Review

Gas chromatography of fatty acids

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ABSTRACT

Lipids in foods contain a wide variety of fatty acids differing in chain length, degree of unsaturation, position and configuration of double bonds and the presence of special functional groups. Modern capillary gas chromatography offers excellent separation of fatty acids. Fused-silica capillary columns with stationary phases of medium polarity and non-polar methylsilicone stationary phases successfully separate most of the natural fatty acids. Special applications, such as the separation of complex *cis–trans* fatty acid mixtures and cyclic fatty acids, require particular chromatographic conditions, including the use of very long capillary columns or more polar stationary phases. The derivatization methods for the preparation of fatty acid esters also need to be optimized to obtain accurate quantitative results. This paper reviews the derivatization techniques, capillary columns and stationary phases commonly used in the gas chromatography of fatty acids in food.

CONTENTS

1. INTRODUCTION

The gas chromatography (GC) of fatty acid methyl esters with flame ionization detection (FID) is the most often used method for the analysis of fatty acids. The advent of wall-coated open-tubular (WCOT or capillary) chromatographic columns, now available with different stationary phases, offer excellent resolution capabilities regardless of the sample type. The differing nature of fatty acids present in various food groups, such as vegetable oils, including hydrogenated oils, dairy and meat fats and fish oils, requires different conditions in their GC analysis.

In this review, the various aspects of the transesterification of lipids, involving different reagents and derivatization techniques, are discussed. The performance of the most commonly used packed and capillary GC columns in the analysis of fatty acids in foods is evaluated.

The analysis of fatty acids involves mainly two steps: the preparation of the fatty acid methyl esters (FAMEs) and the analysis of the FAMEs by GC.

2. PREPARATION OF FATTY ACID METHYL ESTERS

It is necessary to optimize the derivatization step in order to obtain accurate quantitative results. In a study of the myocardial metabolism of fatty acids, by the GC analysis of FAMEs using boron trifluoride (BF$_3$)–methanol as esterification reagent, Al Makdessi et al. [1] concluded that the sample preparation procedure directly affected the quantitative results.

The common procedural deficiencies associated with ester preparation include (i) incomplete conversion of the lipids to FAMEs; (ii) alteration of the original fatty acid composition during esterification, including the formation of positional and/or geometric isomers; (iii) formation of artifacts which can be wrongly identified as fatty acids; and (iv) contamination and subsequent damage of the GC column resulting from traces of the esterification reagent (if not washed properly) or an unclean sample. Incomplete sample clean-up may result in the build-up of refractory material in the column and detector, which in turn will affect the resolution and quantitation.

Esterification reagents (referred here to reagents that convert free fatty acids to esters) and transesterification reagents (those which convert acylglycerols to fatty acid esters), generally fall into two categories: "acid-catalyzed" and "base-catalyzed" reagents. An early excellent review by Sheppard and Iverson [2] discussed in detail the various esterification and transesterification reagents. After a survey of the various reagents and methods, they concluded that "... there is no method of esterification that is ideal". They agreed, however, that most of these methods are acceptable if used properly. Bannon et al. [3] reviewed in detail base methoxide-catalyzed transesterification, which they referred to as methanolysis. They pointed out the various pitfalls and discrepancies associated with the published methods and proposed a suitable methodology taking into consideration the desirable features of the existing procedures.

2.1. Base-catalyzed reagents

Sodium methoxide in methanol is generally accepted for the conversion of the acylglycerols to methyl esters. Nevertheless, this technique is not very useful when large amounts of free fatty acids (FFAs) are present in the sample [4]. This transesterification method is rapid and can be carried out at room temperature. Under such mild conditions the reagent does not cause any isomerization of double bonds, nor does it liberate the aldehyde from the plasmalogens, which could complicate the chromatogram. The sphingolipids and cholesterol esters need more vigorous conditions for transesterification with sodium methoxide [4]. A disadvantage with this base-catalyzed transesterification reagent is that it does not convert the FFAs into FAMEs. The conditions need to be anhydrous as the presence of water causes saponification, thereby resulting in a loss of fatty acids. Negligent and prolonged use of this reagent may cause alterations in the fatty acid composition. Use of high concentrations of base and high temperatures could result in the formation of conjugated fatty acids.

Potassium hydroxide in methanol is another transesterification reagent [5] commonly employed in the transesterification of oils. Bannon et al. [3] discussed the superiority of the sodium methoxide over the potassium hydroxide in methanol method, taking into consideration that the latter could result in saponification. They found the methoxide–reflux

method (which takes only 2 min) to be suitable for the determination of fatty acids in a wide variety of fats. However, this technique is not accurate for the determination of short-chain fatty acid esters such as methyl butyrate and caproate, which are present in considerable amounts in dairy fat. The removal of the excess of sodium methoxide by washing with brine solution before GC analysis is strongly recommended, as the reagent tends to interfere in the analysis [3]. Sodium methoxide is a popular methylating agent in the oil industry, especially for refined oils, where the FFAs have already been removed during the refining stage [6]. A series of publications [3,6–9] from the Unilever group in Australia are devoted to alkali-catalyzed transesterifications and the accuracy of the GC analysis of fatty acids.

More recently, guanidine and its alkylated derivatives in methanol have been used to catalyze the esterification of oils and fats [10]. This basic reagent causes the complete methanolysis of oils, converting both the acylglycerols and the free fatty acids into methyl esters. The FFAs are said to form the salt guanidinium carboxylate, which in the presence of excess of methanol forms the methyl ester. Again, the excess of reagent needs to be extracted before the sample is injected into the GC column. Schuchardt and Lopes [10] compared guanidine-catalyzed methanolysis with the Ce 2-66 (20% BF$_3$–methanol) AOCS method [11] and the Hartman and Lago method (NH$_4$Cl–H$_2$SO$_4$ in methanol) [12] to study the fatty acid composition of several seed oils. The results with the first method were comparable to those obtained by the Ce 2-66 method, the linoleic acid concentration being slightly higher than that obtained by the Hartman and Lago method [12]. The reagent is inexpensive and mild, causing no isomerization of double bonds. This derivatization reaction is rapid, requiring 2 min of heating in a boiling water-bath.

2.2. Acid-catalyzed reagents

Among the various acid-catalyzed reagents (such as methanolic hydrochloric acid, sulfuric acid in methanol and acetyl chloride in methanol), boron trifluoride in methanol has found wide application as a good reagent to convert both the acylglycerols and the FFAs into methyl esters. The popularity of this reagent may be due, in part, to its early accept-

ance in the American Oil Chemists' Society (AOCS) method [8]. The Association of Official Analytical Chemists (AOAC) procedure [13] involves the saponification of the acylglycerols using sodium hydroxide in methanol followed by esterification with BF$_3$–methanol to give the FAMEs. This method has been stated to be not useful for fatty acids containing unusual functional groups such as epoxy, cyclopropenyl, cyclopropyl, acetylenic or conjugated unsaturation. Although the first step is generally considered to be saponification, Ackman et al. [14] clarified that the first step, which involves the use of alcoholic sodium hydroxide, is a base-catalyzed transesterification and not saponification. Christie [4] is of the opinion that BF$_3$–methanol has been overrated as a transesterification reagent; it is known to form artifacts and is not particularly suitable for seed oils containing unusual fatty acids. The reagent reacts with the plasmalogens to liberate the aldehyde, which is then converted into dimethyl acetals. The dimethyl acetals (depending on the chain length) may interfere in the fatty acid analysis by GC [15]. The reagent reacts with cholesterol to give cholesteryl methyl ether and cholestadiene, which also interfere in the GC analysis of fatty acids. The reagent is expensive and has a limited shelf-life if not refrigerated. Use of old or concentrated reagents has been shown to result in loss of polyunsaturated fatty acids (PUFA) [4]. In spite of these various disadvantages, BF$_3$–methanol is currently accepted as being of great value for the rapid methylation of fatty acids.

Aluminum chloride in methanol has been used to transesterify a number of lipid samples [16]. With milk and vegetable oils the transesterification reagent was added directly without prior extraction of lipids. This reagent was comparable to BF$_3$–methanol for the transesterification of cholesteryl esters. The product of methylation obtained by using this reagent showed no artifact when analyzed by TLC, whereas the BF$_3$–methanol reagent showed some extra spots on the TLC plates. One of the disadvantages with the aluminium chloride reagent is that it does not esterify the FFAs. Hence it appears that in spite of being a Lewis acid, like boron trifluoride, it does not behave in a similar way.

2.3. Diazomethane

Diazomethane is a rapid esterification reagent

which esterifies FFAs to FAMEs in the presence of methanol [4]. The reaction is almost instantaneous at room temperature, the excess of reagent being easily eliminated by evaporation under nitrogen. However, diazomethane is extremely toxic. Moreover, diazomethane is known to form artifacts by reacting with double bonds or carbonyl groups [17]. The high reactivity of this reagent is said to work to its own limitations [17]. It is recommended that diazomethane be used only if absolutely necessary [4].

2.4. Other esterification reagents

The past decade has witnessed the emergence of a number of methods for the preparation of FAMEs. Most of the methods involved modification of existing procedures or incorporation of new reagents, and were invariably accented towards the rapidity of the method. With the large number of samples that need to be analyzed, there was a general tendency to seek rapid methods for esterification and analysis.

Strongly basic quaternary salts of ammonia such as m-trifluoromethylphenyltrimethylammonium hydroxide (TFMPTAH) in methanol [18], trimethylphenylammonium hydroxide (TMPAH) [19] and tetramethylammonium hydroxide (TMAH) [20–22] have found applications as transesterification catalysts for acylglycerols. The FFAs which may be present in the sample are converted into quaternary ammonium salts, which under the pyrolytic conditions of the gas chromatograph give the corresponding methyl esters. The advantages of these methods include a single-step transesterification, instead of the conventional saponification followed by esterification. These reagents do not require an extraction step, which is of particular concern when dealing with short-chain fatty acids. Metcalfe and Wang [20] have shown that with the use of TMAH as reagent, one can separately determine the fatty acid composition of the glycerides and the FFAs. With a slight modification the total fatty acid composition can be determined. However, the sample has to be neutralized before injection into the GC system. The high alkalinity and the relatively high column temperature in the pyrolytic methylation step often cause isomerization of the double bond, resulting in the formation of conjugated acids. The excess of TMAH breaks down to give trimethylamine, methanol and dimethyl ether with accept-

able GC properties [23]. TMTFTAH on breakdown gives m-trifluorotolyldimethylamine, which has been shown to interfere in the separation of medium-chain fatty acids [23]. Trimethylsulfonium hydroxide (TMSH) is another single-step transesterification reagent that requires no additional extraction. The product of its pyrolysis, dimethyl sulfide, elutes with the solvent peak during GC analysis [23].

Emphasis has also been placed on a one-step digestion, extraction and esterification of lipid samples, which has been referred to as "direct transesterification" by most researchers [24,25]. This method circumvents the extraction and isolation of lipids, thus saving considerable amounts of time and chemicals. The sample is taken in a solvent and treated with acetyl chloride in methanol to give FAMEs. This method has been modified in that different solvents have been used. The transesterification reagent, heating time and temperature have also been modified. This direct method has been applied to determine the fatty acid composition of different tissues, plasma, feeds, faeces, milk fat, etc. [24–26]. Sukhija and Palmquist [26] carried out the experiment both in benzene and in chloroform. However, when using chloroform one should be careful because the ethanol present as a stabilizer in chloroform could result in the formation of ethyl esters, which would interfere in the GC of FAMEs [27]. Chloroform, in the presence of base and heat, forms dichlorocarbene, which reacts with the double bond of unsaturated fatty acids [4]. The hazardous benzene can be replaced with toluene.

The direct method has been compared with the traditional extraction, Folch wash procedure [28] by Lepage and Roy [24]. This method led to an increase in total fatty acids for plasma, faeces, bile and rat liver as compared with the Folch wash. They concluded that the direct esterification method was more efficient than the prevailing methods because of its simplicity, rapidity and higher accuracy.

Bitman [25] applied the direct transesterification method using acetyl chloride in methanol to human and cow milk fat and compared it with the NaOH–methanol, BF$_3$–methanol and acetyl chloride–methanol methods for the lipids extracted by the traditional Folch wash. The results compared well for most fatty acids in cow milk. The only exception

was a significantly lower mass percentage of C_4 acid by the direct transesterification as compared with the other methods. This derivatization technique was comparable to the BF_3–methanol method applied to Folch extracts for the analysis of human milk fatty acids. Browse *et al.* [29] used hot methanolic hydrochloric acid to digest the leaf tissue and to convert the fatty acids directly to FAMEs. The FAMEs were then extracted into the organic layer and analyzed by GC. This procedure resulted in a lower (10–20%) yield of fatty acids as compared with the conventional extraction, saponification and methylation steps to obtain FAMEs.

2.5. Preparation of esters other than methyl

Esters other than methyl can be prepared for a variety of reasons [4], *e.g.*, butyl esters [30,31] for the analysis of short-chain fatty acids present in dairy fats. The wide range of fatty acids (C_4–C_{26}) present in dairy fat and the volatility and solubility of the short-chain fatty acids require special attention in the preparation of FAMEs and GC analysis. The best methods for transesterification of such fats would be those in which no heating or solvent evaporation (which could result in the loss of volatile fatty acids) or extraction with water (resulting in the loss of soluble short-chain fatty acids) is involved. The Christopherson and Glass method [32] using sodium methoxide in methanol is generally recommended; however, the preparation of fatty acid butyl esters has been shown to give better recoveries of short-chain fatty acids. The method was discussed in detail by Iverson and Sheppard [30,31].

It is apparent that most of the reagents discussed above would serve the purpose depending on what type of sample is to be analyzed. However, care must be taken to follow the method exactly as specified because even a minor modification such as the use of cold instead of tepid water for extraction of the reagent [8], shaking manually instead of vortex mixing the solution [8] or heating for an extra few minutes [14] could account for an inaccurate fatty acid composition. With the newer reagents, especially where no extraction of the transesterification reagent is involved, their long-term effect on the GC column should be assessed. When introducing a new reagent it is not always practical to test its prolonged use on the GC column. To inject a sample 80 times into the GC system just to show the detri-

mental effect of excess of transesterification reagent on the GC column [3], even after predicting the effect, would be a misuse of resources. Hence it would be useful to receive information from different workers using such new reagents. The accuracy of the method should be demonstrated on primary standards, short- and long-chain and polyunsaturated fatty acids. It should be carefully assessed whether the method results in a preferential loss of certain fatty acids or causes the formation of conjugated or different geometric isomers. This point cannot be more stressed, taking into consideration that the conjugated linoleic acid (CLA), especially the 9-*cis*,11-*trans*-isomer, has been shown to exhibit anticarcinogenic activity [33–35]. CLA, which occurs in a number of foodstuffs (cheeses, milk fat, beef, etc.) has mostly been determined by the GC analysis ·of the FAMEs [33–36]. The differing amounts of CLA reported in the same foodstuffs could be due to a number of factors [34,36]; a possible reason could be that CLA was either formed or destroyed during esterification. We are currently assessing various esterification methods to study their applicability to correctly determine the different isomers of conjugated dienoic fatty acids present in dairy and meat products. The preferred reagent is that which does not cause any positional or geometric isomerization of the double bonds and gives a total recovery of the short-chain fatty acids.

3. GC COLUMNS AND STATIONARY PHASES

The superior resolution capability of capillary columns allows the routine identification of fatty acids on the basics of retention time alone, especially if the analysis is performed with stationary phases of different polarities. A wealth of data on the retention times and related parameters of most common fatty acids are available [37–39].

The use of packed columns for the analysis of fatty acids in food products and other samples is well known. Packed GC columns are still acceptable for preliminary analysis or screening procedures, but the increasing concern for the detection of specific fatty acid components in plant and animal tissues requires a much higher resolving power. Problems in maintaining a steady flow and the development of multiple flow paths of the carrier gas in packed columns limit the total column length to

about 6 m. On the other hand, the permeability of capillary columns allows for columns up to 100 m long, with *ca.* $4 \cdot 10^5$ theoretical plates [40].

The development of fused-silica capillary columns was a breakthrough in GC. Capillary columns made of stainless steel or borosilicate glass exhibited similar polarities and elution properties to modern fused-silica supports [41]. Stainless-steel or glass supports, however, presented problems of chemical reactivity or fragility, respectively [42]. Fused-silica columns provide a chemically inert support for the stationary phase. Modern fused-silica capillary columns are treated with a polyamide to decrease the possibility of damage due to an increase in flexibility. As this is essentially an external coating, the process does not modify the column polarity.

Despite their long history and analytical superiority, WCOT capillary columns have been widely accepted only in the last decade. Difficulties were based on the fact that they can be easily overloaded, resulting in a considerable decrease in resolution and quantification capabilities. Capillary columns demand more careful laboratory practices, a higher detector response (for sharp and rapidly eluting peaks) and a more sensitive detector for small sample sizes. Ironically, capillary columns have been criticized for giving "too much information" [42].

Resolution is a function of the square root of the length of the column. Therefore, large increases in column length (and retention times) will be required in order to improve the resolution significantly if other chromatographic conditions remain unchanged. Thus, most analyses are performed with an intermediate column length (*e.g.*, 30 m). The use of longer columns would only be necessary for very complex mixtures (about 100 individual components or more) and special applications (discussed later).

The inner diameter of the column also determined the column efficiency, retention time and column capacity. For columns with identical stationary phases, the smaller diameter would provide better peak resolution. In general, and when sample overloading is not a problem, the use of columns of 0.25 mm i.d. is recommended.

In theory, the only part of the column that will interact with the injected sample is the stationary phase. Therefore, the film thickness of the station-

ary phase is a primary factor in determining the retention of the sample components. Thick films will produce longer retention times and better resolution. Thick films are recommended for volatile solutes such as short-chain (*i.e.*, C_2-C_8) fatty acids. Most analyses, however, can be accomplished with the standard film thickness of 0.25 mm and an i.d. of 0.32 mm.

3.1. Polar versus non-polar stationary phases

While column selection in packed-column GC was crucial to obtaining satisfactory results, the greater resolution capacity of capillary columns made column selection less important. Most of the commercially available columns offer satisfactory separations of major fatty acids. In fact, capillary columns coated with a few stationary phases have replaced dozens of different types of packed columns [40]. Table 1 lists the frequently used stationary phases for capillary columns. Careful column selection in capillary GC is still important for special applications, as will be described later. As a general rule, the use of the least polar phase which provides satisfactory results is recommended. Non-polar phases exhibit superior lifetimes over polar columns.

Capillary columns are commercially available with a variety of stationary phases of different chemical compositions. A convenient way of indicating a GC column characteristic is the column polarity index (CP), which ranges between 0 and 100, from the extremely non-polar (squalene, CP = 0) to the most polar material available (cyanoethyl-silicone, CP = 100) [43]. Methylsilicones are among the most widely used non-polar stationary phases, and they were the first to be developed for capillary GC [41]. Some of the advantages of methylsilicone columns are high thermal stability, a wide range of operating temperatures and chemical inertness. Most FAME mixtures can be separated with methylsilicone columns. In particular, very low polarity phases such as purified methylsilicone are preferred. On non-polar columns FAMEs are eluted in order of their boiling points, and the unsaturated components elute ahead of the corresponding saturated component of the same chain length. This elution order is the reverse of the elution order in columns with intermediate and highly polar stationary phases [41].

TABLE 1

CHEMICAL COMPOSITION OF MOST COMMONLY USED STATIONARY PHASES FOR CAPILLARY GC OF FOODS

Chemical composition	Brand names	Polarity[a]	Applications
100% Cyanopropylsilicone	CP-Sil-88 (Silar-10c)	VP	Soybean oil [61]
100% Cyanoethylsilicone oil	SP-2340, OV-275	VP	Milk fat [51], hydrogenated vegetable oils [62]
Methylsilicone polymer, 25% Cyanopropyl–25% phenyl–50% methyl	OV-225, DB-225, SP-2300	P	Meat fat [67]
Methylsilicone polymer, 1% vinyl–5% phenyl	SE-54	NP	Kernel oil [84]
Methylsilicone	SPB-1	NP	Fish oils [79]
	SPB-5	NP	Seed oils [58]
68% Biscyanopropyl–32% dimethylsiloxane	SP-2330	VP	Butter oil, vegetable oils [62]
	SP-2560	P	Hydrogenated vegetable oils [62]
Polyethylene glycol	DB-WAX, Supelcowax-10, Omegawax, Carbowax 20M	P	Yeasts [48], butterfat [66], marine oils [73], [68], blackcurrant seed oil [83], soybean oil [65] furan fatty acids [75], cottonseed oils [55]
95% Dimethyl–5% diphenylpolysiloxane	DB-5, SPB-5, CP-SIL 8CB	NP	Cottonseed oil [55]
100% Dimethylpolysiloxane	DB-1, Rt-1, SPB-1, SP-2100, OV-1, OV-101, CP-SIL 5CB	NP	Yeasts [48]
86% Dimethyl–14% cyanopropylphenylpolysiloxane	DB-1701	P	Yeasts [48]
Acidified PEG	Nukol, FFAP	P	Dairy products [51,53]

[a] VP = Very polar; P = polar; NP = non-polar.

The major drawback with non-polar phases is the co-elution of some unsaturated FAMEs. For instance, linoleic acid $(18:2\omega 6)^a$ partially overlaps with 18:1 and 18:3 fatty acids (Fig. 1). C_{20} and C_{22} fatty acids present similar overlapping problems [41]. Because many C_{18} and C_{20} fatty acids have important nutritional roles, polar or intermediate stationary phases are more often the choice in the analysis of food products.

Stationary phases of intermediate polarity, such as polyethylene glycol (PEG, Carbowax 20M) are widely used in the GC of FAMEs. Carbowax 20M is especially suitable for the separation of PUFA

and it is being tested in an official method of the Association of Official Analytical Chemists for the analysis of lipids of marine origin [41]. In polar columns, each group of fatty acids, e.g., 18:0, 18:1ω11, 18:1ω9, 18:1ω7, 18:2ω6, 18:3ω6, 18:3ω3 and 18:4ω3, will elute in this order (Fig. 2) [44]. This sequence would expand with increasing column polarity so that the more unsaturated FAMEs will elute after the next highest even-carbon saturated acid (in this example 18:4ω3 will elute after 20:0). In contrast to methylsilicone phases, PEG phases have lower temperature stability. A maximum of 250°C is the recommended temperature for isothermal GC on recently developed high-molecular mass PEG phases.

None of the polar or non-polar columns can completely separate all fatty acids normally present in a complex mixture. Therefore, a laboratory can benefit from the operation of two columns with different polarities.

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[a] For the nomenclature used for fatty acids, consider the example 18:2ω6. In this notation, 18 refers to the total number of carbon atoms, 2 is the number of ethylenic bonds, and ω6 corresponds to the number of carbons from the center of the ethylenic bond furthest removed from the carboxyl group up to and including the terminal methyl group.
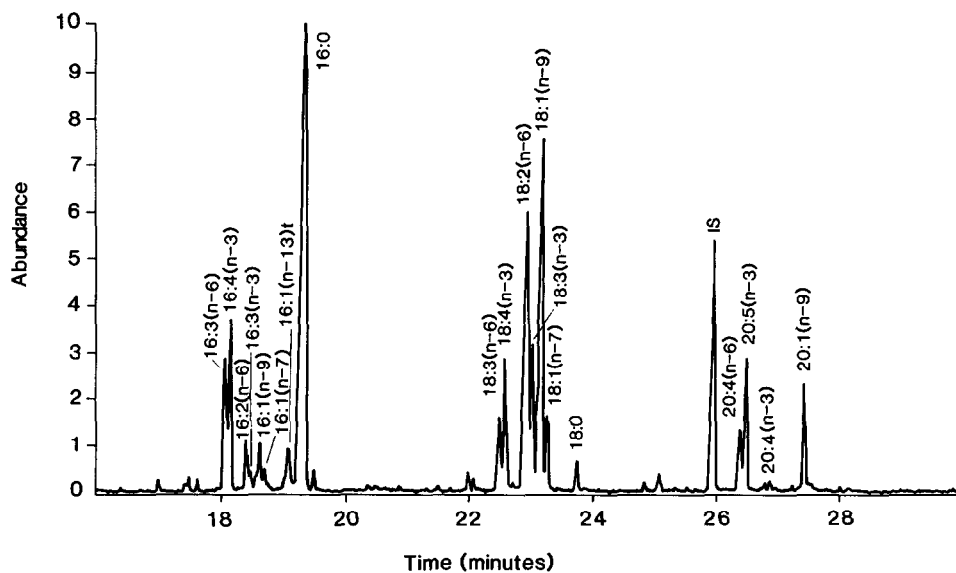
Fig. 1. Reconstructed chromatogram of FAMEs from the unicellular alga *Tetraselmis suecica*, obtained by GC–mass spectrometry using a 50-m methylsilicone fused-silica capillary column. From Volkman *et al.* [74].
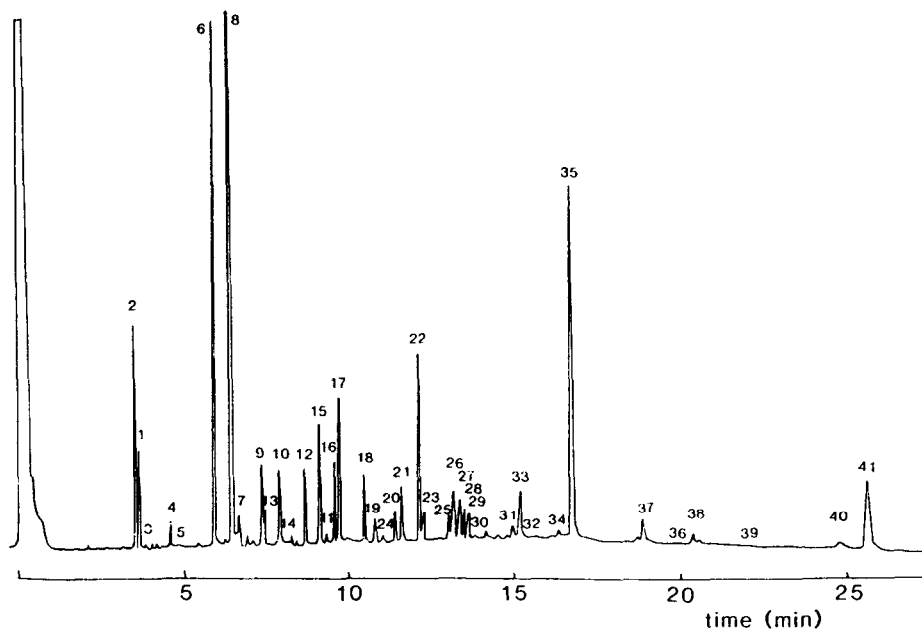

Fig. 2. Separation of FAMEs from the mussel *Mytilus galloprovincialis* on a GC capillary column coated with Silar 5CP. Peaks: 1 = 4,8,12-trimethyl-13:0; 2 = 14:0; 3 = 14:1ω5; 4 = iso-15:0; 5 = 15:1ω7; 6 = 16:1ω7; 7 = iso-16:0; 8 = 16:1ω5; 9 = 16:2ω4; 10 = 16:3ω4; 11 = 16:3; 12 = 16:4ω1; 13 = 17:0; 14 = 17:1ω8; 15 = 18:0; 16 = 18:1ω9; 17 = 18:1ω7; 18 = 18:2ω6; 19 = 18:2ω4; 20 = 18:3ω6; 21 = 18:3ω3; 22 = 18:4ω3; 23 = 18:4ω1; 24 = 19:1ω8; 25 = 20:1ω13; 26 = 20:1ω11; 27 = 20:1ω9; 28 = 5,11 20:2; 29 = 5,13 20:2; 30 = 20:2ω6; 31 = 20:3ω6; 32 = 20:3ω3; 33 = 20:4ω6; 34 = 20:4ω3; 35 = 20:5ω3; 36 = 21:5ω3; 37 = 7,15 22:2; 38 = 22:3ω6; 39 = 22:4ω6; 40 = 22:5ω3; 41 = 22:6ω3. After Christie *et al.* [44].

4. APPLICATIONS IN FOOD ANALYSIS

4.1. Microbiology

The GC of fatty acids is a useful technique in microbial chemotaxonomy [45-47]. In some instances significant and consistent differences in fatty acid compositions were used to identify genus, species and even strains of valuable microorganisms in the food industry [48]. Most bacteria contain a relatively simple fatty acid composition. Bacterial fatty acids normally are no longer than 18 carbons and have no more than one double bond [49]. Therefore, the use of packed columns for the analysis of fatty acids of microbial origin is still common. For example, a short GC column packed with SP-1200 and 1% H_3PO_3 was used in a rapid and simple procedure to determine concentrations of short-chain volatile fatty acids [50]. The use of a short column in this study significantly decreased the time of analysis of fermented products, and maintained an acceptable degree of resolution for underivatized short-chain (C_2-C_6) fatty acids. The screening or identification of different stains of fungi and bacteria requires a more refined separation and identification of fatty acids. Particular strains mainly differ in the proportion of their minor (< 1%) fatty acids [48]. In this comparative study thirteen strains of *Saccharomyces cerevisae* were successfully classified by comparing their fatty acid profiles in a series of capillary columns of different polarities such as PEG, dimethylpolysiloxane (DB-1) and dimethylcyanopropylphenylpolysiloxane (DB-1701).

4.2. Dairy products

Dairy products and especially butterfat are a challenge for the chromatographer as they present a very complex mixture of acyl lipids. The fatty acid chain length spans from C_2 to C_{26}, including straight and branched structures, with zero to six ethylenic bonds, and *cis-trans* geometric isomers. An additional complication is the presence of short-chain free carboxylic acid which may be lost during sample preparation. Free volatile fatty acids (FVFAs) in dairy products are present at low concentrations, but the level of these acids increases during ripening and they are important organoleptic components.

FVFAs were determined in the past on packed columns, using a variety of liquid phases (see ref. 51 for older references). More recently, FVFAs were determined by GC in underivatized form using a relatively short (*i.e.*, 15 m) capillary column coated with an acidified PEG (Nukol) stationary phase using crotonic acid as an internal standard [51].

Even though packed columns give reduced information [52], it is still suitable for the analysis of dairy products such as when analyzing a fraction of the total fatty acids, or to give an estimate of the total content of acyl lipids. However, for a more detailed study, the use of capillary columns is essential. De Jong and Badings [53] used a 25-m fused-silica capillary column coated with the polar phase FFAP-CB and a 15-m column coated with FFAP for the complete separation of FFA in cheese and milk (Fig. 3).

A problem during the analysis of underivatized FFAs is their adsorption on the first part of the capillary column. This problem is particularly serious with long-chain FFAs (16:0, 18:0 and 18:1), leading to selectively low responses. The adsorption of these fatty acids can be prevented by the use of capillary columns with thicker (1.0 μm) stationary phases [53].

4.3. Vegetable oils

Seed oils may contain a large number of minor monounsaturated fatty acids and positional isomers. Although many of these fatty acids can be separated by conventional capillary GC, their structural elucidation requires a more complicated treatment or derivatization. For instance, GC–mass spectrometry with capillary columns of medium polarity (*e.g.*, PEG) was used to separate and locate the position of the double bonds of picolinyl ester derivatives of fatty acids in borage (*Borago officinalis*) seed oil [54].

Seed oils are also a source of many uncommon fatty acids such as cyclopropenoid, epoxy or conjugated acids. Cyclopropenoid fatty acids such as malvalic (2-octyl-1-cyclopropene-1-heptaenoic acid) and sterculic (2-octyl-1-cyclopropene-1-octanoic acid) are common constituents of the plants of the order Malvales [55,56]. Interest due to possible health hazards [57] prompted the need for identifying and determining cyclic fatty acids in edible oils. PEGs or stationary phases of higher polarity, such as dimethyldiphenylpolysiloxane, were successfully used for the separation of dimethyloxazoline derivatives of cyclopropenoid fatty acids [55].
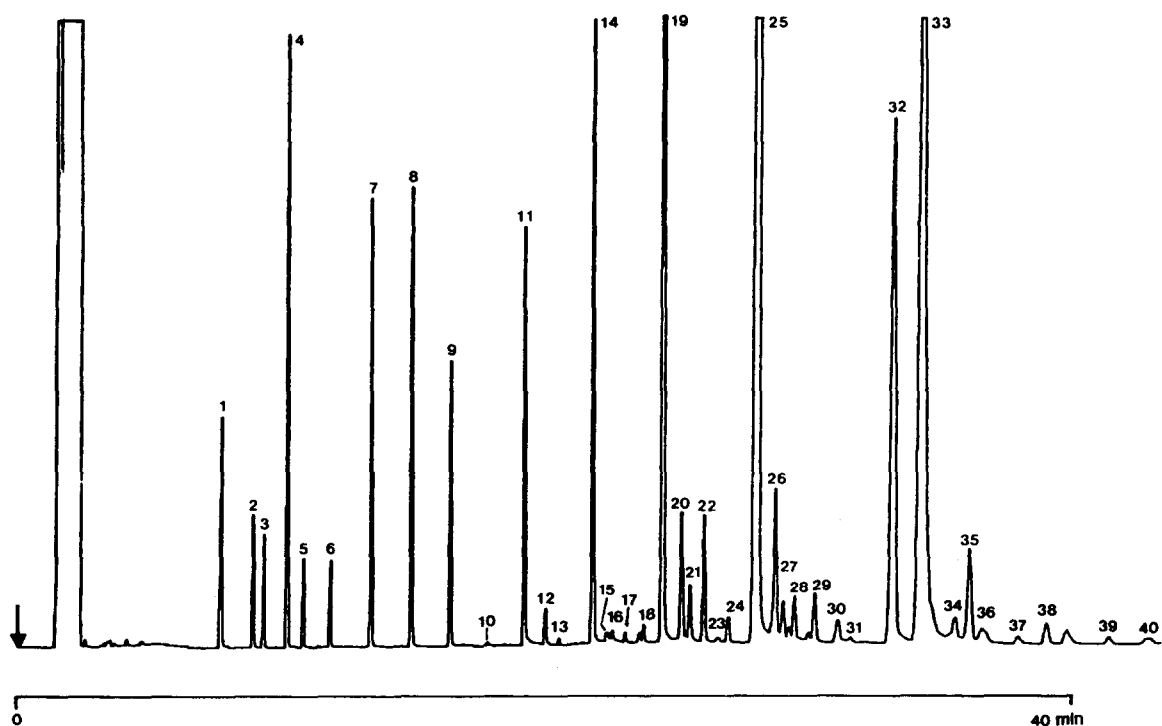
Fig. 3. Gas chromatogram of FFAs from cheese spiked with an FFA reference mixture (2:0, 3:0, 2-CH_3-3:0, 5:0, 3-CH_3-4:0 and 7:0). Peaks: 1 = C_2; 2 = C_3; 3 = 2-CH_3-C_3; 4 = C_4; 5 = 3-CH_3-C_4; 6 = C_5; 7 = C_6; 8 = C_7; 9 = C_8; 10 = C_9; 11 = C_{10}; 12 = C10:1; 13 = C_{11}; 14 = C12:0; 15 = C12:1; 16 = C_{13}-iso; 17 = C13:0; 18 = C_{14}-iso; 19 = C14:0; 20 = C14:1 + C15-iso; 21 = C_{15}-anteiso; 22 = C15:0; 23 = C15:1; 24 = C_{16}-iso; 25 = 16:0; 26 = C16:1; 27 = C_{17}-iso; 28 = C_{17}-anteiso; 29 = C17:0; 30 = C17:1; 31 = C_{18}-iso; 32 = C18:0; 33 = C18:1; 34 = C18:2; 35 = C18:2; 36 = C19:0; 37 = 18:3; 38 = C18:2 conjugated; 39 = C20:0; 40 = C20:1. After De Jong and Badings [53].

Epoxy fatty acids such as cis-12,13-epoxy-cis-9-octadecenoic acid, are present in high concentrations in a number of seed oils (e.g., *Amaranthus cruentus*, a West African vegetable crop). These acids are susceptible to suffer a loss of functional groups and show poor recoveries when subjected to standard methylation procedures. A mild and rapid preparation of FAMEs with a methanolic solution of sodium methoxide was developed for the GC of epoxy fatty acids, and the FAMEs were separated with a 30-m SPB-5 column of low polarity [58].

Special applications of capillary GC to vegetable oils include the determination of individual oils in a blend. This objective can be achieved by a statistical analysis of GC data on fatty acids and other components. This approach was tested with a blend containing two, three or six oils (*i.e.*, corn, soybean, sunflower, palm, cottonseed, palm kernel, sesame,

coconut and olive oils) [59]. The column used to analyze the FAMEs was a 2-m glass column packed with 10% diethyleneglycol succinate (DEGS) on Chromosorb W.

Trans fatty acids are minor components in natural foods, but relatively large concentrations of C_{18} *trans* fatty acids are formed by the industrial hydrogenation of vegetable oils in the processing of margarine [60]; 8-*trans*,12-*trans*-18:2 is a common product of the partial hydrogenation of vegetable oils. The successful analysis of this type of sample could represent a chromatographic problem, especially when the aim of the analysis is unequivocally to identify and determine individual components. Polar capillary columns coated with cyanopropylsiloxane are recommended for this purpose [61,62]. Complete fatty acid separations, however, can only be achieved by complicated procedures involving

more than one analytical technique [63]. Such a complex analysis is illustrated by the work of Ratnayake *et al.* (Fig. 4) [62]. This work shows the incomplete separation of several *trans*-octadecenoic acids even with the use of a very long (100-m) capillary column coated with a polar SP-2560 stationary phase.

Thermal oxidation of oils also produces a number of artifacts. Special attention has been paid to the production of fatty acid dimers and high polymers with different degrees of polarity. The separation of these components requires the combined use of gas, high-performance liquid and thin-layer chromatography. A complex mixture of synthetic dimers was analyzed by both packed and capillary column GC [64]. The separation of non-polar fatty acid dimers and isomers presented a difficult task.

The mixture could at the best be partially resolved using 50-m long, low-polarity columns (*e.g.*, SPB-1, methylsilicone) [64].

The sensitivity of modern capillary GC–FID permits the routine determination of the fatty acid composition of a large number of small seed tissues. This information facilitates the genetic selection and further genetic manipulation of the lipid composition of seed oils. To simplify the analysis and to reduce the costs of column replacement, routine determinations are usually done on packed columns [65]. Simplified one-step extraction–derivatization and transesterification coupled with the rapid determination of FAMEs permitted inexpensive screening procedures for up to 2000 samples per month [65].

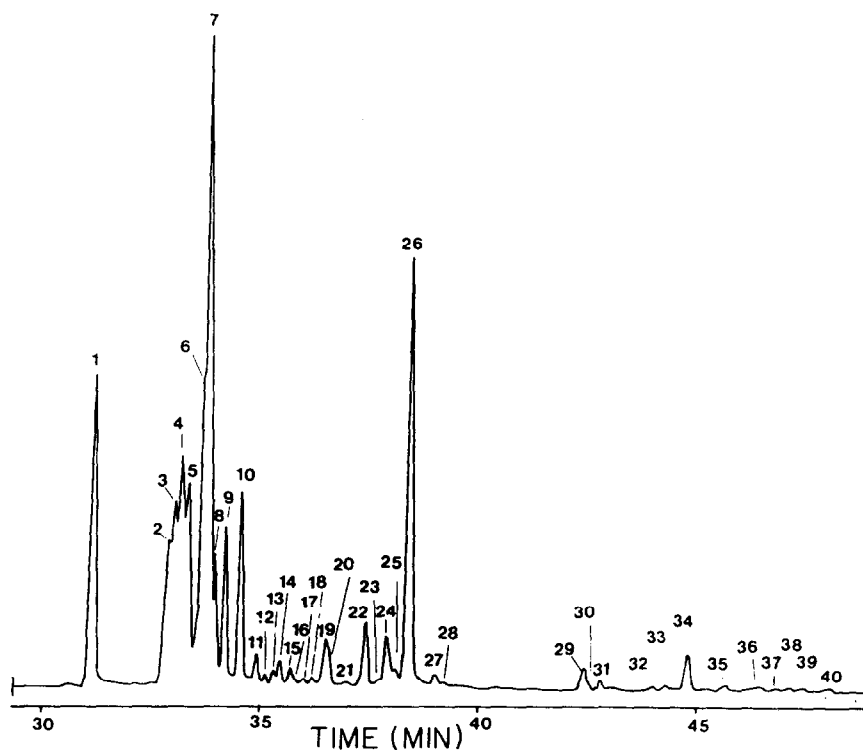The high resolving power and reproducibility of

Fig. 4. Partial gas chromatogram of the C_{18} region of soybean oil margarine FAMEs on a 100-m SP-2560 capillary column. Peaks: 1 = 18:0; 2 = *trans*-6–8-18:1; 3 = *trans*-9-18:1; 4 = *trans*-10-18:1; 5 = *trans*-11-18:1; 6 = *trans*-12-18:1; 7 = 9-*cis*-18:1 + 13-*trans*-18:1 + *trans*-14-18:1; 8 = 10-*cis*-18:1; 9 = 11-*cis*-18:1; 10 = 12-*cis*-18:1 + 15-*trans*-18:1; 11 = 13-*cis*-18:1; 12 = *trans*–*trans*-18:2; 13 = 14-*cis*-18:1; 14 = *trans*–*trans*-18:2; 15 = 15-*cis*-18:1; 16 = *trans*–*trans*-18:2; 17 and 18 = *trans*/*cis*–*cis*/*trans*-18:2; 19 = *trans*-9-*trans*-12-18:2; 20 and 21 = *trans*/*cis*–*cis*/*trans*-non-methylene-interrupted (NMI)-18:2; 22 = *cis*-9-*trans*-12-18:2; 23 = ?; 24 = *trans*-9-*cis*-12-18:2; 25 = ?; 26 = *cis*-9-*cis*-12-18:2; 27 = 9-*cis*-15-*cis*-18:2; 28 = ?; 29 = 20:0; 30 = unidentified 18:3 isomer; 31 = *cis*-9-*cis*-12-*trans*-15-18:3; 32 = *trans*-9-*cis*-12-*cis*-15-18:3; 33 = *cis*-9-*trans*-12-*cis*-15-18:3; 34 = *cis*-9-*cis*-12-*cis*-15-18:3; 35 = 20:1; 36–40 = 18:2 conjugated ?. After Ratnayake *et al.* [62].

capillary columns helps in the design of methods for efficiently monitoring the quality of fats and oils. This approach is currently used to predict the adulteration of animal fats in butter–margarine blends with a high degree of certainty [66]. This type of analysis demands a positive identification of a wide variety of fatty acids ranging from short to long chain and *cis–trans* geometric isomers and also requires accurate determination. Modifications of this basic approach were recently developed in order to detect the presence of pork in processed meats [67] and to determine the content of clinically important fatty acids in fish oils [68].

4.4. Fish oils

Fish oils and in general marine lipids have a diverse fatty acid composition (typically an even-carbon chain length of C_{14}–C_{22} and with 0–6 methylene-interrupted double bonds). Marine lipids also contain minor amounts of less common fatty acids with non-methylene-interrupted double bonds, branched hydrocarbon chains or chain lengths longer than 22 carbons. Clinical and epidemiological studies in the last decade indicated that the consumption of fish or fish oils can improve or prevent certain human cardiovascular conditions [69]. This potential therapeutic property of marine lipids renewed interest in investigating the lipid contents and fatty acid compositions of fish and shellfish species around the world [70]. Studies were especially concentrated on the accurate determination of the content and availability of marine fatty acids of medical interest (eicosapentaenoic acid, EPA, 20:5ω3; and docosahexaenoic acid, DHA, 22:6ω3). The fatty acid compositions of many seafood products and edible algae and encapsulated fish oil products have been reviewed [71–73].

Typically, fatty acids of marine origin are analyzed with capillary columns of medium polarity, such as Carbowax 20M. Earlier work with packed columns presented serious problems of overlapping between different even-carbon fatty acids [39]. Of special interest is the coincidence of 22:1 with 20:5ω3 on medium-polarity packed columns, and 22:1 with 20:4ω6 on higher polarity packed columns [39]. There are a number of reports on the capillary GC of marine lipids using non-polar stationary phases [74]. Low-polarity capillary columns, however, present some partial overlapping

problems, especially with the pairs 18:2ω6–18:3ω3 and 20:4ω6–20:5ω3. PEG columns, on the other hand, have shown reasonably good thermal and chemical stability [41]. They offer excellent resolution of all major fatty acids of marine lipids and of a number of minor but ubiquitous components of fish oils. For example, capillary columns coated with Carbowax 20M separated furan fatty acids of salmon roe [75], EPA geometric isomer artifacts in heated fish oils [76] and branched-chain fatty acids in fish oil concentrates [77].

5. QUANTIFICATION

Quantification errors can be introduced in many different ways and each step in the analysis of fatty acids has the potential for sample loss. Sources of problems for the determination of fatty acids by GC–FID arise during sample preparation (extraction and derivatization), on the injector, on the column, in the detector and during data collection.

Quantification errors originating as a consequence of column reactivity can be reduced by selecting highly pure stationary phases and inert supports. In all instances it is recommended to perform routine checks on selective adsorption or losses of FAMEs in the column. Calibration of the GC system should be performed by using FAME mixtures similar in composition to the sample being analyzed. Partial overlapping and co-elution of different components may generate other quantification problems [78].

Quantification of PUFA presents the additional problems of losses due to oxidation during sample handling. Losses of PUFA can be easily determined by comparing the fatty acid composition of the sample before and after total hydrogenation [39].

With capillary columns problems arise as a consequence of defective injection procedures. Christie [79] developed a series of simple and effective injection techniques, such as hot needle and cold trapping injection. He indicated that an injection temperature of about 375°C improved the reproducibility of the analysis without causing any adverse effect on the recovery of PUFA. Regardless of the preferred injection technique, the use of automatic injection is highly desirable for minimizing the introduction of random errors.

When the determination of FAMEs with FID re-

quires high accuracy, correction factors should be applied to compensate for the fact that the carboxyl carbon is not ionized during combustion [80]. In addition, the degree of unsaturation of a FAME mixture also requires the use of small correction factors. The magnitudes of these factors are greater for very short-chain or highly unsaturated fatty acids [79].

There are a few reports on quantitative aspects of the capillary GC of fatty acids. Not unexpectedly, the results of inter-laboratory studies have shown very poor reproducibility for the analysis of fatty acid mixtures containing a wide variety of molecules [80]. Inter-laboratory studies rely on the availability of reference samples and standards. Standardized FAME mixtures are available from the National Institute of Health and several commercial suppliers, but unfortunately they do not cover all areas of application.

A linear FID response is generally assumed for long-chain fatty acids. Therefore, absolute concentrations are determined by adding odd-chain saturated fatty acids as internal standards (e.g., 17:0, 19:0 and 23:0). Recently, 24:1ω9 (nervonic acid) has been used as internal standard for the quantitative analysis of fish oils [81]. The addition of a single internal standard assumes no discrimination of the different fatty acids during the processes of isolation, derivatization and actual chromatography.

A more refined approach was recently developed, consisting in the use of a series of odd-chain fatty acid standards. The determination of a naturally occurring even-carbon fatty acid was compared with the two adjacent odd-carbon fatty acids ("bracketing" method) [82]. It has been demonstrated that this bracketing method is superior to the use of a single fatty acid as an internal standard [82].

6. CONCLUSIONS

GC is the method of choice for the determination of fatty acids. Food products contain a wide variety of lipids and their fatty acids differ in chain length and degree of unsaturation and in the presence of special functional groups and positional and geometric isomers.

Procedures for the derivatization of fatty acids need to be optimized to achieve good quantitative results. In general, most transesterification methods, whether acid- or base-catalyzed, or direct methods would meet the purpose depending on the nature of the sample to be esterified. New reagents and procedures, especially where no removal of the reagent is recommended, should be assessed for their long-term effect on the GC column.

Contemporary capillary GC offers excellent separations of most naturally occurring fatty acids. These capillary columns, especially those of medium polarity (e.g., Carbowax 20M), and the non-polar methylsilicone, successfully separate FAMEs ranging from C_2 to C_{24} and with 0–6 ethylenic bonds. Special applications, such as the separation of complex cis–trans fatty acid mixtures (characteristic of partially hydrogenated oils), cyclopropene or epoxy fatty acids (common in some seed lipids), require particular chromatographic conditions. These include the use of very long capillary columns (e.g., 100 m) and/or more polar stationary phases composed of cyanoethyl- or cyanopropylsiloxanes.

7. ACKNOWLEDGEMENTS

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