FAMEs based on anion exchange resin Amberlite IRA-904 catalyzed transesterification of vegetable oil/fat with iodomethane as alkylating reagent. The vegetable oil and animal fats used were sunflower oil, palm oil, vanaspati (hydrogenated vegetable oil), olive oil, tallow and butter. The analytical results for the FAMEs showed a good agreement when comparing the resin based proposed method with the conventional BF_3-MeOH method, thus indicating the possibility of using Amberlite IRA-904 based transesterification instead of intensive treatments inherent with the conventional time-consuming methods [186].

More recently, Juárez et al. made a comparison of four different extraction-derivatization combinations to determine intramuscular FA content in meat. The methods tested were one *in situ* method, a saponification method, the classic Folch method and one method consisting in a combination of the classic Folch extraction and methylation with trimethylsilyldiazomethane (TMS-DM) [187]. Results showed that the *in situ* method extracted less total fat than the saponification and classic methods,

showing the lowest recovery values. The saponification method showed the best balance between recovery and variation values, especially for the PUFAs, as compared to the other three methods. It has the lowest variation in most PUFAs included in CLA. Also, TMS-DM has been reported not to change the original isomer distribution nor alter the geometric configuration of conjugated double bonds, and does not produce the methoxy artifacts associated with base-catalised [188]. The results from the classic method showed the lowest recovery values and the highest inter-day and inter-laboratory variation values for PUFAs. However, as previously stated, the alkali-based transesterification has the shortcoming that the FFAs remain partially unreacted. The combination of classic extraction and TMS-DM methylation showed good recovery values, but it had the highest intra-day, inter-day and inter-laboratory variation values compared to the other three methods. Nevertheless, the formation of trimethylsilyl esters or ethers is one of the most popular and versatile derivatization techniques available for GC because it improves chromatographic properties such as volatility and thermal stability, of the compounds. In this sense, Durant et al. developed an *in situ* silylation method for the determination of FFAs of canola oil using a mixture of hexamethyldisilazane (HMDS), pyridine and trifluoroacetic acid in a reaction at 60 ^{o}C for 1 h [189].

Méndez Antolín et al. evaluated five derivatization methods for the determination of the composition of a mixture of VLCFAs ($C_{24:0}$-$C_{36:0}$) [190]. The methods used diazomethane, sulphuric acid-methanol, hydrochloric acid-methanol, BF_3-methanol and N-methyl-N-trimethylsilyltrifluoroacetamide as derivatizating reagents, respectively. Results showed that methods were similar on account of the fatty acid content determined (84.2 – 86.6 %) however, whereas the hydrochloric acid-methanol method needed 90 min to complete the derivatization, the other methods only required 10 min. Considering costs, speed, safety and GC response, the method using sulphuric acid-methanol was found the most appropriate for determining these fatty acids.

Methylation is not efficient for analyzing carboxylic acids of medium or short chain (<C12) as their volatility can lead to unquantifiable losses. Derivatization methods forming propyl or butyl esters have been known for a long time [191, 192] but gained popularity recently. Due to their decreased volatility, butyl esters allow the simultaneous analysis of both low- and high-molecular weight fatty acids. The relative

efficiency of different butylation procedures was recently examined by Hallman et al. [193]. A mixture of 14 organic acids standards dissolved in DCM was processed under varying conditions of esterification to compare fatty acid butyl ester yields. Each reaction scheme was conducted with three different amounts of n-butanol and two different catalysts (BF_3 or H_2SO_4). The most efficient recovery for fatty acids was obtained using n-butanol/BF_3 (10%, w/w) at 100 ^oC for 2 h, however optimum derivatization conditions vary strongly for different acids and no single derivatization protocol can be employed without certain losses.

The popular BF_3 procedure is not an obvious choice if automated FAME sample preparation is the goal, since it involves heating to boiling point, the use of condensers and also large amounts of reagents. Alternative approaches to achieve methylation in a methanol medium, with the aid of acidic and/or alkaline catalysts include the aforementioned TMSH, treatment with sulphuric acid or potassium hydroxide [194] and treatment with sodium methylate, $NaOCH_3$ [195]. De Koning et al. have designed a $NaOCH_3$-based robotic sample preparation procedure for the determination of FAMEs and cis/trans methyl ester composition of fats and oils [196]. After weighing and manual addition of heptane, a XYZ robotic autosampler was used for all remaining work, which includes reagent addition, agitation, sample settling and the final injection into the gas chromatograph. The total sample preparation, including weighing and heptane addition takes 12 min. This automated $NaOCH_3$ procedure was based to the method of Schulte and Weber but in this case the sample preparation and analysis are synchronized which means that each sample preparation is performed “just in time” to be ready for injection at the moment the GC returns to the ready status. The novel procedure was found to be much faster (12 min versus 1 h) when compared to the classical BF_3 method and manual sample handling was drastically decreased.

3.1.2. Gas chromatography of fatty acids – stationary phases and columns

Capillary gas chromatography is the traditionally used technique for the analysis of FAMEs in a wide number of research areas. FAME analysis demands high chromatographic resolution especially to provide evidence of positional and geometrical isomers of unsaturated fatty acids in complex mixtures. To meet these requirements, polar stationary phases are normally used for the separation of complex FAME mixtures,

since they enable separation according to unsaturation and carbon number. In recent reviews, the esterification methods, injection techniques and analytical columns, as well as the analysis and identification of fatty acid isomers have been described in detail [197, 198]. Nowadays, GC analysis of fatty acids (as methyl esters) in oils (fish oils, olive oil), fat (e.g. milk) and processed (hydrogenated and refined) oils and fats is a well established technique [199, 200]. Guil-Guerrero et al. analyzed the amount of γ-linolenic acid (18:3, ω-6) and stearidonic acid (18:4, ω-3) of seed oils from nineteen species of the plants of the genus Echium using two types of capillary columns: a highly polar SP-2330 column coupled to FID detection, and a methyl silicone HP-1 column coupled to MS detection to verify the double bounds [201]. Verleyen et al. developed a GC method to analyze the deodorized distillate of different vegetable (soybean, canola, sunflower and corn) oils without saponification. Separations were performed with cold on-column injection on a capillary column CP-Sil 8 CB Low bleed/MS (15 m x 0.25 mm x 0.25 µm) with FID detection [202]. Similarly, FAMEs from cod liver oil and commercial ethyl ester capsules, as well as milk powder samples were analyzed on a CP-Sil 88 (100% poly[biscyanopropylsiloxane]) capillary column (50 m x 0.32 mm x 0.2 µm) with direct on-column injection and FID detection [203]. These kind of polar stationary phases based on cyanoalkyl polysiloxane, like the above mentioned CP-Sil 88, and the CP-Sil 52 have also been employed in the determination of the fatty acid composition of five wild edible mushrooms from Northeast Portugal [204], and more recently for the detection and quantification of long chain fatty acids of liquid and solid samples collected from anaerobic digesters [205]. Other highly polar stationary phases such as SP-2380 (90% poly[biscyanopropyl/ 10% cyanopropylphenyl siloxane]) and SP-2560 (100% poly[byscyanopropylsiloxane]) have been used in the determination of the fatty acid composition of foals and seed oils from plants [206, 207], and in the FA determination of pasture-reared fryer rabbit meat and meat from reptiles [208, 209]. GC with a different polar stationary phase (DB-WAX (30 m x 0.25 mm x 0.25 µm) has also been used to determine the stability of a fatty acid mixture of blackcurrant oil as a function of age and storage [210]. These polar DB-WAX columns have also been employed in the determination of the fatty acid composition of green crab from Mediterranean coasts [211]. Other examples of polar stationary phases used in the analysis of fatty acids are: the Supelcowax-10 fused silica capillary columns (60 m x 0.32 mm x 0.25 µm), in the fatty acid profiling of oil seeds from different plants such as chia (*Salvia hispanica* L.) and flase flax (*Camelina sativa* L.) [212, 213]; the Innowax

columns (30 m x 25 mm x 0.25 µm), in the identification and quantification of fatty acids from Celta pig [214]; the CP wax 52 CB capillary columns (50 m x 0.32 mm x 1.2 µm), in the analysis of FAMEs from seeds of *Pinus pinea* L. [215]; and the Omegawax fused silica capillary columns (30 m x 0.32 mm x 0.25 µm), in the analysis of the total fatty acid content in infant foods used in the transition from breast milk to an adult diet [216].

On the other hand, weakly polar columns, routinely used in capillary GC for analyzing sterols, food additives, etc., can also be used for separating FAMEs. Very recently, Yamamoto et al. used a narrow bore (30 m x 0.25 mm x 0.25 µm) and wide bore (30 m x 0.53 mm x 0.50 µm) SPB-50, BPX-50 and DB-17 weakly polar columns to separate FAMEs from soybean oil, lanolin and fish capsules by the degree of unsaturation [217]. Authors found out that unsaturated FAMEs eluted after the corresponding saturated ones, within a chain length, being the elution pattern very similar to those obtained with medium polar columns.

Fast analysis by GC could be achieved by optimizing key operating parameters such as carrier gas type and velocity, length and diameter of the capillary column [218]. The reduction of the diameter of the column led to the emergence of a new generation of open-tubular capillary columns named fast columns. The use of fast GC has been reported in previous studies [219, 220]. Masood et al. published a study on the separation of FAMEs by fast GC in which the authors optimized the GC conditions to increase the throughput of plasmatic fatty acid composition in the frame of large clinical trials [221]. Bondia-Pons et al. quantified the conjugated isomers of linoleic acid in human plasma by means of fast GC [222], and Mondello et al. used fast GC to analyze the fatty acid composition of fish oils and different edible oils and fats [223]. From the results obtained so far it is clear that the use of fast columns will impact significantly the analysis of lipids. More recently, Destaillats and Cruz-Hernández published a very interesting study on the separation of complex FAMEs preparations using a short and highly polar BPX-70 (10 m x 0.1 mm x 0.2 µm) fast capillary column [224]. The GC parameters were optimized to achieve separation of FAMEs ranging from 4:0 (butyric acid) to 24:1 with a total run time of 4.9 min; samples studied were milk fat, cocoa butter and tuna oil.

GC has also been employed in the analysis of FFAs from different sources [225-227]. Some authors have studied the FFA profiles of different cheeses and their evolution during ripening. The hydrolysis of milk TG into FFAs by microorganisms and native milk lipases is an important phenomenon in the development of flavour during cheese ripening. Perotti et al. analyzed the FFAs from C6:0 to C18:2 from Reggianito Argentino cheeses at different ripening times using a fused silica capillary column PE-Wax (polyethylene glycol, 30 m x 0.25 mm x 0.25 µm) [228]. Fernández-García et al. studied the extent and pattern of lipolysis of different Spanish protected designation of origin semi-cured and cured cheeses [229]; they used a polar FFAP (CP-Wax 58) column (30 m x 0.32 mm x 0.25 µm) with a temperature program starting at 65 ^{o}C, up to a final temperature of 240 ^{o}C at 10 ^{o}C/min. Authors found significant differences among all cheeses in all FFAs concentrations, except for the medium chain fatty acids (C10:0 to C14:0). Results also showed that all individual FFAs concentrations increased with ripening time. Similarly, Poveda and Cabezas studied the FFAs composition of different regionally-produced Spanish goat cheeses and they found that the most abundant FFAs were oleic, palmitic, stearic and myristic acids which together accounted for roughly 85% of total FFAs [230]. Morin-Couallier developed a GC method to enable the simultaneous quantification of deformed peaks of FFAs and less polar compounds from aqueous distillery effluents. A 30 m x 0.25 mm x 0,25 µm Rtx®-200 mid-range polarity column was used for the analysis and, although ideal chromatographic conditions were not achieved, calibration curves for the FFAs could be produced and rigorously validated, making quantification possible [231]. GC has also been applied in the analysis of FFAs of blood and plasma. Under metabolic stress, like during a febrile illness, fatty acids are mobilized for energy and their concentrations increase in blood. Kimura et al. developed a GC method to measure FFAs in dried blood spots employing a weakly polar fused silica DB-5 (30 m x 0.25 mm x 0.25 µm) capillary column [232] and, much more recently, Kangani et al. analyzed the plasma FFAs by GC/FID as well as with combustion-isotope ratio mass spectrometry (GC-C-IRMS) in ^{13}C-enrichment experiments [233]. In this case, authors employed different columns depending on the detection system: a SPD-1 (30 m x 0.53 mm x 0.01 µm) for the GC/FID, and a DB-5 (30 m x 0.25 mm x 0.25 µm) for GC-C-IRMS, both operating with the same temperature oven program.

As previously described, long-chain fatty acids are important metabolites, intermediates in biological processes. Due to the importance of LCFA, new methods, using new and different columns, are being developed; for example, Maile et al. compared two different types of gas chromatographic columns in the analysis of LCFA from insect exocrine glands: a low polarity BPX5 (5% phenyl-95% methylsiloxane phase) (12 m x 0.32 mm x 0.25 µm), and a polar Stabilwax (polyethylene glycol phase) (15 m x 0.32 mm x 0.25 µm) [234]. Authors found that the Stabilwax column, at injector temperatures around 250 ^{o}C, showed the best results. One of the main problems for the analysis of n-3 LC-PUFA is that PUFAs are rather unstable, so that calibration cannot be performed by using quantitative standards. The official methods of the Association of Analytical Chemists (AOAC) [235] and the American Oil Chemists´s Society (AOCS) [236] provide clear guidelines for accurate quantification of n-3 LC-PUFAs in marine oils. Both methods describe the use of C23:0 (methyl tricosanoate) as internal standard and wax-type capillary columns are mandatory. Moreover, split injection at 250 ^{o}C and a split rate of 1:50 is recommended as the preferred injection technique in both methods. Nevertheless, the procedure leaves the possibility to apply other injection techniques such as on-column injection. It has also been suggested that the injection technique, especially in vaporizing injectors, is the main source of error in quantitative GC [237]. Discrimination effects inside the injector are mainly caused by different volatilities of analytes. Therefore, it is likely to assume that suboptimal performance of the injection system could affect long-chain saturated fatty acids (internal standards) to a different extent as PUFAs. In this sense, Schreiner published a study on the factors affecting accuracy in the quantification of LC-PUFAs [238]. He used two different GC systems: one equipped with manual on-column injector and two with manual split injection. Split injection was performed at 250 ^{o}C with a split ratio of 1:50. Also, two different types of capillary columns were used in the study: a Supelcowax 10 (10 m x 0.10 mm x 0.1 µm), and a RTX 225 (30 m x 0.25 mm x 0.25 µm). Results confirmed that the on-column injection system produced the most accurate results for PUFAs. The reason why C23:0 is normally considered as the standard of choice for comparison with n-3 LC-PUFAs is because it usually elutes without interference with other components. Using a low polarity column like RTX 225, the internal standard C23:0 eluted in the range of C22:4ω6 and C22:5ω6. However, this problem can be solved by fine-tuning instruments parameters, such as temperature program or flow rate. Systems employing wax-columns were optimized and C21:0 FA eluted between C20:3ω6 and C20:4ω6.

Schreiner found that the selection of the appropriate internal standard (C19:0 for unsaturated C20 FAMEs and C21:0 for unsaturated C22 FAMEs) improves both, accuracy and precision for the analysis of PUFAs.

Alternative ways to circumvent coelutions and to enhance resolution are the use of several different temperature and pressure programs on a single (polar and middle polar) column or the application of two-dimensional gas chromatography (GC x GC) by serial coupling of two capillary columns [239]. Comprehensive GC x GC and GC x GC-MS have been already applied to the analysis of FAMEs [240-242], mostly to profile the complex fatty acid composition in fish oils. Recently, GC x GC was applied to the profiling of fatty acids in plasma, resulting in about 60 detected fatty acids [243]. Recently Härtig developed a multidimensional approach based on GC/MS for the identification of FAMEs [244]. Mass spectra and retention data of more than 130 FAME from various sources (chain lengths in the range from 4 to 24 carbon atoms) were collected in a database. To verify the identity of single species and to ensure an optimized chromatographic resolution, the database was compiled with retention data libraries acquired on columns of different polarity (HP-5, DB-23, and HP-88). For a combined use of mass spectra and retention data, standardized methods of measurement for each of these columns were developed and always applied under the conditions of retention time locking (RTL), a technique which uses electronic pressure control to calibrate column head pressure versus retention time, to fix the retention time of a chosen locking compound. RTL allowed an excellent reproducibility and comparability of absolute retention times. Moreover, as a relative retention index system, equivalent chain lengths (based on the homologous series of saturated straight chain FAMEs) of FAMEs were determined by linear interpolation. As demonstrated, the use of retention data and mass spectral information together in a database search leads to an improved and reliable identification of FAME (including positional and geometrical isomers) without further derivatization. The same year, Akoto et al. used a GC x GC with time-of-flight mass spectrometry detection to profile the fatty acid composition of whole/intact aquatic microorganisms such as the common fresh water green algae *Scenedesmus acutus* and the filamentous cyanobacterium *Limnothrix* sp. strain MRI without any sample preparation steps [245]. With this approach new mono- and diunsaturated fatty acids were found in the C16 and C18 regions in both of the aquatic microorganisms, which have not been detected with the conventional one-dimensional

GC analysis of these species due to either co-elution and/or low concentration of such compounds in the sample. Table 2 shows some examples of the methods applied in the GC determination of bioactive fatty acids

3.1.3. Gas chromatography of trans-fatty acids

The analysis of *trans* fatty acids is extremely challenging and complex because of the wide range of positional monoene, diene and triene isomers. The analytical methods mainly include infrared spectroscopy, ^{13}C nuclear magnetic resonance spectroscopy, GC, TLC, HPLC and mass spectrometry or combination of them [246, 247]. Analysis of cis and trans isomers is best carried out by GC using 100 m long, flexible, fused silica capillary columns coated with highly polar cyanopolysiloxane stationary phases, containing various polar substituents. A variety of cyanopolysiloxane capillary columns are available from chromatographic suppliers and marketed under trade designations such as BPX-70, HP-88, CP-Sil 88, SP-2340 SP-2560, SP-2380, Silar 10C, etc [248]. These cyanopolysiloxane columns enable the analysts to separate the geometric and positional isomers of fatty acids (as their FAMEs) that cannot be resolved by columns coated with non-polar or moderately polar phases. In cyanopolysiloxane capillary columns, the *trans* isomer always elute before the corresponding *cis* isomer, for example, 9t-18:1 elutes before 9c-18:1, 11t-18:1 before 11c-18:1, and 12t-18:1 before 12c-18:1. As a general rule, with any type of cyanopolysiloxane capillary column, the isomers from 4t-18:1 to 11t-18:1 are readily separated from cis 18:1 isomers. But after 11t-18:1 (i.e. *trans* isomers from 12t-18:1 to 16t-18:1) overlapping problems start to occur with some cyanosilicone capillary columns [249]. This overlapping problem primarily depends on two factors: the length of the capillary column and the GC operating parameters. With a 100 m capillary column, the overlaps between the *cis* and *trans* isomers can be minimized, provided that the proper operating conditions are selected. For instance, Ratnayake et al. has shown that on 100 m SP 2560 and CP-Sil 88 capillary columns, the best separation of fatty acids of partially hydrogenated vegetable oils is achieved when the column temperature is operated isothermally at 180 ^{o}C [250]. Also, Mjøs investigated the gas chromatographic properties of the EPA and DHA isomers with one and two *trans* double bonds on GC columns with BPX-70 (70 m x 0.25 mm x 0.25 µm) and SP-2560 (100 m x 0.25 mm x 0.20 µm) cyanopropyl stationary phases. Mjøs applied different temperature and pressure programs to introduce

variations in the retention indices of the isomers. The retention indices of all the *trans* isomers showed a strong linear correlation to the retention indices of the equivalent all *cis* isomer, but the slopes for corresponding linear regression lines varied with the number of trans double bonds in the molecule. For DHA on BPX-70, and for EPA on both columns, it was possible to find windows where isomers with one *trans* double bond can be resolved from the corresponding all-*cis* isomers. Mjøs found that, in general, BPX-70 seemed to have a more suitable selectivity for the analysis of these isomers than SP-2560 [251]. Information on *trans* fatty acids or unsaturated fatty acids that contain at least one double bond in the trans configuration is generally lacking from food composition tables. Baylin et al. studied the composition on *trans* fatty acids in partially hydrogenated soybean oil consumed as part of Costa Rican diet, using a fused silica *cis/trans* column SP-2560 (100 meters x 0.250 mm x 0.20 µm) [252]. Authors found that in a period of 10 years the amount of total *trans* fatty acids in Costa Rican soybean oil has decreased from an average of 20 to 1.5%. Huan et al. evaluated a simplified gas chromatography method based on the AOAC method 996.06 to analyze the *trans* fat content in food samples. They used an Alltech AT™-Silar-90 capillary column (30 m x 0.25 mm x 0.20 µm). Ten types of the *trans* fatty acid standard were separated completely from the *cis* standard, and the *trans*-18:1, *cis*-18:1, *cis*-18:2, and *cis*-18:3 were the major fatty acids found in the shortening sample tested with this method [253]. Although the flexible fused silica capillary columns coated with polar cyanoalkyl polysiloxane stationary phases are widely used to determine *cis* and *trans* isomers, the greatest limitation of these highly polar is that they may bleed, as they are not chemically bonded. In this case, the column separation efficiency is reduced and so is its lifetime. Recently, a column with a polar bonded phase called CP-Select (100 m x 0.25 mm x 0.25 µm) for FAME was developed for the separation of *cis* and *trans* isomers. This column was used by Martin et al. in the quantification of monounsaturated and polyunsaturated *trans* fatty acids in partially hydrogenated fats by GC. The quantification was optimized using equivalent chain length values of FAMEs that could coelute in the temperature range from 155 to 200 ^{o}C. Authors found that the most appropriate temperature for the simultaneous determination of the 18:1, 18:2, and 18:3 *trans* isomers was around 197 ^{o}C [254]. CP-Sil 88 capillary columns (100 m x 0.25 mm x 0.25 µm) has been used by Golay et al. in the direct quantification of *trans* fatty acids in dairy powders [255]. A unique feature of dairy fat is the occurrence of a specific trans fatty acid i.e. trans-11 (vaccenic) 18:1 acid and cis-9,trans-11 (rumenic)

18:2 acid, a conjugated isomers of linoleic acid. Accurate separation of almost all *trans* 18:1 fatty acid isomers could be achieved using highly polar capillary columns having at least 100 m length [256, 257]. Although a pre-fractionation step before GC analysis remains the unique methodology available for accurate measurement of all *trans* fatty acid isomers, an estimation of up to 95% of total 18:1 *trans* fatty acids could be directly achieved using a 100 m CP-Sil 88 capillary column. The effect of the type of carrier gas and flow rate on *cis* and *trans* isomer resolution was studied by Ratnayake et al. [250], who tested the performances of hydrogen and helium as a carrier at different flow rates on the separation and quantification of FAMEs of dietary fats containing *trans* fatty acids. Their study demonstrated the advantage of using hydrogen as a carrier gas at a flow rate of 1 mL/min since the GC run time was shortened 20 min when compared to helium at the same flow rate.

3.1.4. Gas chromatography of conjugated linoleic acid (CLA) isomers

From the mid 1990s onwards, much research into CLA analysis techniques has been undertaken and some reviews on the topic have been published [258, 259]. Nowadays, these techniques generally consist on a sample preparation step (usually lipid extraction plus methylation), followed by the separation, identification and quantification of CLA isomers. Alkali-catalyzed methylation methods (e.g. using $NaOCH_3$ or KOH in methanol at room temperature) are considered the most reliable for determining the distribution of CLA isomers because they cause no isomerization and produce no methoxy artifacts [260]. On the contrary, acid-catalyzed methods, employing BF_3 and HCl enhance extensive isomerization of conjugated dienes and contribute to the formation of allylic methoxy artifacts [261].

GC with flame ionization detection (FID) is by far the most widely used method for the analysis of fatty acids and it remains the only tool employed by many researchers to determine total CLA content. Identification is often based solely on comparison of retention times, with limited availability of standards, and therefore it may be tentative at best. Non-polar capillary columns such as methylsilicone or phenylmethylsilicone phases fail to resolve the isomers of CLA. A polar capillary GC column is absolutely mandatory for the analysis of the geometric and positional isomers of CLA. There are 56 potential geometric and positional CLA isomers, with variations in double bonds

position and geometry (cis or trans configuration) being the *cis*9*trans*11 and *trans*10*cis*12 isomers the only ones which are known to posses biological activity. Many separations have been published using 100 m cyanopropylsilicone capillary columns, being the commercially named CP-Sil 88, SP-2560 and BPX-70 capillary columns the best choice for trying to resolve most of the closely related isomers of CLA. A shorter (50 or 60 m) is more prone to interferences than a 100 m column. Ledoux et al. analyzed the fatty acid composition of 54 butters with especial emphasis on CLA isomers. They used a CP-Sil 88 column (100 m x 0.25 mm x 0.20 μm) and despite the qualities of the long, highly polar columns, some peak resolution remained impossible, so the peak 18:1-*trans* really corresponded to the isomers from *trans*-4 through *trans*-12. In the case of butter, rumenic acid (CLA 18:2 c9,t11) accounted for about 90% of total CLA [262]. Same type of column was used by Glew et al. in the analysis of butter oil with particular focus on CLAs [263]. The contribution that total CLAs made to the fatty acid composition was of 1.45%, and the predominant CLA was rumenic acid (accounting the 90% of the total CLAs), which was in agreement with the previously cited study. Referring to total CLAs, the authors were able to separate the c9,t11, c9,c11, c11,c13, and t11,t13 isomers of linoleic acid (18:2). Adai et al. also used a CP-Sil 88 (100 m x 0.25 mm x 0.20 μm) in the development of a method for the simultaneous determination of a wide range of different fatty acids from intramuscular fat of beef meat, including the two most abundant CLA isomers (*cis*9*trans*11 and *trans*10*cis*12), in the same run along with other well known saturated, monounsaturated and polyunsaturated fatty acids [264]. The complete analysis took 97 min and 47 peaks were detected ranging from C10:0 to C22:6ω-3. Referring to CLA isomers, *cis*9*trans*11 represented a 6.5 times higher quantity than *trans*10*cis*12, being the overall CLA content of 0.24%. The derivatization method, using direct saponification with KOH/methanol followed by a derivatization with (trimethylsylil)diazomethane, did not change the original isomer distribution nor alter the geometric configuration of conjugated double bonds. A fused silica capillary SP-2380 column (100 m x 0.32 mm x 0.20 μm) was used by Zabala et al. for the quantitative analysis of *cis*9*trans*11 and *trans*10*cis*12 isomers of CLA in liver [265]. Authors chose C19:0 and trinonadecanoylglycerol as internal standards for quantification instead n-heptadecanoic (C17:0) acid, widely used in the literature. Kramer et al. [266] suggest C17:0 as internal standard for tissue lipids analysis due the lack of resolution between C19:0 with oleic (C18:1 (*cis*-9)) and γ-linoleic (C18:2 (*cis*-9,12)) acids. However, authors found small

amounts of C17:0 in biological liver. Prandini et al. employed a CP-Select CB capillary column (100 m x 0.25 mm x 0.25 μm) in the determination of the content in CLA of commercial samples of yoghurt, fermented milk and cheese [267]. The only CLA isomer detected with their method was the *cis*9*trans*11 linoleic acid. More recently, a fused silica capillary column CP-7420 Select FAME (100 m x 0.25 mm x 0.25 μm) was used to quantify CLA *cis*9*trans*11 and *trans*10*cis*12 isomers in *Longissimus* muscle of steers of different genetic breeds [268]. CLA isomers were identified by comparison of relative retention times with a CLA O-5632 commercially available mixture. Again, the predominant CLA isomer was CLA *cis*9*trans*11, accounting for more than 60% of total CLA. As mentioned, when analyzing the CLA composition of a given sample, it is important not to modify the original isomer distribution. Base-catalyzed derivatization procedures do not methylate FFAs, whereas acid-catalyzed methylation change the CLA isomer distribution and generate allylic methoxide from CLA. Accurate determination of CLA isomers in biological substrates depends on lipid extraction, on the procedure used to separate the CLA components from the rest of the lipid fraction, and lastly and principally, on the methylation method. Luna et al. used GC-FID to examine the effects of temperature and time on methylation of individual and mixtures of CLA isomers in free fatty acid form using H_2SO_4 as catalysts in methanol [269]. They used a CP-Sil 88 fused silica capillary column (100 m x 0.25 mm x 0.25 μm) for the analysis of the mixture of CLA isomers. H_2SO_4 featured advantages over other derivatizating reagents (HCl or BF_3) like a complete methylation and an absence of isomerization of CLA molecules during the derivatization process. This was in agreement with results from Christie et al. [270], who found that methylating CLA in FFA form using 1% (v/v) H_2SO_4 at 50 ^oC for 1 h generated hardly any artifacts or allylic methoxy esters.

Shorter chromatographic columns have been recently employed in the determination of CLA isomers from different food matrices. Moltó-Puigmartí et al. used a RTX-2330 capillary column (40 m x 0.18 mm x 0.1 μm), containing a 10% cyanopropylphenyl-90%biscyanopropyl polysiloxane nonbonded stationary phase, for measuring *cis*9*trans*11 and *trans*10*cis*12 CLA isomers in human and rat milk [271]. In comparison with the commonly used 100 m x 0.25 mm x 0.20 μm columns, this new type of fast columns allowed the separation of FAMEs with the same resolution but in less time (26 min) (see Figure 2). In this case, the authors chose an alkali-catalyzed methylation

(NaOCH$_3$/MeOH, 10 min) followed by an acid-catalyzed one (BF_3/MeOH, 3 min) at 80 ^oC, which presented the lowest oxidized CLA levels and allowed the total derivatization to FAMEs of the fatty acids in human plasma. This combined method made the fatty acids less susceptible to further oxidation and isomerization by the following reagent (BF_3).

Due to the different relative abundance of FAME in human and rat milk, different levels of separation of *trans*- and *cis*- C18:1 isomers were achieved with this column. C18:1 (*cis*- plus *trans*-) in rat milk accounted for only 14% of total fatty acids, while in human milk it accounted for approximately 40%. Consequently, while in rat milk the separation at baseline of both isomers was easy, there was a coelution of *trans* and *cis*-C18:1 isomers in human milk. To establish the *trans*-C18:1 content in human milk a second sample at lower concentration was mandatory. Same group used the same type of column and methylation procedure for the determination of CLA in human plasma [272] but, as the limited sample capacity is one of the major drawbacks of fast GC techniques [273], different split ratios with different amounts of sample were tested in order to avoid band broadening. A split ratio of 1:30 together with 100 µL of plasma sample turned out to be the best option. The temperature program was optimized and a temperature of 170 ^oC for 20 min (corresponding to the isothermal step) allowed the separation of all the CLA isomers in the minimal elution time to avoid peak overlapping.

Capillary GC-MS combines an efficient separation technique with a sensitive detector that can provide elemental composition, double bond equivalents and mass spectra indicating the location of double bonds for CLA and related compounds. Methyl esters of CLA gas chromatograph well, but the electron impact (EI) mass spectra of individual CLA methyl esters are indistinguishable hence suitable derivatization techniques that produce structurally useful EI data from CLA are mandatory. Spitzer reviewed derivatization techniques that produce structurally useful data from CLA [274]. Derivatives recommended are the dimethyloxazolyne (DMOX), 4-methyl-1,2,4-triazoline-3,5-dione (MTAD), although other derivatives of pyrrolidides and picolinyl esters have also been used [275]. DMOX derivatives are strongly preferred, even though their preparation requires higher temperatures than MTAD. The DMOX react with the acid functionality which permits GC-MS DMOX identification of most fatty acids including CLA, moreover, CLA DMOX separations are influenced by both geometry

and position of the double bonds of the CLA isomers, with the result that these derivatives provide superior CLA-isomer resolution compared to MTAD [258]. DMOX derivatives of fatty acids are very useful for locating double bonds, and have confirmed the positions of double bonds in CLA isomers in a wide range of materials [276, 277]. Roach et al. used a CP-Sil 88 (100 m x 0.25 mm x 0.2 µm) column and under their chromatographic conditions the positional isomers of DMOX CLA spread out over a retention time window (57-63) of approximately 5 min [258]. In the same work Roach described the distinctive electron impact mass spectra of the most common CLA positional isomers (from 7-9 to 12-14). Yurawecz et al. detected the presence of *trans*-7 and *cis*-9 CLA isomers in different food and biological matrices [278], as well as Luna et al. with the *cis*-7 *trans*-9 C18:2 CLA geometrical isomer in ewes milk fat [276]. On the other hand, GC can also be linked to Fourier transform infrared (FTIR) for the determination of the geometrical configuration of double bonds in fatty acids. Infrared spectroscopy differentiates CLA isomers by the geometry of their double bonds. Dispersive infrared spectra were reported about half a century ago to distinguish between the *cis* and *trans* double bond configurations in fats and oils [279] and CLA isomers from complex chemical and biological mixtures could be separated as FAMEs or DMOX derivatives, and measured by FTIR [280, 281]. Table 3 shows a summary of the methods employed in the GC determination of isomeric fatty acids in foods.

3.2 High performance liquid chromatography of fatty acids

3.2.1. Recent developments in derivatization, analysis and detection of fatty acids

Besides GC, different types of HPLC (in reversed and normal phase) have been used in the analysis and separation of fatty acids. Reversed phase mode is generally used in the analysis of organic acids, hydroxyl-polyunsaturated fatty acids, prostaglandins, leukotrienes, other polar lipids and lipoproteins. Normal phase combines partition effects of polar solid phase and non-polar mobile phase to separate compounds efficiently. The solid phase consists of polar functional groups chemically linked to silica particles or plain silica. Other functional groups are NH_2 > diol > CN (order of polarity). NH_2 and CN are available for both normal and reversed phase separation modes depending on the polarity of the mobile phase. HPLC, in reverse-phase mode

with UV-absorbing or fluorescent derivatives, is the only technique that can compare with GC for the analysis of fatty acid derivatives [282]. However, HPLC in normal-phase mode was found to be efficient in the separation of isomers by silver-ion chromatography [283] in which the separation occurs according to the degree of unsaturation. In reversed-phase HPLC (RP-HPLC) the separation depends on both, the chain length of the FAs and the degree of unsaturation. Successful resolution of free fatty acids and acylglycerols was affected by an aqueous gradient elution system acetonitrile-water combined with non-aqueous 2-propanol-hexane mobile phase [284] and gradient elution system acetonitrile-water combined with 2-propanol-acetonitrile [285]. Generally, fatty acids have no strong ultraviolet (UV)/visible-absorbing chromophores and fluorophores. In contrast with GC, the use of HPLC allows the fatty acids to be converted to a large number of different derivatives, and, in this sense, several HPLC methods have been developed for the analysis of saturated and unsaturated FAs, employing pre-column derivatization techniques to increase the sensitivity and selectivity of the detection [286 – 288, to name a few]. Derivatization can overcome some problems such as tailing peaks, and low detector sensitivity by the formation of less polar compounds, which can be more easily analyzed by HPLC. However, some of the drawbacks of those methods are the lengthy and cumbersome clean-up procedures, such as liquid-liquid extraction, the need for quantitative isolation of FAs from biological materials prior derivatization, the long analysis times, and the uncompleted separation of biologically important PUFAs such as γ-linolenic acid, EPA, AA and DHA. New derivatization methods with or without saponification of the samples, and without sample work-up steps have been nicely reviewed by Miwa [289]. In that review, the determination of FFAs by HPLC in conjunction with a direct derivatization technique of FFAs to form 2-nitrophenylhydrazines is described. The derivatization reagent 2-nitrophenylhydrazine hydrochloride (2-NPH·HCl) has previously been used in the determination of mono-, poly- and hydroxycarboxylic acids in food and beverages [290]. All of the FAs, including saturated and mono- and polyunsaturated FAs react sensitively with 2-NPH·HCl using 1-EDC·HCl as a coupling agent to give acid hydrazides. Those compounds show strong absorption at around 230 nm, thus allowing their monitorization with a UV detector. Also, they gave absorption maxima at 400 nm in acidic medium being detectable photometrically at this wavelength. One of the advantages of using this derivatization reagent is that an excess of the reagents and reaction by-products does not interfere with the HPLC analyses in

the visible range, because they do not absorb visible radiation at 400 nm and elute before any of the FA hydrazines. Moreover, by using visible detection, chromatograms are simpler and more selective, in spite of approximately four-fold lower sensitive than when UV detection is used [291]. Miwa was able to successfully separate a mixture of saturated and mono- and polyunsaturated FA hydrazines (C8:0 – C22:6), including cis-trans isomers and double bond positional isomers, using a J´sphere ODS-M 80 column (particle size 4 μm, 250 mm x 4.6 mm i.d.) and acetonitrile-water (86:14, v/v) as the eluent at a flow rate of 2.0 mL/min [289].

In the last decade, a variety of derivatization reagents with chromophores, fluorophores or electrophores have been reported [292 – 294]. Fluorescence (FL) derivatization reagents, which have a fluorophore group and a reactive functional group for carboxylic acids, have been developed and applied to the trace analysis of fatty acids in biological samples. In this sense, Miwa published a complete review on the tagging methods using FL detection reagents for physiologically important carboxylic acids including fatty acids in HPLC [289]. FL derivatization fall into two categories due to the reaction timing before and after the chromatographic separation: off-line pre-column and on-line post-column, being the pre-column derivatization the most frequently used for sensitive detection by HPLC. On the other hand, FL reactions can be classified into following two groups: (1) FL generation, which is generally due to the production of the FL compound from the reaction of non-FL reagent with target compound, resulting in the generation of fluorescence, and (2) FL tagging, based in the covalent binding formation between the reactive functional groups in FL reagent and target compound. A large number of FL reagents have been developed for the determination of carboxylic acids involving FFAs, among them, the bromoalkyl reagents (Br-MMC, Br-MDC, Br-MA,…), diazomethane reagents (ADAM, PDAM, DAM-MC), amine reagents (NEDA, 9-AP, MBPA,…), alcohol reagents (PTM, HMA, DNS-AE,…), sulfonate reagents (NE-OTf, AE-Otf,…), etc. Recently, You et al. developed a new FL reagent 1-[2-(p-toluenesulfonate)-ethyl]-2-phenylimidazole-[4,5-f-]-9,10-phenantrene (TSPP) as tagging reagent with fluorescence detection in the determination of LCFAs (C_{20} – C_{30}) from plant extracts [295]. Optimal derivatization conditions were obtained at temperatures of 90 ^{o}C for 30 min, using DMSO and K_2CO_3 as co-solvent and basic catalyst, respectively. The LCFAs derivatives were successfully separated on a reversed-phase Eclipse XDB-C8 column (150 mm x 4.6 mm, 5 μm) by a gradient

elution where the eluent A was pure acetonitrile and eluent B was a mixed solvent of acetonitrile and DMF (1:1, v/v). Similarly, Chung et al. developed an HPLC method for the analysis of free LCFAs (docosanoic, tetracosanoic and hexacosanoic) in human plasma as fluorescent derivatives [296]. The FL reagent was a sulfonate type reagent 2-(2-naphthoxy)ethyl-2-(piperidino)ethanesulfonate (NOEPES), and the separation was performed in a phenyl-hexyl column (250 mm x 4.6 mm, 5 μm) with a mobile phase consisting in methanol-water-tetrahydrofuran (87:6:7, v/v) at a flow rate of 1.2 mL/min. In this case, authors used toluene as reaction solvent since it was reported from previous studies to be a good solvent for derivatizing carboxylic acids [297]. On the other hand, HPLC-chemiluminescence (CL) detection has also been applied for the measurements of many kinds of biologically important compounds including carboxylic acids [298, 299]. CL is the emission of light as the result of electronic excitation of the luminescing species by a chemical reaction of a precursor of the species. CL derivatization methods and peroxyoxalate CL (PO-CL) methods have been mainly used for the determination of fatty acids. In CL derivatization methods, the reagents having both a chemiluminogenic group and a reactive functional group for the carboxylic acid are used. The CL derivatives of fatty acids are generally separated by a reversed-phase HPLC and produce CL by a post-column CL reaction. PO-CL detection method is known as one of the most efficient and versatile CL systems, which is based on the oxidation by hydrogen peroxide of aryl oxalate in presence of suitable fluorophores such as FL derivatives of fatty acids. Liquid chromatography of fatty acids with chemiluminiscence detection has been extensively reviewed by Ohba et al. [300]. *N*-(4-amidobutyl)-*N*-ethylisoluminol (ABEI), 6-[*N*-(4-aminobutyl)methylamino]-2,3-dihydro-1,4-phthalazinedione (ABMI), and more recently 6-[N-(3-propionohydrazino)thioureido]benzo[g]-phthalazine-1,4-(2*H*,3*H*)-dione (PRBO), has been employed as CL derivatization reagents. ABMI has been used for the derivatization of PUFAs [301] and PROB derivatives of FAs such as linoleic, oleic and stearic acids were successfully separated by a C18 column [302].

In the last decade, the marriage of liquid chromatography with MS has also allowed the dual separation and identification of organic compounds. However, while there is a significant amount of literature on liquid chromatography-mass spectrometry (LC-MS) of TGs, only a limited amount of work appears to have been conducted on fatty acids in any form. For the determination of free PUFAs, such as EPA and DHA, Sajiki directly

analyzed, for the first time, sea products using RP mode column after employing a solid phase extraction column for purification of FFAs [303]. Suzuki et al. reported an accurate method for the determination of 11 kinds of free PUFAs after ADAM esterification using HPLC with two kinds of columns: Develosil ODS-5 column containing octadecyl phase (250 mm x 4.6 mm, 5 μm), and Capcell Pak CN SG 120 column containing cyanopropyl phase (250 mm x 4.6 mm, 5 μm) [304]. Later on, Sajiki and Yonekubo determined free PUFAs and their oxidative metabolites (eicosanoids) by HPLC-MS with a CrestPack column C18T-5 (250 mm x 4.6 mm, 5 μm) coupled to a mass spectrometer equipped with an electrospray ionization (ESI) interface system [305]. At the same time, a series of chiral columns were developed based on matrices with derivatized amylose or cellulose (e.g., Chiralpak and Chiralcel series), and used for steric analysis of *cis-trans* conjugated hydroxyl acids [306]. These type of columns were employed by Bayer et al., who used HPLC with ESI-MS in the analysis of chiral derivatives of polyunsaturated hydroxyl fatty acids with an enantioselective Chiralpak® AD column (250 mm x 4.6 mm, 10 μm) with amylase tris-(3,5-dimethylphenylcarbamate) coated on silica-gel as the chiral stationary phase [307]. Also, the enantiomers of methyl ester derivatives of hydroperoxyeicosatetraenoic acids (HPETEs) were separated by chromatography on Chiralpak AD in reversed phase mode with excellent resolution of the enantiomers [308]. However, the analytical Chiralpak and Chiralcel HPLC columns have two draw-backs: they are expensive and the immobilized polysaccharide matrices can only be operated at relatively low pressures. The Reprosil Chiral-NR columns contain silica with a covalently bound chiral aromatic selector, and, in many cases, separations are comparable to matrices based on polysaccharides with chiral selectors. Very recently, a Reprosil Chiral-RN column (150 mm, 20 mm), which was eluted at 0.5 mL/min with hexane/acetic acid (1000/0.1, v/v), containing 1-1.5% of an alcoholic modifier (1.2-1.5% isopropanol, 1.2% ethanol, or 1% methanol), was used to separate the enantiomers of 18:2 ω-6 and 20:4 ω-6 as free acids, and analyzed by LC-tandem mass spectrometry (MS/MS/MS) [309]. Other applications involve a gradient LC/MS method using a C18 column (250 mm x 4.6 mm, 5 μm) and ESI source under negative ion mode for the simultaneous analysis of arachidonic acid and its eicosanoid metabolites [310]; the optimization of a stable isotope dilution LC/ESI-MS method for saturated and unsaturated fatty acid analysis using trimethylaminoethyl (TMAE) derivatives (quaternary ammonium salts) of long chain fatty acids [311]; and the LC-tandem mass spectrometry (MS/MS) for fatty acid ethyl

esters such as ethyl oleate, ethyl linoleate, and ethyl arachidonate, among others, using a C8 reversed phase column using water/isopropanol/acetonitrile (20:40:40, v/v/v) as a mobile phase [312].

Atmospheric Pressure Chemical Ionization (APCI) is a robust and dependable interface for LC-MS. Kusaka et al. used anilide derivatives of fatty acids for LC/APCI-MS of fatty acids [313]. Yamamoto et al. reported the LC/APCI-MS analysis of FFAs [314] and Rezanka demonstrated that PUFAs could be successfully analysed by LC/APCI-MS as methyl esters although this work was not combined with UV detection [315]. In 2002, Nichols and Davies determined the fatty acid composition of *Shewanella pealeana* by the analysis of the FAMEs via GC-MS and fatty acid 2-oxo-phenylethyl esters via LC/APCI-MS and LC combined with UV detection [316]. Results showed a good agreement between the percentage composition of components determined by GC-MS and LC-UV analysis. However, LC-MS analysis using APCI demonstrated a dramatically enhanced detection of unsaturated fatty acid 2-oxo-phenylethyl esters. The degree of enhancement was proportional to the degree of unsaturation. Test with a pure PUFA standard gave an absolute detection limit in full scan mode of 200 pg. In samples, the selectivity of MS over UV gave a significantly lower detection limit due to lack of chemical interferences. Wang et al. [317] determined the free fatty acid composition of the seed oil of *Nitratia tangutorum* with a pre-column derivatization method using a sulfonate type reagent BDETS as labeling reagent. The target compounds were identified by LC/APCI-MS with a reversed-phase C8 column (150 mm x 4.6 mm, 5 μm) and a vaporizer temperature of 350 ^{o}C, nebulizer pressure of 60 psi and a capillary voltage of 3500 V as APCI conditions. Yang-Jun et al. developed a method for the identification of long chain unsaturated fatty acids in deep-sea fish oil by LC/APCI-MS using BDEBS as pre-column derivatization reagent [318]. The HPLC column used was a reversed-phase C18 column (200 mm x 4.6 mm, 5 μm) and a vaporizer temperature of 450 ^{o}C, a nebulizer pressure of 60 psi and a capillary voltage of 3500 V. More recently, Rezanka et al. identified by LC/APCI-MS very-long-chain PUFAs and odd-numbered very-long-chain PUFAs from two different algae (see Figure 3) [319, 320]. In both papers they use HIRPB-250AM columns (250 mm x 2.1 mm, 5 μm) with APCI conditions of vaporizer temperature of 400 ^{o}C.

Evaporative light scattering detection (ELSD) using two symmetry C-18 reversed-phase columns connected in series and acetonitrile and water as the mobile phase has been used in the analysis of methyl esters of epoxidized FAs [321]. For example, Orellana-Coca et al. have developed a method for the analysis of FAs and their various epoxidation products, including regio-isomers, using HPLC with ELSD [322]. The FAs and their epoxydation products were separated on a C-18 reversed phase column using methanol-water containing 0.05% acetic acid as mobile phase. With this method, the saturated fatty acids, palmitic (C16:0) and stearic (C18:0) were eluted after 30.7 and 34.2 min respectively, whereas the unsaturated fatty acids, oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acid eluted after 30.7, 28.1 and 26.0 min, respectively. Palmitic and oleic acid thus co-eluted and their separation based on retention time was not possible, however, the compounds could be distinguished by MS and tandem mass spectrometry (MS/MS). More recently, ELSD was used in the determination of 11 standards such as oleic, linoleic, methyl linoleate, stearic, palmitic acids, among others, by ultra high performance liquid chromatography (UPLC) [323]. The separation column was an UPLC BEH Phenyl C18 (100 mm x 2.1 mm, 1.7 μm) and the mobile phase consisted of acetonitrile-water (3:1, v/v) in isocratic elution mode. The flow rate was of 0.3 mL/min and the column was heated at a temperature of 40 ^{o}C. Under these conditions the sample was detected by ELSD in only 5 min.

High performance size-exclusion chromatography (HPSEC) offers the possibility to quantify groups of oxidation compounds differing in molecular weight or size. The main advantage of this technique is the accurate quantification achieved of the total non-volatile oxidation compounds formed, of greatest nutritional significance because they are retained in the food and hence ingested. This methodology has been recently proposed to quantitate primary and secondary oxidation compounds in methyl esters of oleic acid, 9-cis,12-cis-linoleic acid, linolenic acid and nonadecanoic acid [324]. The separation was performed on two 100 and 500 Å Ultrastyragel columns (25 cm, 0.77 cm I.D.) packed with porous, highly cross-linked styrene-divinylbenzene copolymers connected in series, with tetrahydrofuran as the mobile phase.

Silver ion chromatography (Ag^{+}-HPLC), has been successfully applied to all lipid classes in every area of lipid investigation, including lipid chemistry, food industry (in the development of structured lipids), plant and animal physiology, medicine and

pharmacy. Ag^+-HPLC, utilizing columns packed with silver ions bonded to a silica or similar substrate, competes with GC in the analysis of FA isomers, especially in the analysis and semipreparative separation/isolation of cis and trans geometric and positional FAMEs and TG isomers [325, 326]. Ag^+-HPLC has been widely applied to the separation/quantitation of cis/trans fatty acids [246, 327], FAME positional isomers from partially hydrogenated vegetable oils [328], FAME or TG mixtures containing FA of widely-differing chain lenghts [329] or non-methylene –interrupted double bonds [330]. In the case of medium chain length mono-unsaturated fatty acids, Ag^+-HPLC provided remarkable separation based on the double bond geometric configuration, partial separation based on double bond position, and limited separation based on the FA chain length [331]. Adlof, who has extensively worked on Ag^+-HPLC, recently used two ChromSpher Lipids columns (250 mm x 4.6 mm, 5 μm particle size, silver ion impregnated) connected in series and immersed in a low-temperature bath for the analysis of FAMEs (zero to six double bonds) and TGs [332]. The mobile phases consisted of isocratic solvents (0.3% to 0.7% acetonitrile in hexane) and the Ag^+-HPLC column temperatures were programmed from +20 ^oC to -20 ^oC, and by the addition of dry ice, to -40 ^oC. Results showed that, while FAMEs elution times continued to decrease from 0 ^oC to -10 ^oC, they began to increase at -20 ^oC. A similar situation was noted for the TG isomers, except that retention times began to increase below 0 ^oC. similarly, three Ag^+-HPLC columns (ChromSpher 5 Lipids, 250 mm x 4.6 mm, 5 μm) were used in series, with a mobile phase consisting in 0.15% MeCN in isooctane at 1.0 mL/min, for the fractionation of cis/trans heptadecaenoic (C17:1) FAs [333]. Very recently, Nikolova-Damyanova has nicely reviewed the retention of different classes of lipids in Ag^+-HPLC [334].

The composition in essential fatty acids α- and γ-linolenic acids of different seed oils has recently been studied using HPLC with ^1H NMR (Nuclear Magnetic Resonance spectroscopy) detection. Bártlova et al. used two analytical columns C8 and C18 in tandem mode for the separation of FFAs of blackcurrant seed oil [335]. The mobile phase consisted of acetonitrile-chloroform-d-mixture 90:10 (v/v) and the quantitative analysis was performed via on-flow ^1H NMR differentiating between individual signals of CH_3 groups. When using a second analytical approach (reversed phase HPLC system with multi-stage non-linear gradient elution using methanol and *tert*-butyl methyl ether) they found that the α- and γ-linolenic acids co-eluted. On the contrary, those fatty acids

were distinguished, at least in the form of free acids, with the use of the LC-NMR method. These results were in agreement with those obtained by Sýkora et al. in the analysis of naturally occuring mixtures of FFAs by LC-NMR [336]. When co-elution threatens, HPLC can be accomplished by using structure sensitive detection assuming that the co-eluting compounds differ in the number of double bonds, their positions and the length of the chain. Then combining HPLC with NMR, individual components in the group with the same retention time could be identified and quantified simultaneously by NMR spectroscopy. Using a bi-dimensional HPLC with a C8 and a C18 reversed phase columns in series, in conjunction with ^{1}H NMR spectra measurements, the authors were able to simultaneously determinate and quantify co-eluted components without the need of any derivatization. Table 4 shows some examples of the methods recently applied in the HPLC determination of fatty acids (samples, columns, detection, etc.).

3.2.2. Liquid chromatography of conjugated linoleic acid (CLA) isomers

The complementary use of Ag^{+}-HPLC with GC is currently the most effective way to separate and quantitate individual isomers of CLA. A fairly comprehensive listing of recent applications of silver ion chromatography (TLC and HPLC) to the analysis of CLA in a variety of substrates has been presented by Adlof [337], Roach et al. [258], and, more recently, by de la Fuente et al. [259]. Sehat et al., based on a method developed by Adlof [338], were the first to use Ag^{+}-HPLC to complement GC in the assay of CLA [339]. CLA FAMEs are selectively detected by their characteristic UV absorbance at 233 nm and the identifications of isomers in HPLC chromatograms are based on co-injections of known reference materials obtained from commercial sources or synthesized [259]. The advantage of using Ag^{+}-HPLC in the analysis of CLA isomers is that compounds are separated into a *trans,trans*-, a *cis,trans/trans,cis*- and *cis,cis*-group depending on the configuration of double bonds and on the double bond position within each group. However, the geometrical isomers *cis/trans* and *trans/cis* have been reported to be difficult to resolve [259]. The resolution of most CLA isomers from natural and commercial products could be improved when operating from one to six Ag^{+}-HPLC columns in series [340, 341] being the use of three columns the best compromise to achieve, in a timely manner, resolution of most CLA isomers in biological matrices. Furthermore, three columns could resolve the 11-13 pair of

cis/trans geometric CLA isomers [259]. Composition of the mobile phase is a potential source of error to address when trying to obtain reproducible results between Ag^+-HPLC runs. In this sense, Müller et al. evaluated 13 solvent systems with respect to stability of retention times and resolution using a commercial CLA mixture and two ChromSpher 5 Lipids columns in series [342]. They found that 0.2% propionitrile in hexane showed the highest stability compared with the reference acetonitrile system, although it did not give as good resolution of CLA as acetonitrile, besides of being a toxic reagent. Delmonte et al. proposed an alternative mobile phase consisting on 2% acetic acid/hexane which allowed the discrimination of the two cis/trans 10-12 isomers and partial resolution for the cis-6 trans-8 isomer from the trans-7 cis-9 isomer [343]. Müller et al. also tested the combination of atmospheric pressure photoionisation (APPI) and Ag^+-HPLC for the detection of CLA FAMEs and their elongation and β-oxidation metabolites by tandem-mass spectrometry [344]. They employed three ChromSpher 5 Lipids columns (250 mm x 4.6 mm, 5 μm) in series, and 0.2% propionitrile in n-hexane as mobile phase. As APCI has been successfully used in combination with Ag^+-HPLC for TGs analysis both interfaces were tested to elucidate differences in the sensitivity of analysis of a CLA mixture. Flow injection analysis of a CLA mixture showed that CLA are very susceptible for APPI analysis resulting in an increase in signal-to-noise ratio by a factor of 40 in comparison to APCI. This sensitivity was sufficient for common analysis of CLA isomers, their metabolites and isomerization products, and moreover, the eluent of 0.2% propionitrile in n-hexane did not influence sensitivity. Results showed that no isomerization of *cis*9*/trans*11 CLA and its elongation and β-oxidation product to other *cis/trans* isomers occurred but small isomerization of C18:2 and C16:2 *trans/trans*-isomers occur. Very recently, Rodríguez-Alcalá et al. studied the influence of high pressure homogenization, a novel technology that promotes fat globule size reduction and microbial inactivation, in the content in CLA isomers of milk samples [345]. Authors used an HPLC system equipped with a diode array detector to detect and quantify individual CLA isomers present in milk fat samples. Absorbance was scanned from 190 to 300 nm wavelenght and 233 nm used for isomer quantification. The CLA methyl esters were separated using a Ag^+-HPLC ChromSpher 5 Lipid column (250 mm, 4.6 mm, 5 μm), and the major positional isomer determined was the C18:2 *cis*9/*trans*11 (rumenic acid) (81-85% total CLA), being the C18:2 *cis*7/*trans*9 isomer the second most relevant CLA accounting for 10, 5 and 8% of total CLA for cow, goat and ewe milks, respectively. No significant alteration either in quantity or distribution

of these major CLA isomers was observed as a result of the processing of milk using high pressure homogeneization.

Conclusions

In this review, an overview of the more recent methods developed to analyze bioactive fatty acids in different matrices has been presented. The revision has focused on the sample preparation methods (extraction, fractionation) and in the new methodologies for FAs derivatization previous to be analyzed by either GC or HPLC. New columns and detectors used in GC and HPLC to selectively separate and to detect/identify bioactive fatty acids are discussed. Special attention is paid to trans fatty acids and to the separation of CLA isomers, thus, these types of fatty acids are discussed separately. Due to the importance of FAs in food and health, this review attempts to be a guide to analysts who start in this new challenging area of research.

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2092 Figure Legends.

2093

2094 Figure 1 Total ion chromatogram obtained from DMC/titanium silicate PY-GC-MS of a)
2095 olive oil; b) linseed oil and c) walnut oil. Redrawn from [180]. (*) Isomers of linoleic
2096 and linolenic methyl esters; c: contaminant.

2097

2098 Figure 2. Fast-GC chromatogram of human milk FAMEs. Peak identification: (1) C8:0;
2099 (2) C10:0; (3) C12:0; (I.S.) C13:0; (4) C14:0; (5) C14:1; (6) C15:0; (7) C16:0; (8)
2100 C16:1n−9; (9) C16:1n−7; (10) C17:0; (11) C17:1; (12) C18:0; (13, 14) C18:1n−9 c + t;
2101 (15) C18:1n−7; (16) C18:2 t9,t12; (17) C18:2 c,t/t,c; (18) C18:2n−6 c; (19) C18:3n−6;
2102 (20) C20:0; (21) C18:3n−3; (22) CLA c9,t11; (23) C20:1n−9; (25) CLA t,t; (26)
2103 C20:2n−6; (27) C20:3n−6; (28) C22:0; (29) C20:4n−6; (30) C22:1n−9; (31) C20:5n−3;
2104 (32) C22:2; (33) C24:0; (34) C22:4n−6; (35) C24:1; (36) C22:5n−6; (37) C22:5n−3; (38)
2105 C22:6n−3. Redrawn from [271].

2106

2107 Figure 3. LC–MS/APCI chromatogram of FAMEs of very long chain PUFAs
2108 (VLCPUFA) of marine dinoflagellate *Amphidinium carterae* treated with Ag+–TLC to
2109 separate FAMEs with seven and eight double bonds. Redrawn from reference [319].

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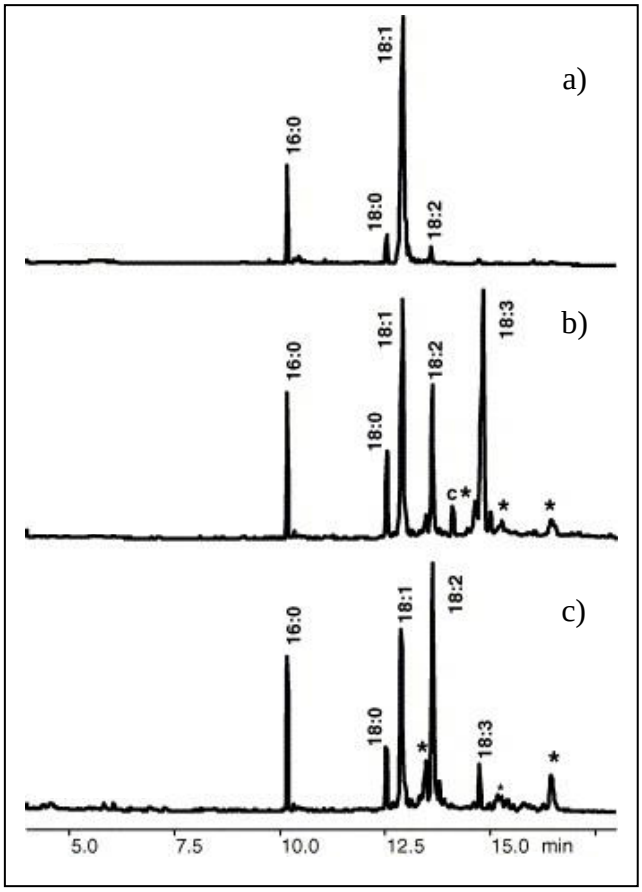
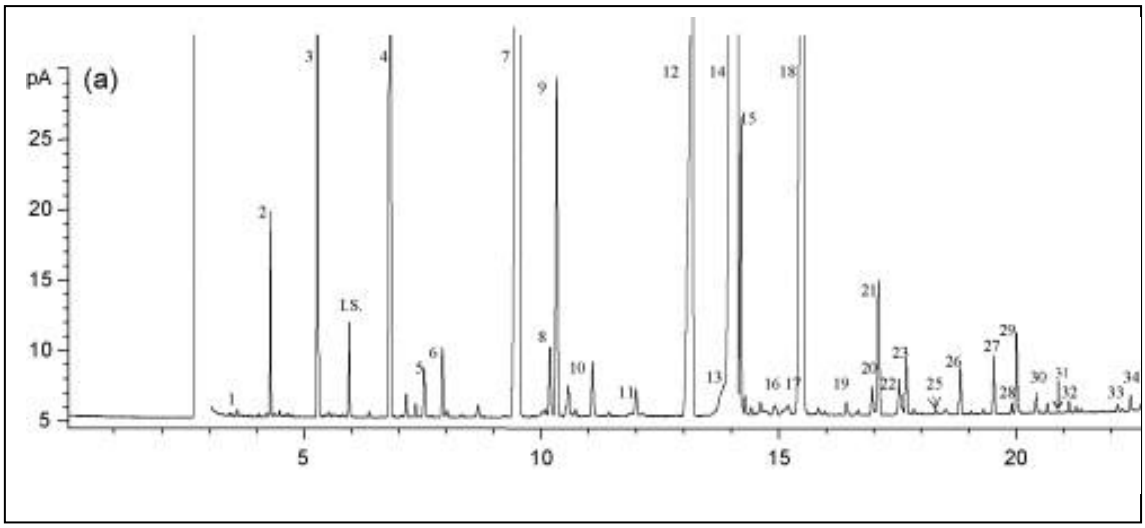
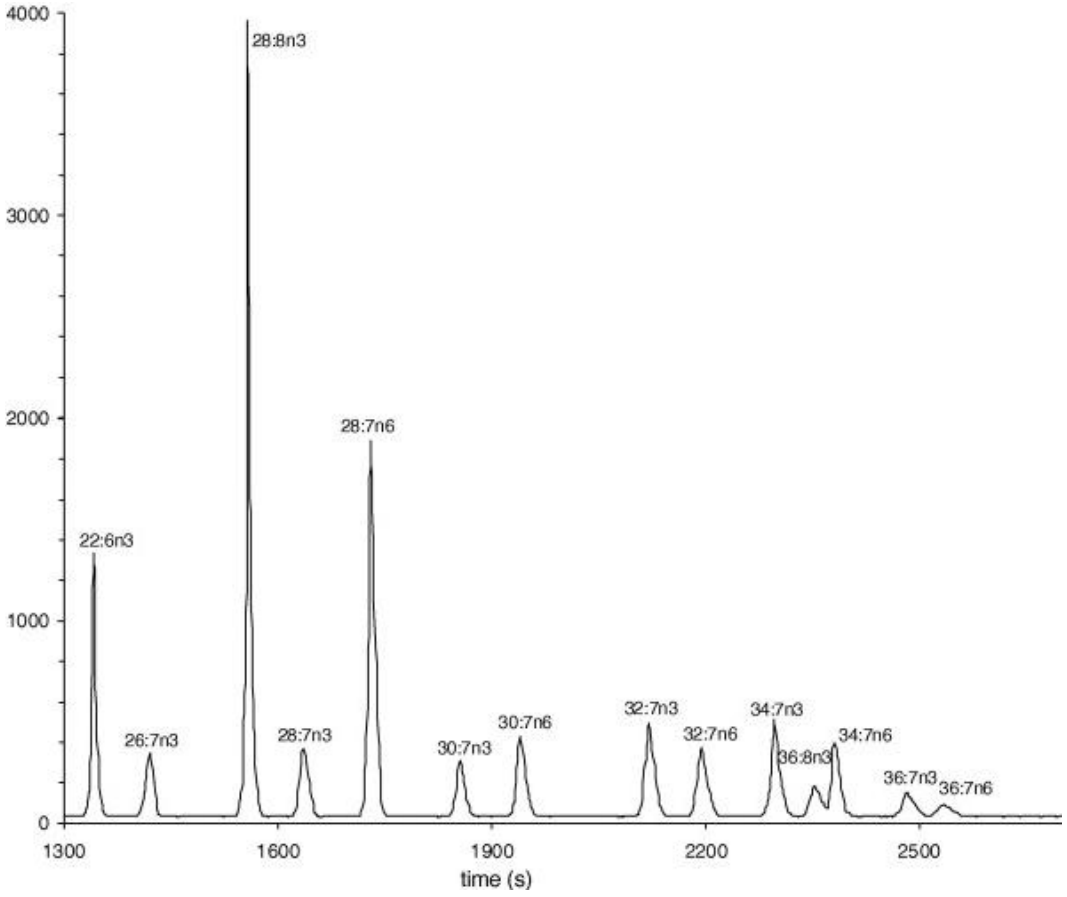




2115


4000
3000
2000
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0
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time (s)
22:6n3
26:7n3
28:8n3
28:7n3
28:7n6
30:7n3
30:7n6
32:7n3
32:7n6
34:7n3
36:8n3
34:7n6
36:7n3
36:7n6

2116

Table 1. Novel extraction techniques recently employed in the extraction of fatty acids from different food matrices.

Food lipids	Extraction method	Solvent employed	Fatty acid analysis	Reference
Oleaginous seeds, meat, bakery products	Microwave-integrated Soxhlet (MIS)	n-hexane	GC-MS	[83, 84]
Cheese	Simultaneous distillation-extraction (SDE)	Water, dichloromethane	GC-FID	[88]
Cereals	Automated acid hydrolysis-extraction (AHE)	Petroleum ether	GC-FID	[91 – 93]
Cereals, egg yolk, chicken breast muscle, poultry meat, spice leaves, pistachio oil, mate leaves	Pressurized liquid extraction (PLE)	Chloroform/methanol (2:1, v/v), isopropanol/n-hexane (2:3 v/v), n-hexane/2-propanol (3:2 v/v), n-hexane, n-hexane/acetone (9:1 v/v)), chloroform/methanol (1:4 v/v), n-pentane, ethanol, chloroform/methanol/phosphate buffer (1:2:0.8 v/v/v), chloroform/methanol (1:2 v/v)	GC-FID GC-MS	[101 – 108]

2133 Table 2. Examples of the methods recently applied in the GC determination of bioactive fatty acids.

Lipid Source	*Extraction/Separation*	*Derivatization*	*Capillary Column*	*Identification/quantitation*	*Reference*
Sunflower seed oil crude wax	n-hexane, TLC	0.7 N HCl/MeOH, BF_3/MeOH (85 ^oC, 10 min)	SPB^{TM}-1 30 m	GC-MS	[120]
Olive oil, sunflower oil, Palm oil, coconut oil, pork fat	CMTS/propanol (90 ^oC, 20 min)	KOH/BF_3-MeOH (75 ^oC, 5 min) BF_3/MeOH (80 ^oC, 60 min)	SP-2330 30 m	GC-FID, Internal standard (I.S.)	[170]
FFA from cheese	Acetonitrile (45 ^oC, 10 min) Cl_2CH_2 (50-110 ^oC, 3h)		FFAP 25 m DB-Wax 30 m	GC-MS, I.S., Distribution coefficients (K)	[88, 89]
PUFAs from individual zooplankters		0.25 M TMSH/MeOH 2.2 M TMAH/MeOH	Ultra ALLOY-CW 30 m	GC-MS	[176]
Rabbit meat	Alcoholic NaOH	BF_3/MeOH	SP2560 100 m	GC-FID, FAMEs standards	[208]
Beer (MCFA)	Stir bar sorptive extraction with Cl_2CH_2/hexane (50:50 v/v)		Phenomenex 2B-Wax 30 m	GC-FID, Octanol/water partition coefficient ($K_{o/w}$)	[149]
Flax seeds	Soxhlet extraction with n-hexane (48 h)	BF_3/MeOH (90 ^oC, 2 min)	SGE BP20 25 m, DB-5 30 m	GC-MS, FAMEs standards	[226]
Soybean oil, lanolin, fish oil capsules	TLC	0.5 M KOH/BF_3-MeOH	SPB-50 30m, BPX-50 30 m, DB-17 ms 30 m, DB-17 30 m	Retention time (Rt), GC-MS, equivalent chain-length	[217]
Vegetable oil deorodizer distillates	Diethyl ether	Pyridine/BSTFA/TMCS (70 ^oC, 20 min)	CP-Sil 8 CB 15m x 0.25 mm x 0.1 μm	GC-FID, Rt	[202]
Blackcurrant oil	Chloroform/MeOH (1:1 v/v) (20 h) x 2, TLC	MeOH/Chloroform (3:2) plus Acetyl chloride (70 ^oC, 90 min)	DB-Wax 30 m	GC-FID, equivalent chain-length	[210]
Milk fat, cocoa butter, tuna oil		Sodium methoxide/MeOH (room temperature, 3.5-4 min)	BPX-70 10 m x 0.1 mm x 0.2 μm	GC-FID, FAMEs standards	[224]
LCFA from anaerobic reactors	Cl_2CH_2	HCl/1-propanol	CP-Sil 52 CB 30 m	GC-FID, FAMEs standards, I.S., GC-MS	[205]
Edible mushrooms	Soxhlet with petroleum ether	ISO 5509 (2000) transesterification method	CP-Sil 88 50 m	GC-FID, FAMEs standards, relative retention times	[204]
D003 (VLCFA mixture)		Hydrochloric acid/MeoH (80 ^oC), BF_3/MeOH (60 ^oC, 5 min), MSTFA (60 ^oC), H2SO4/MeOH (80 ^oC)	BPX-5 25 m	GC-FID, I.S., FAMEs standards	[190]

2134

2135 Table 3. Examples of the methods applied in the GC determination of isomeric fatty acids in foods.
2136

Food Lipids	Extraction	Derivatization	Capillary column	Identification/quantitation	Reference
Cereals	Diethyl ether, Petroleum ether	0.5 N NaOMe, BF_3/MeOH	Rtx®-2330 30 m	GC-FID, Rt, I.S.	[91]
Linseed oil	Chloroform/MeOH	Ammonium chloride/MeOH/H_2SO_4 (1:30:1.5 m/v/v)	CP-Select CB-FAME 100 m	GC-FID, equivalent chain length, FAMEs standards	[254]
Shortenings	n-hexane	0.5 M sodium methoxide/MeOH	AT™-Silar-90 30 m	GC-MS, Rt	[253]
Oils, butters, margarines, Creams	Hexane/isopropanol (3:2)	Acetyl chloride/MeOH	SP2560 100 m	GC-FID, Rt, FAMEs standards	[252]
CLA in human milk	Agitation in ultrasonic bath	$NaOCH_3$/MeOH (80 °C, 10 min) 1.25 M HCl/MeOH (80 °C, 3 min)	RTX-2330 40 m	GC-FID, FAMEs standards	[271]
CLA in rumen fluid	Chloroform/MeOH	H_2SO_4/MeOH (35 °C, 30 min)	CP-Sil 88 100 m	GC-MS, I.S.	[269]
CLA in beef	Chloroform/MeOH	ISO Method 5509	CP-7420 Select FAME 100 m	GC-FID, relative retention time, FAMEs standards	[268]
CLA in yoghurt, fermented milk, cheese	Chloroform/MeOH	2N methanolic-potassium-hydroxide (20 °C, 6 min)	CP-Select CB 100 m	GC-FID, Rt, FAMEs standards	[267]
CLA in cow milk, butter oil	Chloroform/MeOH	0.5 N NaOH/MeOH, BF_3/MeOH	CP-Sil 88 100 m	GC-FID, FAMEs standards	[263]
CLA in beef	Petroleum spirit	2M TMS-DM/hexane	CP-Sil 88 100 m	GC-FID, Rt, FAMEs standards	[264]
CLA in butter	Petrroleum ether	2N sodium methoxide (room temperature, 20 min), BF_3/MeOH (room temperature, 20 min)	CP-Sil 88 100 m	GC-FID, FAMEs standards	[262]

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2138 Table 4. Some examples of the methods recently applied in the HPLC determination of fatty acids.

Lipid source	Extraction/derivatization	Columns	Mobile Phase composition	Detection/quantification	Reference
ALA and GLA from blackcurrant seed oil	Supercritical CO_2 extraction (28 Mpa, 45 ^oC) and then dissolved in light petroleum/acetone	C8 Luna (150 mm x 4.6 mm, 5 μm) C18 Luna (250 mm x 4.6 mm, 5 μm) , connected in series	Acetonitrile:chloroform (90:10 v/v), isocratic elution	1H NMR, External standard method	[335]
LCFAs from plant Extracts	Distillation extraction with 1:1 (v/v) Chloroform/MeOH. Labelling with TSPP at 90 ^oC in the presence of K_2CO_3 and DMF	Eclipse XDB-C8 (150 mm x 4.6 mm, 5 μm)	A: acetonitrile; B: acetonitrile/DMF (1:1 v/v). Gradient: 100% A to 100 % B (kept for 10 min) in 15 min	Fluorescence detection, HPLC/APCI-MS, I.S.	[295]
FFAs from seed oil	Supercritical CO_2 extraction (20 Mpa, 40 ^oC). Labelling with BDETS at 90 ^oC in the presence of K_2CO_3 and DMF	Eclipse XDB-C8 (150 mm x 4.6 mm, 5 μm)	A: acetonitrile:ammonium formate (1:1 v/v); B: acetonitrile. Gradient: 0-40% (B) from 0 to 12 min; 40-80% (B) from 12 to 15 min; 80–100% (B) from 15 to 25 min, to 100% (B) (kept for 10 min)	Fluorescence detection, HPLC-APCI-MS	[317]
LCFAs from fish oil	2 M KOH/EtOH solution, followed by hexane and acetonitrile/DMF extractions. K_2CO_3 and DMF (85 ^oC, 45 min). BDEBS as pre-column derivatization reagent.	Hypersil BDS-C18 (200 mm x 4.6 mm, 5 μm)	A: acetonitrile:formic acid (1:1 v/v); B: acetonitrile. Gradient: 0-100% (B) from 0 to 40 min, then kept at 10 min	HPLC-APCI-MS	[318]
VLCFAs from Algae	$CHCl_3$/MeOH (2:1 v/v), followed of disollution in toluene and 1% H_2SO_4 in MeOH (50 ^oC, overnight). Esters were then extracted with n-hexane	HIRPB-250AM (250 mm x 2.1 mm, 5 μm) x 2 (in series)	A: acetonitrile (AcN), B: dichloromethane (DCM), C: propionitrile (EtCN). Gradient: initial: AcN/EtCN/DCM (60:30:10 v/v/v) linear from 5 to 50 min AcN/EtCN/DCM (30:40:30 v/v/v), to 60.5 min.	HPLC-APCI-MS, comparison with synthesized standards.	[319]
11 FAs standards mixture		UPLC BEH Phenyl C18 (100 mm x 2.1 mm, 1.7 μm)	Acetonitrile:water (3:1 v/v), isocratic elution.	UPLC-ELSD	[332]
ALA and GLA in artificial FFAs Mixtures		C8 Luna (150 mm x 4.6 mm, 5 μm) C18 Luna (250 mm x 4.6 mm, 5 μm) , connected in series	Acetonitrile:chloroform (90:10 v/v), isocratic elution	1H NMR, External standard method	[336]
CLA in rumen fluid	Chloroform/MeOH. H_2SO_4/MeOH (35 ^oC, 30 min)	Three ChromSpher 5 Lipis Ag^+-HPLC (250 mm, 4.6 mm, 5 μm), in series	Isocratic: 0.1 % (v/v) acetonitrile and 0.5% (v/v) diethyl ether in hexane.	Ag^+-HPLC with diode array detection at 233 nm. FAMEs standards.	[269]
LA, ALA, GLA, AA, EPA, DHA from atherosclerotic plaques	Chloroform/MeOH (2:1 v/v). TMAE esters were prepared using dimethylaminoethanol and a iodide/MeOH mixture (1:1 v/v)	Varian Pursuit Diphenyl column (150 mm x 2 mm, 3 μm)	A: 5 mM ammonium acetate in water; B: 5 mM ammonium acetate in acetonitrile. Gradient: 40% B at 0 min, 40% B at 3 min, 60% B at 13 min, 80% B at 15 min, 80% B at 20 min, 40% B at 25 min, 40% B at 35 min	HPLC/ESI-MS and HPLC/MS/MS	[311]

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