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Journal of Chromatography B, 671 (1995) 113–131

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Review

Gas chromatographic analysis of fatty acid methyl esters

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Abstract

The full process of fatty acid methyl ester (FAME) analysis consists of esterification of lipids, and of injection, separation, identification and quantitation of the FAMEs. In order for the required accuracy and precision to be attained, each of these steps has to be optimized.

Esterification of lipids can be carried out with several reagents based on acid-catalysed or base-catalysed reactions. The advantages and disadvantages of these reagents are discussed. The most critical step in the gas chromatographic analysis of FAMEs is sample introduction. The classical split injection technique, which is the most widely used technique in the analysis of FAMEs, has the potential disadvantage of boiling-point-dependent sample discrimination. Cold injection of the sample, either on-column or by programmed-temperature vaporization, does not present this problem and should therefore be preferred.

Modern, commercially available fused-silica capillary columns offer excellent separation of FAMEs from biological samples. Very polar stationary phases give excellent separation of all FAMEs but have relatively low thermal stability, resulting in long retention times. Non-polar phases have a much greater thermal stability but inferior selectivity. For many analyses, phases of intermediate polarity, which combine the advantages of a relatively high resolution capability with relatively high thermal stability, are the most suitable.

FAMEs can be identified by comparison of their retention times with those of individual purified standards or secondary standards based on lipids that have been well characterized in literature. Relative retention times and equivalent chain-length values also provide useful information. FAMEs can be quantitated by peak areas via calibration factors, and absolute concentrations can be determined by adding an internal standard.

Among numerous applications in biomedical research, the analysis of fatty acids from body tissues may contribute to the understanding of the link between the dietary intake of fatty acids and the diseases with which these acids are associated.

Contents

0378-4347/95/$29.00
SSDI 0378-4347(95)00142-5

List of abbreviations

BHT	Butylated hydroxytoluene
DMA	Dimethyl acetal
ECL	Equivalent chain length
FAME	Fatty acid methyl ester
FID	Flame ionization detector
GC	Gas chromatography
LDL	Low-density lipoprotein
NEFA	Non-esterified fatty acid
PTV	Programmed-temperature vaporizer
PUFA	Polyunsaturated fatty acid
RRF	Relative response factor
RRT	Relative retention time
WCOT	Wall-coated open tubular

1. Introduction

Fatty acids play important roles in biological tissues. For example, fatty acids as constituents of lipids in biological membranes influence membrane properties such as fluidity, integrity, permeability, and the activities of membrane-bound enzymes [1]. Fatty acids as components of foods serve as a source of energy for man and animals. The type of fatty acids consumed has important implications for human health, especially with respect to concentrations of serum lipids and the risk of coronary heart disease [e.g., 2–5]. C_{20}-polyunsaturated fatty acids such as arachidonic acid, eicosapentaenoic acid or dihomo-γ-linolenic acid located in biological membranes serve as precursors for eicosanoids [6,7].

Fatty acids in foods and biological samples are commonly analysed by gas chromatography of their fatty acid methyl esters (FAMEs), although there are some reports dealing with gas chromatographic separation of underivatized fatty acids [8]. The total gas chromatographic analysis of FAMEs consists of several steps, including fatty acid esterification and the injection, separation, identification and quantitation of FAMEs. In order for high accuracy and precision of analysis to be attained, each of these steps has to be optimized. The present paper describes methods for the preparation of FAMEs, injection techniques and their advantages and disadvantages, columns and conditions for FAME separation, and methods for FAME identification and quantitation.

2. Preparation of fatty acid methyl esters

In order for lipid-bound fatty acids to be analysed by gas chromatography (GC), the fatty acids have to be split off and converted into derivatives with lower boiling points, such as alcoholic esters. Formerly, the fatty acids were split off by saponification with sodium hydroxide or potassium hydroxide, then methylated with either boron trifluoride–methanol reagent [9,10] or with methanolic acids [11]. However, saponification proceeds very slowly, and therefore the saponification–esterification procedure requires very long reaction times [12]. Direct transesterification of lipids ("alcoholysis") proceeds far more rapidly than saponification, with hydrolysis and esterification taking place in one step, and

only one reagent is necessary. It is therefore not only faster but also simpler than saponification–esterification.

However, in order for accurate quantitative results to be obtained by transesterification, the procedure has to be optimized. Problems associated with ester preparation include [13]: (1) incomplete conversion of lipids into FAMEs; (2) changes in the original fatty acid composition during transesterification; (3) formation of artefacts which can be wrongly identified as fatty acids or overlap with FAME peaks in the GC analysis; (4) contamination of, and subsequent damage to, the GC column resulting from traces of the esterification reagent; (5) incomplete extraction of FAMEs after transesterification; and (6) losses of highly volatile short-chain FAMEs. Even methods generally accepted officially do not necessarily produce accurate results, especially when the sample contains low-molecular-mass FAMEs [14,15]. Moreover, various lipid transesterification methods described in literature give far from complete recovery of FAMEs [16]. Therefore, the accuracy of any method should be checked using primary lipid standards prior to analysis of biological samples. If recovery of FAMEs from lipid standards is not complete, analytical conditions have to be optimized for the sample to be transesterified.

Yields of FAMEs from lipids are influenced by a number of experimental factors, including the molar ratio between methanol and the lipid, the reaction temperature and time [17–19]. All the reagents used for methylation are composed mainly of methanol. Non-polar lipids such as triglycerides and cholesterol esters, in particular in large quantities, are not soluble in the transesterification reagents, and will not react in a reasonable time. In order to dissolve these lipids, solvents such as benzene, toluene, chloroform or tetrahydrofuran [16,18,20,21] have to be added. However, chloroform can only be used in this way if it does not contain ethanol as a stabilizer because ethanol competes with methanol for esterification. In addition, all the solvents mentioned require careful handling because of their toxicity.

A general point of attention is the risk of decomposition of polyunsaturated fatty acids (PUFAs) during the analytical procedure. The risk of autoxidation can be reduced by handling lipids in a nitrogen atmosphere. If a transesterification method is used that requires heating for completion of the reaction, it is advisable to protect PUFAs from autoxidation by adding antioxidants such as butylated hydroxytoluene (BHT).

In most of the procedures, FAMEs have to be extracted after transesterification. Most biological samples as well as foods contain predominantly long-chain fatty acids. The corresponding FAMEs are very non-polar, and thus can be extracted with alkanes such as *n*-pentane [18], *n*-hexane [16] or iso-octane [12]. Extraction of FAMEs should be carried out twice for complete recovery [16,18], and the ratio between the volume of the extraction solvent and that of the transesterification reagent must be sufficient. Water or salt solutions are added to increase recovery of FAMEs [15,16,18]. In the procedure described by Morrison and Smith [18], FAMEs were extracted twice by two volumes of pentane and one volume of water. This extraction results in 97–99% recovery of FAMEs [18]. Because methyl esters of short-chain fatty acids such as those present in dairy products are highly volatile and are partially soluble in water, quantitative recovery is very difficult [22]. Therefore, for samples which contain short-chain fatty acids, the preparation of butyl esters, which have higher boiling points and are less water-soluble than methyl esters [23,24], is recommended.

Several types of reactions are suitable for the direct transesterification of lipids. Acid-catalysed and base-catalysed transesterification are described in the following.

2.1. Acid-catalysed transesterification

Common reagents used for acid-catalysed transesterification are methanolic, hydrochloric and sulfuric acid, and boron trifluoride in methanol. All are suitable for lipid transesterification and also free-fatty-acid methylation. However, neither acid-catalysed nor boron-fluoride-catalysed reactions proceed at ambient temperature; both types of reaction require heating. Among

the reagents mentioned, boron trifluoride–methanol reagent (12–14% w/v) is the most often used for transesterification of all types of lipids [14] and it has been shown to be a very useful reagent for lipid esterification [13,14,22].

Morrison and Smith [18] determined the optimum conditions for esterification of triglycerides, sterol esters, monoglycerides, diglycerides, phosphoglycerides, sphingolipids, and free fatty acids. Under the conditions recommended (heating at 100°C), transesterification is complete within 2 min for free fatty acids, within 10 min for phosphoglycerides, within 30 min for triglycerides and within 90 min for sphingomyelin. In the author's laboratory, boron fluoride–methanol reagent is used for transesterification of lipids occurring in biological samples under the following conditions: a small aliquot of the lipid extract (dissolved in chloroform) is fed into a screw-capped tube (Teflon cap liner); 0.5 to 1 ml boron fluoride–methanol reagent (140 g/l, containing an adequate amount of BHT as antioxidant) is added; the tube is then closed and heated at 90°C for 2 h. This method has proved to give complete transesterification of all lipids; the addition of BHT totally prevents decomposition of PUFA. After the transesterification has been completed, FAMEs are extracted twice by adding *n*-hexane and water. This procedure has been found to be very simple and effective.

In spite of its wide popularity, boron trifluoride–methanol has a few disadvantages. Unless refrigerated, the reagent has only a limited shelf life. The use of old or too concentrated solutions may result in the production of artefacts or loss of PUFAs [22]. If the sample contains plasmalogens, aldehydes are liberated by the reagent and are converted into dimethyl acetals (DMAs), which are virtually impossible to separate from some major fatty acid methyl esters such as methyl palmitate [16,25,26].

Anhydrous methanolic hydrochloric acid and methanolic sulfuric acid have been used for lipid esterification under very different conditions, in particular, different acid concentrations, different reaction temperatures and different reaction times [27–30]. Methanolic acids like boron fluoride–methanol methylate free fatty acids very rapidly and can be used to transesterify all the lipids which are typically constituents of biological samples. Complete transesterification with 5% methanolic hydrochloric acid can be carried out by heating the sample in the reagent under refluxing for about 2 h. The reaction can also be carried out at 50°C overnight [22]. A solution of 1–2% (v/v) concentrated sulfuric acid in methanol can be used in the same way for the transesterification of lipid samples. Like boron trifluoride–methanol, methanolic, hydrochloric and sulfuric acids also have the disadvantage that DMAs are formed from plasmalogens during transesterification.

Methanolic, hydrochloric and sulfuric acids have been shown to transesterify sphingolipids faster than boron trifluoride–methanol reagent [16,19]. Peuchant et al. [31] described a very useful method for the combined extraction and esterification of lipids using isopropanol and sulfuric acid. In the first step, lipids are extracted from the tissue with isopropanol, which is sufficiently apolar for the purpose. In the second step, sulfuric acid is added to an aliquot of the extract, and on heating isopropyl esters are produced which can be analysed by gas chromatography under similar conditions to methyl esters.

Other reagents used for transesterification include acetyl chloride [20,32,33] and aluminium chloride [34]. Both reagents have been added to samples without prior extraction of the lipid and have been shown to give complete transesterification. However, aluminium chloride has the disadvantage that it does not esterify free fatty acids.

2.2. Base-catalysed transesterification

Popular reagents used for base-catalysed transesterification of lipids are sodium methoxide and potassium hydroxide in methanol. Several papers describing procedures involving the use of these reagents under different reaction conditions have been published [15,16,23,35–37].

In contrast to all the acid-catalysed reactions, transesterification with sodium methoxide

proceeds at ambient temperature. Therefore, the risk of decomposition of PUFAs is lower, and transesterification at ambient temperature does not require BHT. This is undoubtedly an advantage because in some instances, in the gas chromatographic analysis of FAMEs, it has proved impossible to separate methyl myristate or palmitoleate from BHT and its derivatives [38]. Another advantage of sodium methoxide transesterification is that it proceeds very rapidly. Under reasonable conditions, transesterification of triglycerides and phosphoglycerides is typically completed within a few minutes [23,35,36,39–41]. Moreover, sodium methoxide does not liberate aldehydes from plasmalogens; thus no DMAs are formed during esterification [16,26]. This is especially of significance in the fatty acid analysis of samples containing relatively large amounts of plasmalogens, such as erythrocyte membranes.

On the other hand, sodium methoxide has also some disadvantages. It does not esterify free fatty acids and it does not transesterify sphingolipids. Therefore, samples containing considerable amounts of free fatty acids or sphingolipids have to be treated with other reagents [16]. Badings and de Jong [36] proposed a combined procedure for the conversion of free and glyceride-bound fatty acids. In the first step sodium methoxide is used for the transesterification of lipid-bound fatty acids, and in the second step methanolic hydrochloric acid for the esterification of free fatty acids. It should further be noted that, with sodium methoxide as a reagent, the conditions have to be anhydrous because water causes saponification as a side reaction.

Another reagent used for the transesterification of lipids is potassium hydroxide in methanol [42]. However, this has the disadvantage of a potential risk of saponification during transesterification [14].

In the past decade, some strongly basic quaternary salts of ammonia such as trimethyl(*m*-trifluorotolyl)ammonium hydroxide (TMTFTAH) [43], tetramethylammonium hydroxide (TMAH) [44,45], trimethylphenylammonium hydroxide (TMPAH) [46], and trimethylsulfonium hydroxide (TMSH) [21] have been introduced for transesterification of glycerides. After addition of these reagents to the sample, lipid-bound fatty acids are converted into quaternary ammonium salts which, in turn, are pyrolysed to methyl esters in the hot injection port of the gas chromatograph. Major advantages of this one-step reaction are ambient-temperature transesterification and elimination of the extraction step [21]. These points are of special interest when fats with short-chain fatty acids, such as dairy products, are to be analysed. Losses of short-chain FAMEs which occur during heating, solvent evaporation and extraction in other procedures can be avoided by this route [44].

2.3. Specific methylation of non-esterified fatty acids

Under reasonable reaction conditions, most of the reagents described above methylate non-esterified fatty acids (NEFAs) and at the same time transesterify lipid-esterified fatty acids occurring in the sample. However, in many cases the fatty acid composition of the non-esterified fatty acid fraction should be analysed. For this purpose, the NEFA fraction can be separated from lipid esters by thin-layer chromatography or by liquid chromatography prior to methylation [47,48].

However, isolation of NEFAs prior to methylation is very time-consuming. Therefore, some procedures have been developed which allow specific methylation of NEFAs without simultaneous transesterification of esterified lipids. In the procedure described by Pace-Asciak [49], NEFAs from plasma were methylated using diazomethane under mild conditions. Under these reaction conditions, methylation of NEFAs proceeds with virtually no FAMEs being released from esterified lipids. However, diazomethane has the disadvantage of forming artefacts by reacting with double bonds or carbonyl groups and of being highly toxic and potentially explosive [13]. Lepage and Roy [50] reported a procedure in which NEFAs were completely methylated under very mild conditions using a methanol–acetyl chloride reagent without any transesterification of lipid esters. In this procedure, though, great care must be given to the reaction conditions in order to avoid

transesterification of esterified lipids. If the concentration of acetyl chloride or the incubation time is increased, considerable transesterification of esterified lipids occurs, especially of phospholipids.

In the procedure described by Chapman [51], transesterification of esterified lipids by boron trifluoride or boron trichloride is suppressed by methylurea, thus allowing selective methylation of NEFAs. Ciucanu and Kerek [52] have reported a procedure in which selective methylation of NEFAs was achieved by the use of strong anion-exchange resins as heterogeneous basic catalysts. Allen et al. [53] proposed a method for analysing NEFAs after isolation from hexane extracts in aqueous potassium hydroxide and subsequent methylation with methyl iodide.

3. Injection of fatty acid methyl esters

There is general agreement that, in high-resolution gas chromatography, sample injection is the most critical step with respect to achieving high accuracy and precision [54–57].

3.1. Split injection

The technique most often used for the injection of FAMEs is the classical split injection mode, in which the sample is introduced into a hot injector chamber [58–68]. Splitting of the carrier gas has the advantage that samples with relatively large FAME concentrations can be injected without the risk of overloading the stationary phase, causing degradation of column performance. Typical split vent ratios used in FAME analysis range between 1:10 and 1:200.

The major drawback of the classical split injection technique in FAME analysis, which can have serious consequences, is discrimination between high-boiling and low-boiling compounds in the sample [57,69]. Naturally occurring fatty acids have chain lengths between 4 and 24 carbon atoms, and therefore a wide range of boiling points. As a result, the accuracy and precision achieved is often not acceptable [58,63,70].

The problem of sample discrimination associated with split injection may be due to a number of factors, including changes in the splitting ratio caused by pressure waves, selective evaporation in the syringe needle, and incomplete evaporation. A detailed survey of the problem of sample discrimination, as well as recommendations to minimize its effects is given by Grob [57].

Some researchers have been successful in overcoming the sample discrimination problem in the analysis of FAMEs with classical split injection. Bannon et al. [62] investigated the effect of several injection parameters such as split vent flow-rate, concentration of primary standard, injector temperature, speed of injection and injector inserts on analysis quality. They pointed out that excellent accuracy and precision can be achieved provided three points are considered. First, sample discrimination in the syringe needle must be avoided. Second, the sample must be vaporized rapidly. Third, the vaporized sample must be thoroughly mixed with the carrier gas. These requirements can be fulfilled by high-speed injection, small sample volumes, low sample concentrations, high injector temperatures (375°C) and good injector insert designs. Bannon et al. showed that this high injector temperature does not cause pyrolysis of FAMEs, even when they are highly unsaturated.

In contrast to the recommendations given by these authors, typical injector temperatures described in literature for FAME injection are in the range 250–300°C [60,64–66,70–78] or even below 250°C [25,79,80].

Inaccuracy and low reproducibility caused by sample discrimination can usually be avoided by automatic injection. Injection in automatic gas chromatographs is performed at high speed; thus some potential causes of discrimination are avoided. Moreover, automatic injection eliminates much of the variation caused by the injection technique of the individual operator, and therefore, systematic effects caused by sample discrimination can be corrected via calibration [81].

Van der Steege et al. [58] introduced correction of injection-related discrimination by the bracketing method. In this method, equal masses

of several odd-carbon-numbered FAMEs over a wide range of chain lengths are added to the sample as internal standards for correction of sample discrimination. The authors have shown that this method allows accurate and precise quantitation of the naturally occurring even-carbon-numbered fatty acids. Precision of analysis of FAMEs can be also improved by correcting sample discrimination by using three internal standards covering a wide range of boiling points such as $C_{11:0}$, $C_{17:0}$, and $C_{21:0}$ [70].

3.2. Cold sample injection

The problems associated with classical split injection can be overcome by the use of cold sample injection: for example, on-column injection or programmed-temperature vaporizing (PTV) injection.

3.2.1. On-column injection

In the on-column technique, the sample is directly introduced into the cool section of the column. In contrast to the split and splitless techniques, prior vaporization in a hot injector chamber is not required. As no sample splitting is performed and as no evaporation of the sample occurs in the syringe needle, sample discrimination can be completely avoided. Therefore, samples with a wide range of component volatilities can be determined with high accuracy and precision [82,83].

There is wide acceptance that the on-column injection technique is the most reliable method of introducing a sample into a capillary column to optimize quantitation [84]. This is also true for FAMEs over a wide range of chain lengths [85]. Modern gas chromatographic systems offer temperature-programmed on-column injection. Thus, by heating the head of the column, accumulation of highly volatile components, which was a problem in earlier on-column injectors, can be avoided.

One major disadvantage of the on-column technique, however, is that some solvents could damage certain columns [86].

3.2.2. Programmed-temperature vaporizing (PTV) injection

In PTV injection, the sample is introduced into a cold vaporizing chamber which is packed with deactivated glass or quartz wool for homogeneous mixing of sample and carrier gas. After withdrawal of the syringe needle and after evaporation of the solvent, the vaporizing chamber is very quickly heated to 250–350°C for evaporation of the higher-boiling compounds. One main advantage of this technique is that, as in the on-column injection technique, sample evaporation inside the syringe needle does not occur.

PTV even has some advantages over the on-column technique. First, it can be used in three different operation modes: cold split injection, cold splitless injection and solvent elimination injection [70,87]. Second, for PTV injection no special syringes are needed. Third, the glass insert can be exchanged and cleaned for removal of non-volatiles [88].

A disadvantage of PTV injection is that the glass wool used as insert can serve as a potential surface for decomposition of PUFA methyl ester, especially at a high injector temperature.

Cold split injection offers the advantage that samples with high FAME concentrations can be injected without the risk of the capacity of capillary columns being exceeded.

PTV split injection has the advantage over the classical hot split injection that non-linear splitting caused by pressure waves does not arise because evaporation of the solvent happens before evaporation of the compounds to be analysed.

Some authors have shown that boiling point-dependent discrimination of *n*-alkanes can be totally avoided by PTV split injection [87–91].

PTV split injection also has been shown to give accurate and precise results in the analysis of FAMEs with more than 10 carbon atoms [70,90]. Analysis of FAMEs with less than 10 carbon atoms is not as accurate as that of long-chain FAMEs [70]. The reason for this might be that, even from the cold vaporization chamber, the vaporization and transfer of more volatile compounds begins before the injection temperature

Table 1
Chromatographic conditions used for separation of FAMEs from various tissues by gas chromatography (data as given by the authors)

Tissue	Column (brand name), length/I.D./film thickness	Carrier gas, flow	Temperature program	Injection	Time of analysis (min)	Reference
Very polar phases						
Milk lipid classes	CP-Sil 88, 50 m/0.25 mm/n.n.	Nitrogen, 1.5 bar	175 to 210°C, 5°C/min	Split, 1:60	30	[64]
Erythrocyte phospholipid classes	CP-Sil 88, 50 m/0.25 mm/0.20 μm	Hydrogen, 2.0 ml/min	50 to 160°C, 30°C/min 160 to 200°C, 15°C/min 200 to 225°C, 10°C/min	PTV, Split 1:2	13	[102]
Plasma, erythrocytes, cerebrospinal fluid	CP-Sil 88, 25 m/0.25 mm/0.25 μm	Helium, 0.65 ml/min	150 to 200°C, 1°C/min	Split, 1:15	40	[63]
Phospholipid classes from cod tissues	CP-Sil 88, 50 m/0.32 mm/n.n.	n.n.	n.n.	On-column	n.n.	[138]
Milk	CP-Sil 88, 50 m/0.32 mm/0.20 μm	Helium, 1.8 ml/min	30 to 230°C, 5°C/min	On-column	n.n.	[36]
Separation of isomers of linolenic acid	CP-Sil 88, 50 m/0.33 mm/0.24 μm	Helium, 0.8 kg/cm^2	Isothermic, 150°C	Split, ratio n.n	45	[74]
Erythrocyte membranes	SP-2560, 100 m/0.25 mm/0.20 μm	Helium, 0.67 ml/min	80 to 220°C, 8°C/min	Split, 1:30	52	[25]
Polar phases						
Fish oil, serum	FFAP, 25 m/0.25 mm/0.25 μm	Nitrogen, n.n.	80 to 160°C, 6°C/min 160 to 200°C, 0.5°C/min	Split, 1:25	110	[98]
Erythrocytes	FFAP, 25 m/0.32 mm/n.n.	Helium, 75.8 cm/s	60 to 170°C, 15°C/min	On-column	40	[85]
Rat-liver microsomes, plasma, fish oil	Durabond-Wax, 15 m/0.32 mm/0.15 μm	Hydrogen, 50 cm/s	160 to 185°C, 5°C/min 185 to 240°C, 8°C/min	Split, 1:186	10	[60]
Pig-muscle phospholipid classes	Durabond-Wax, 30 m/0.25 mm/0.15 μm	Hydrogen, 1.8 ml/min	100 to 240°C, 10°C/min	Split, 1:50	20	[99]
Human adipose tissue	Supelcowax-10, 60 m/0.25 mm/0.25 μm	Helium, 2.0 ml/min	200 to 260°C, 2°C/min	Split, 1:25	n.n.	[71]
Plasma lipids	DB-225, 30 m/0.25 mm/0.25 μm	Hydrogen, 1.3 ml/min	140 to 180°C, 20°C/min 180 to 240°C, 3°C/min	Split, 1:9	23	[73]
Non-polar phases						
Plasma lipid classes	Ultra 1, 50 m/0.2 mm/0.11 μm	Helium, 0.52 ml/min	160 to 240°C, 2°C/min 240 to 290°C, 10°C/min	Split, 1:20	35	[76]
Human milk samples	CP-Sil 5, 50 m/0.20 mm/n.n.	Helium, 0.52 ml/min	60 to 200°C, 4°C/min 200 to 285°C, 5°C/min	Split, 1:20	48	[58]
Myobacterial culture	SPB-1, 15 m/0.25 mm/1 μm	Helium, 1 ml/min	175 to 300°C, 8°C/min	Split, 1:50	16	[139]
Soil oligotropic bacteria	SPB-1, 60 m/0.32 mm/0.25 μm	Helium, n.n.	100 to 320°C, 4°C/min	Splitless	n.n.	[140]
Plasma	OV-101, 25 m/0.35 mm/0.2 μm	n.n.	110 to 150°C, 35°C/min 150 to 265°C, 5°C/min	On-column	20	[97]
Candida albicans	OV-1, 25 m/0.20 mm/n.n.	Helium, 1.5 ml/min	90 to 275°C, 4°C/min	Splitless	30	[95]

n.n = Not known.

program is initiated; for these compounds double peaks or peak broadening may occur [82].

Splitless injection in general is useful for the analysis of very dilute solutions. In the analysis of FAMEs, PTV splitless injection is of interest because, in biological research, samples are usually small, and therefore the FAME concentrations to be analysed are very low.

The solvent elimination technique has been developed for the analysis of very dilute samples. It allows the injection of large sample volumes (up to 250 μl) [92,93]. Prior to injection of the sample the split vent is opened and the injector is kept at low temperature. After the solvent is vented to waste, the split valve is closed, the injector heated, and the sample vapours enter the column [89]. This technique is critical for the analysis of fatty acids containing less than 16 carbon atoms because these fatty acids can be lost together with the solvent through the split exit [70,90].

If the glass liner of the injector is packed with materials providing adequate retention characteristics and toluene is used as the solvent injected, methyl myristate, too, can be accurately determined in the solvent elimination mode [94].

4. Separation of fatty acid methyl esters

Today, the high resolution capability of modern capillary columns means that separation of even complex mixtures of FAMEs can be carried out routinely in the laboratory. Several companies offer capillary columns which have been developed especially for the separation of FAMEs over a wide range of chain lengths and are highly suitable for this purpose. Packed columns have the advantage of having a very high capacity, but the disadvantage of a relatively low resolution capability. Packed columns are still acceptable for the analysis of simple FAME mixtures such as vegetable oils, which contain virtually no fatty acid with more than 18 carbon atoms. With the introduction of the flexible fused-silica columns, however, packed columns became virtually obsolete.

Capillary columns have several advantages over packed columns, the most important being the very high resolution capacity. Modern capillary columns with a length of 50 m and more have 10^5 or more theoretical plates. A disadvantage of capillary columns, on the other hand, is that they can be easily overloaded, which will reduce resolution and quantitation capabilities. They also demand more careful laboratory practices, a higher detector response and a more sensitive detector [13]. The resolution capability of a wall-coated open tubular (WCOT) capillary column depends on several factors such as the polarity of the stationary phase, column length, internal diameter and film thickness.

4.1. Stationary phases

Separation of FAMEs can be carried out with three different types of capillary columns, i.e. with non-polar [e.g., 59,76,95–97], polar [e.g., 60,61,71,73,75,98,99] and very polar [e.g., 25,63,64,66,68,70,72,74,80,100–102] stationary phases. The polarity of the stationary phase influences retention times of FAMEs, especially those of polyunsaturated types. The column should be chosen according to the sample to be analysed. Equivalent chain-length (ECL) values of FAMEs tabulated in literature for several columns are a useful aid in the choice of column for a particular separation problem [103–105]. In general, the resolution capability for FAMEs, especially for unsaturated types, is highest in columns with very polar phases. This type of column should be chosen if complex FAME mixtures such as those from biological membranes are to be analysed. However, very polar phases have a shorter lifetime than non-polar phases. In many cases, non-polar phases provide sufficient separation of FAMEs [13] and should be chosen in this case. Table 1 shows examples of chromatographic conditions used for separation of FAMEs from various tissues on different column types.

4.1.1. Very polar phases

The most important very polar stationary phases are composed of 100% cyanoethylsilicone oil (SP-2340, OV-275), 100% cyanopropylsilicone

(CP-Sil 88) or 68% biscyanopropyl–32% dimethylsiloxane (SP-2330) [13]. The very rapid separation of FAMEs from the liver of rats fed either a diet with a coconut oil–safflower oil mixture or a diet containing linseed oil on a CP-Sil 88 phase is shown in Fig. 1, and that of human erythrocyte membrane FAMEs on a SP-2560 phase is shown in Fig. 2.

The main advantage of polar phases compared to non-polar phases is their high resolution capability of unsaturated FAMEs. Under reasonable separation conditions on very polar phases all FAMEs, even from very complex samples, such as cod-liver oil or originating from pig testes, can be excellently separated [105]. If a critical pair of FAMEs such as 20:3 $n-3$ and 20:4 $n-6$ coelutes on a specific phase, resolution can be achieved in most cases by use of a more polar phase.

Given a constant chain length, every additional double bond increases the retention time. At a constant number of double bonds, FAMEs in which the last double bond is located farther away from the ω-end elute before those in which the last double bond is located closer to the ω-end. In the fatty acid group with 18 carbon atoms the elution order is: 18:0, 18:1 ($n-9$), 18:1 ($n-7$), 18:2 ($n-6$), 18:3 ($n-6$), 18:3 ($n-3$), 18:4 ($n-3$). This sequence expands with increasing polarity of the column. Therefore, in very polar columns, some polyunsaturated FAMEs elute after the next highest even-carbon saturated

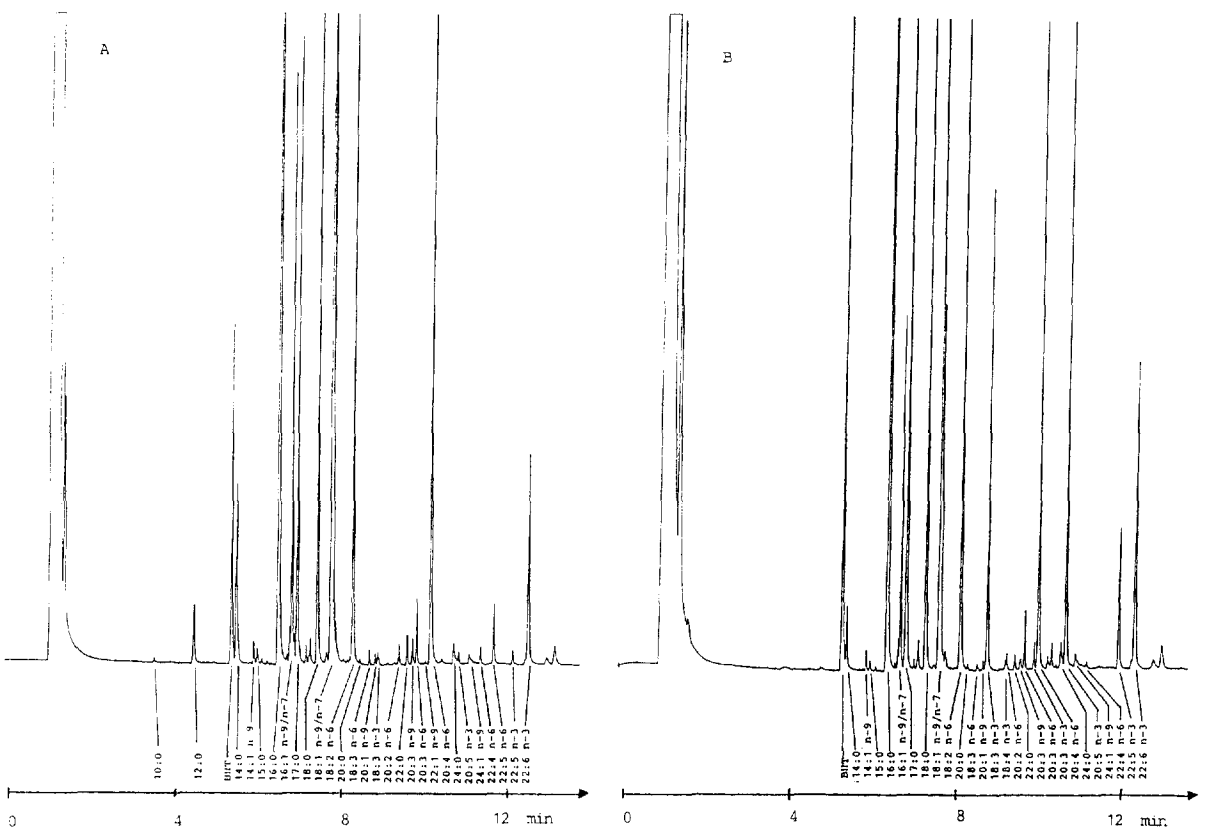

Fig. 1. Separation of FAMEs from rat liver total lipids on a CP-Sil 88 fused-silica capillary column (50 m × 0.25 mm I.D., 0.2 μm film thickness) within 14 min. The diets of the rats contained either (A) a coconut oil–safflower oil mixture or (B) linseed oil as the fat source. Gas chromatographic conditions: injection by PTV (split ratio 1:50); oven temperature program: 90°C for 1 min, 30°C/min to 160°C, 15°C/min to 200°C, 200°C for 1.5 min, 10°C/min to 225°C, 225°C for 15 min; carrier gas: hydrogen (2 ml/min). $C_{17:0}$ added as internal standard.

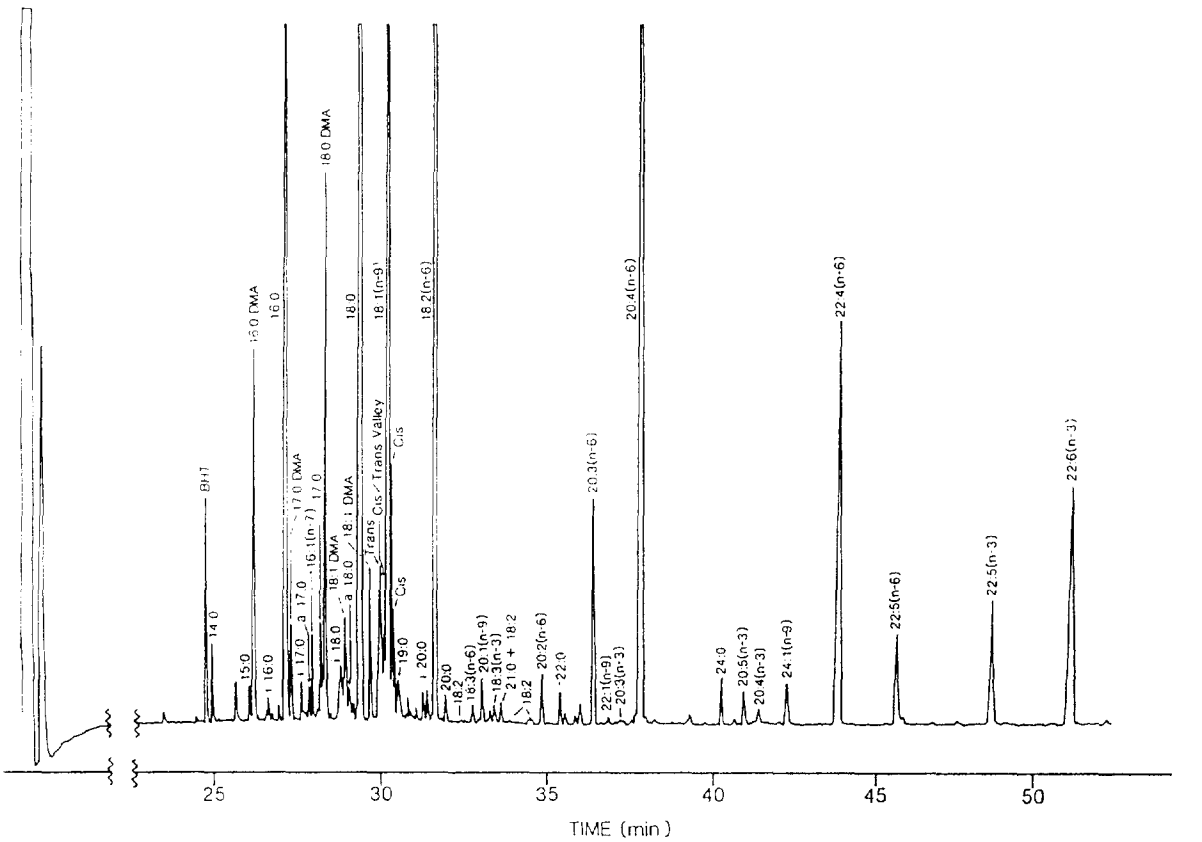

Fig. 2. Separation of human erythrocyte membrane FAMEs on a SP-2560 fused-silica capillary column (100 m × 0.25 mm I.D., 0.2 μm film thickness). Chromatographic conditions: split injection (split ratio 1:30); oven temperature program: 80°C for 2 min, 8°C/min to 220°C, 220°C for 32 min; carrier gas: helium (0.67 ml/min). DMA = dimethyl acetal derivatives of fatty aldehydes; i = iso; a = anteiso. Reproduced from Alexander et al. [25] with permission.

FAME (e.g., 18:3 *n*–3 after 20:0, 20:3 after 22:0, 22:4 after 24:0).

Apart from the predominantly occurring straight-chain FAMEs, GC profiles of methylated extracts from typical biological samples also include branched-chain isomers, double-bond positional isomers and DMAs [25]. Branched-chain isomers of FAMEs can be easily separated from the straight-chain FAMEs. Both on polar and on non-polar capillary columns, the iso form is eluted first, followed by the anteiso form and the straight-chain FAME [12]. Using a SP-2560 fused-silica capillary column, relative retention times (RRTs) were 0.977 for iso-18:0 and 0.987 for anteiso-18:0 relative to straight-chain 18:0 [25]. Geometrical double-bond isomers can also be separated on very polar capillary columns [25,72,79,101,106,107].

The analysis of *trans* fatty acids in foods is becoming of considerable importance because of the implications of these acids for human health [108–111]. Fatty acids with the *trans* configuration occur mainly in partially hydrogenated oils and fats and in ruminant fats. On very polar columns the C_{18} monoethylenic *trans* fatty acids elute before the monoethylenic *cis* fatty acids [25,72,79,110]. However, the *trans* fatty acids with a low omega value (*n*–3) can overlap with the *cis* fatty acids with a high omega value (*n*–11) [112].

Wolff [74] succeeded in separating all geometrical isomers of linolenic acid methyl ester

on a CP-Sil 88 coated capillary column under isothermic conditions within 45 min. As in the monoethylenic FAMEs, the inclusion of *cis* double bonds increased retention times compared with *trans* double bonds. The elution order of the geometrical isomers of linolenic acid methyl ester was (t = *trans*, c = *cis*): ttt < ctt < tct < cct < ttc < ctc < tcc < ccc; ECL values ranged between 19.45 and 19.89.

The occurrence of DMAs originating from plasmalogen phospholipids can seriously interfere with some FAMEs [16,25,26]. Even with very polar capillary columns no satisfactory separation of all the DMAs from FAMEs can be achieved [25]. On very polar columns the pair 15:0 FAME and 16:0 DMA and the pair 17:0 FAME and 18:0 DMA cannot be resolved at all or can only be resolved partially [25,26]. This is particularly a problem if 17:0 FAME is used as an internal standard. The problem of coelution of FAMEs with DMAs can be overcome by using a methylation method that does not produce DMAs. Treatment of plasmalogens with acids prior to methylation also prevents formation of DMAs by hydrolysis of the labile ether bond [99,102].

Very polar stationary phases have the disadvantage of less thermal stability compared with non-polar phases. For the CP-Sil 88 column, the maximum isothermic temperature given by the manufacturer is 225°C. The long retention times for highly unsaturated fatty acids such as 22:5 *n*–3 and 22:6 *n*–3 would not be necessary if columns were resistant to higher temperatures [25,63,72].

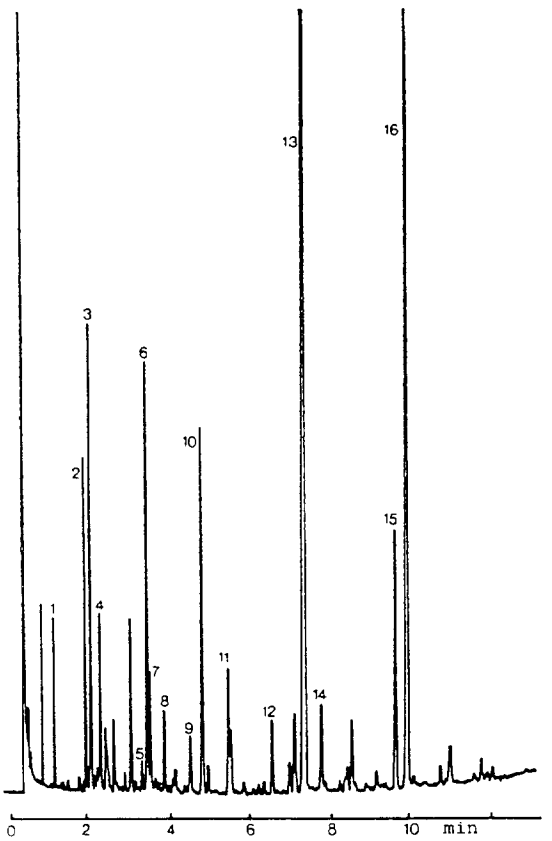

Fig. 3. Separation of fish oil FAMEs on a Durabond-Wax capillary column (15 m × 0.32 mm I.D, 0.15 μm film thickness). Chromatographic conditions: split injection (split ratio 1:186); oven temperature program: 160°C for 1 min, 5°C/min to 185°C, 8°C/min to 240°C, 240°C for 10 min; carrier gas: hydrogen (50 cm/s). Peaks: 1 = 14:0, 2 = 16:0, 3 = 16:1, 4 = 16:2, 5 = 18:0, 6 = 18:1 (*n*–9), 7 = 18:1 (*n*–7), 8 = 18:2, 9 = 18:3, 10 = 18:4, 11 = 20:1, 12 = 20:4, 13 = 20:5, 14 = 22:1, 15 = 22:5, 16 = 22:6. Reproduced from Welz et al. [60] with permission.

4.1.2. Polar phases

The most important stationary phases of intermediate polarity are polyethylene glycol (DB-Wax, Supelcowax 10, Carbowax 20M), acidified polyethylene glycol (FFAP), 86% dimethyl–14% cyanopropylphenylpolysiloxane (DB-1701), and methylsilicone polymer, 25% cyanopropyl–25% phenyl–50% methyl (OV-225, DB-225, SP-2300) [13].

The relatively fast separation of FAMEs from an encapsulated fish oil on a Durabond-Wax capillary column is shown in Fig. 3. The elution order is different from that from the very polar columns (e.g., 20:4 and 20:5 elute before 22:1; 18:3 and 18:4 elute before 20:1).

Intermediate-polarity columns in most cases allow acceptable separation of FAMEs from biological samples such as plasma [73], adipose tissue [71], erythrocyte membranes [86], or marine oils [60,61]. Thus, these columns combine the advantages of a relatively high resolution capability with those of a relatively high thermal stability.

4.1.3. Non-polar phases

The most important non-polar stationary phases are based on methylsilicones (SPB-1, SPB-5), 95% dimethyl–5% diphenylpolysiloxane (DB-5, SPB-5, CP-Sil 8CB) or 100% dimethylpolysiloxane (DB-1, Rt-1, SPB-1, SP-2100, OV-1, OV-101, CP-Sil 5CB) [13].

The separation of FAMEs from a human milk sample on a CP-Sil-5 cross-linked methylsilicone-coated column is shown in Fig. 4. The nature of the separation attained on non-polar phases is rather different from that with polar phases. In non-polar columns, FAMEs are eluted according to their boiling points. Therefore, unsaturated FAMEs elute before saturated FAMEs. This elution order is the reverse of that in very polar and polar columns. Isomeric fatty acids differing in the position of the double bonds such as 18:1 (*n*–9) and 18:1 (*n*–7) or 18:3 (*n*–3) and 18:3 (*n*–6) are usually separated on non-polar phases under appropriate operating conditions [105].

The main disadvantage of non-polar columns

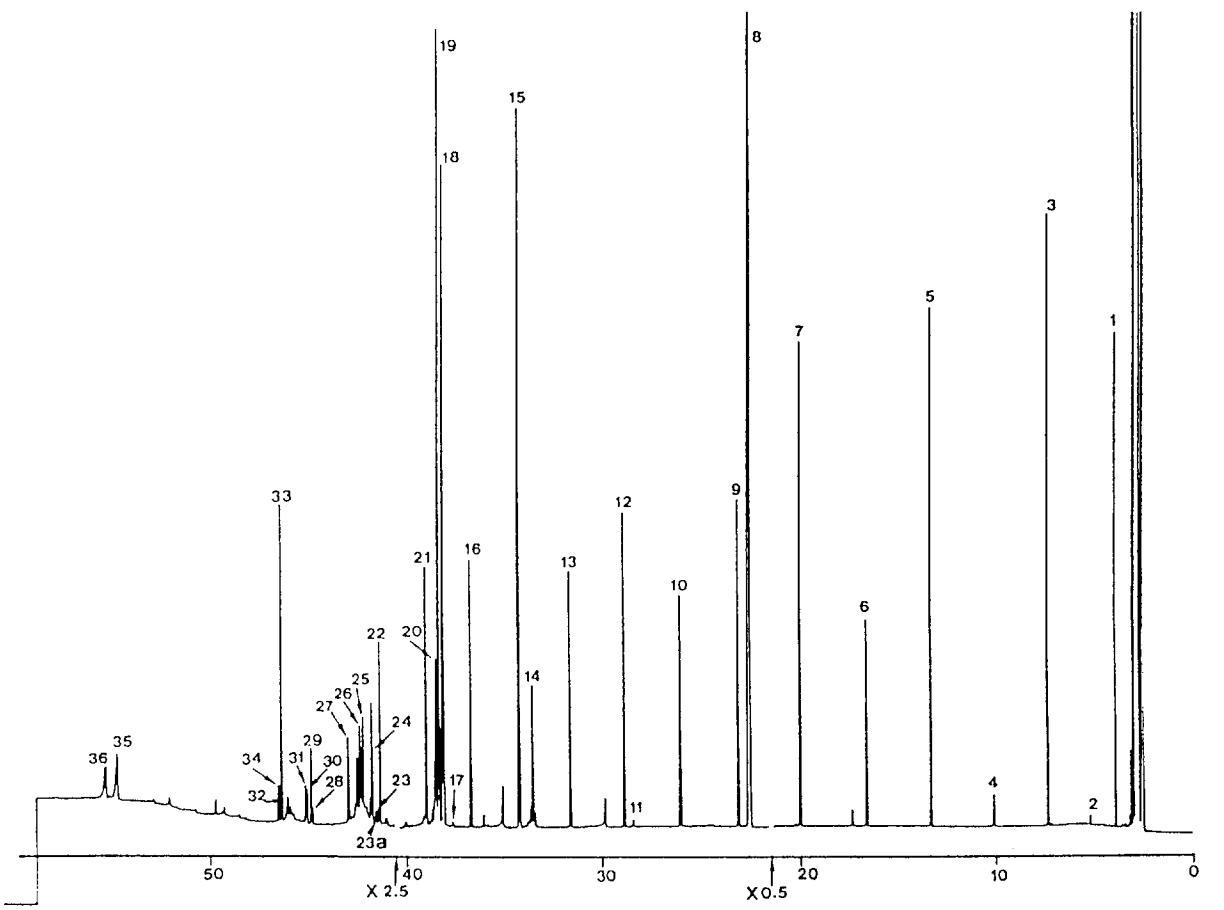

Fig. 4. Separation of FAMEs from a human milk sample from Curaçao on a CP-Sil-5 cross-linked methylsilicone-coated capillary column (50 m × 0.20 mm I.D.). Chromatographic conditions: split injection (split ratio 1:20); oven temperature program: 60°C, 4°C/min to 200°C, 50°C/min to 285°C, 285°C for 15 min; carrier gas: helium (0.52 ml/min). Peaks: 1 = 5:0, 2 = 6:0, 3 = 7:0, 4 = 8:0, 5 = 9:0, 6 = 10:0, 7 = 11:0, 8 = BHT, 9 = 12:0, 10 = 13:0, 11 = 14:1, 12 = 14:0, 13 = 15:0, 14 = 16:1 (*n*–7), 15 = 16:0, 16 = 17:0, 17 = 18:3 (*n*–6), 18 = 18:2 (*n*–6) + 18:3 (*n*–3), 19 = 18:1 (*n*–9), 20 = 18:1 (*n*–7), 21 = 18:0, 22 = 20:4 (*n*–6), 23 = 20:5 (*n*–3), 23a = 20:3 (*n*–9), 24 = 20:3 (*n*–6), 25 = 20:2 (*n*–6), 26 = 20:1 (*n*–9), 27 = 20:0, 28 = 22:5 (*n*–6), 29 = 22:6 (*n*–3), 30 = 22:4 (*n*–6), 31 = 22:5 (*n*–3), 32 = 22:0, 33 = 24:1 (*n*–9), 34 = 24:0, 35 = 5β-cholestan-3α-ol, 36 = cholesterol. Reproduced from Van der Steege et al. [58] with permission.

is partial overlapping of some important unsaturated FAMEs. On non-polar phases, usually 18:2 $n-6$ is not fully resolved from 18:1 $n-9$; 18:2 $n-6$ and 18:3 $n-3$ overlap completely; similar resolution problems occur in the C_{20} and C_{22} compounds [105]. Because these unsaturated fatty acids play an important role in biological samples and are of great significance in lipid research, the use of a more polar column is recommended for the analysis of such samples.

Advantages of the non-polar phases are high thermal stability, a wide range of operating temperatures and chemical inertness [13]. Non-polar capillary columns have a temperature limit of 340°C or above. Therefore, non-polar phases have advantages in analysis of fatty acids with higher molecular masses.

4.2. Capillary column dimensions and film thickness

4.2.1. Column length

The length of the column is an important parameter for resolution capability. Increases in column length improve the resolution capability, but also increase retention times. Therefore, a compromise should be found which minimizes the duration of the whole analysis while at the same time providing acceptable separation. This means that the column length which gives an acceptable resolution is a function of the stationary phase.

Relatively short very polar phases often give better resolution of unsaturated FAMEs than relatively long non-polar phases. For instance, excellent separation of all the unsaturated FAMEs from various tissues has been demonstrated on a 25-m very polar (CP-Sil 88) column [63], whereas on a 50-m non-polar column based on methylsilicone separation of all the important unsaturated fatty acids could not be achieved [58,59].

Most of the columns described in literature for FAME separation range between 25 and 50 m in length [58,59,68,70,73,85,97,98,102,113]. Very long capillary columns (100 m) have been used for the resolution of complex FAME mixture from foods [72] and human erythrocyte membranes [25]. Relatively short columns, 25 m or shorter, can be used for rapid analysis of relatively simple FAME mixtures. A common vegetable oil containing fatty acids with chain lengths between 14 and 18 carbon atoms can be analysed in less than 5 min on a short column [104].

4.2.2. Internal diameter and film thickness

The internal diameter and the film thickness of a WCOT capillary column influence its capacity, resolution capability and FAME retention times.

In general, increasing film thickness increases the sample capacity of the column, the resolution capability and the retention times. In most cases, a film thickness of 0.25 μm will provide sufficient resolution capability (although the resolution capability of course depends primarily on the type of stationary phase) [13].

Decreasing the internal diameter of the column increases the resolution capability of the column but increases retention times and reduces the column capacity.

If narrow-bore capillary columns (with thin films) are used, the amount of sample introduced into the column must be restricted by splitting the carrier gas at a large split ratio. However, most analyses can be performed on medium-bore capillary columns that have an internal diameter of 0.25 or 0.32 mm.

5. Identification of fatty acid methyl esters

The easiest way to identify FAMEs occurring in biological samples is to compare their retention times with those of commercially available individual purified standards. However, some of the very long-chain highly unsaturated FAMEs occurring in some animal tissues are not commercially available. If the sample to be analysed contains such fatty acids, they can be identified by the use of natural products containing a wide spectrum of fatty acids that has been well characterized. Natural products that can be used in this way include cod-liver oil, which contains many different fatty acids, especially those of the $n-3$ family [114], or samples originating from bovine and porcine testes, which

contain a wide range of fatty acids of the $n-6$ series [115,116]. Christie [22,105] recommended a mixture made up of the fatty acids of pig-liver lipids, cod-liver oil and linseed oil. This mixture contains all the major fatty acid classes.

An alternative way of identifying FAMEs is based on their RRTs or ECL values [22,103,105,112]. The RRT is defined as the ratio between the retention time of any FAME and that of a reference FAME (usually $C_{16:0}$ or $C_{18:0}$). Although RRTs vary somewhat with operating parameters such as temperature and flow-rate, RRTs given in tabulation systems in the literature are helpful in the identification of unknown FAME peaks. The concept of ECL is based on Kovats' retention index [117–119]. The use of ECL values results in a ready visualization of the position of a particular FAME relative to saturated straight-chain acids, 16:0, 18:0, 20:0, 22:0, 24:0. For the calculation of ECL values, retention times of FAMEs are determined under isothermal operation. Actual ECL values for FAMEs can then be calculated either from an equation [120–122] or by plotting on a semilogarithmic paper [22] (for an example see Ackman [123]). Unknown FAMEs can be identified by comparing the actual ECL value with ECL values tabulated in literature. In comparison with RRTs, ECL values have the advantage that they are specific for column types but are relatively insensitive to experimental parameters.

If a peak cannot be clearly identified by the above means, in most cases study of its retention on two columns with different polarities will prove useful.

Although identification by comparison with reference substances as well as RRTs and ECL values is widely used, it is only tentative because non-fatty-acid contaminants can behave like FAMEs. Therefore, identification of FAMEs must be confirmed by on-line mass spectrometry.

6. Quantitation of fatty acid methyl esters

For detection of FAMEs, the flame ionization detector (FID) is most convenient. It is very sensitive and has good linearity over a wide range of concentrations provided the recommendations of the instrument manufacturers on gas flows are followed [112]. In general, most serious inaccuracies in the gas chromatographic analysis of FAMEs result not from detection, but from losses during esterification or injection.

The amounts of FAMEs are usually determined via the peak area. The peak area of a given amount of FAME is not influenced by the peak shape provided that extremely strong leading or tailing does not occur. Electronic digital integration is the most accurate and reproducible means of quantifying peak areas. To avoid potential sources of error, it is essential to ensure that the instrumental parameters (especially sampling rates) on the integrator are appropriate to the peak widths at various times during analysis [105].

Modern FIDs under appropriate operation conditions offer a wide range of linearity. Nevertheless, to ensure accurate quantitation, the linearity should be checked with various dilutions of standard mixtures (selected according to the FAME concentrations of the samples to be analysed). The linear response of the FID can be improved if a higher flow-rate than required for optimum sensitivity is used [124].

Quantitation based on peak height has the disadvantage that even minor changes in the peak shape distort the result [82]. Problems of quantitation occur if two peaks are not completely separated, especially if one component is visible only as a minor shoulder or broadening of a major peak. In this case, either the operating conditions must be altered or a column with a higher resolution capability must be used [105].

Quantitation of FAMEs is commonly carried out after calibration of the system with standards containing known amounts of FAMEs. FAME mixtures should be similar in composition to the samples to be analysed [13]. Amounts of FAMEs are calculated via relative response factors (RRFs), giving the detector response between any FAME and a reference FAME, usually the internal standard. Ackman and Sipos [125] calculated the theoretical RRFs by the mass per cent in the molecule of active carbon atoms, which includes all carbon atoms except that of the

carbonyl group. Bannon and coworkers [124,126,127] have shown that these theoretical RRFs are reliable for all FAMEs provided equipment and technique are optimized. In consequence, they concluded that these theoretical FID factors are fundamental constants for all FAMEs. A large difference between actual and theoretical response factors indicates that the equipment is not properly optimized, or that some element of technique is faulty [84]. Typically, boiling-point-dependent sample discrimination caused by hot split injection gives response factors for the high-boiling FAMEs with 22 or 24 carbon atoms that are far below the respective theoretical response factors. Thus, the injection conditions should be corrected until there is agreement between actual and theoretical response factors. In contrast, if discrimination-free sample injection techniques, such as the on-column or PTV technique, are used, in most cases there is good agreement between actual and theoretical response factors [70].

A popular method for absolute quantitation is the addition of a FAME not present in the sample as an internal standard. The internal standard should be selected according to the sample to be analysed. Because in most biological samples fatty acids with 16 and 18 carbon atoms are predominant, $C_{17:0}$ is a popular internal standard in the analysis of FAMEs [60,65,99,102]. If the sample contains $C_{17:0}$, other FAMEs such as $C_{19:0}$ [85] or $C_{21:0}$ [72] can be used as the internal standard. In the analysis of sphingomyelin, which contains large amounts of $C_{24:0}$ and $C_{24:1}$, $C_{21:0}$ has been used as an internal standard [16].

In the analysis of fish oil, which contains large amounts of highly unsaturated fatty acids with 20 and 22 carbon atoms, accuracy may be highest with $C_{24:1}$ as the internal standard [61]. For quantitation of arachidonic acid, dihomo-γ-linolenic acid ($C_{20:3\ n-6}$) can be used as the internal standard, provided that it does not occur in the sample [128]. However, unsaturated fatty acids as internal standards have the disadvantage of a potential loss by autoxidation during sample preparation or injection [105].

If boiling-point-dependent sample discrimination cannot be eliminated by optimization of the equipment and injection technique, the use of more than one internal standard can improve the accuracy and precision of results [70].

7. Fatty acid analysis in biomedical research

Fatty acids in biological tissues have several functions. The fluidity of biological membranes, and related important properties, depend greatly on the fatty acid composition of membrane phospholipids. Saturated fatty acids decrease membrane fluidity, while PUFAs increase it [1,129].

Eicosanoids derive from membrane-bound PUFAs, such as arachidonic acid, eicosapentaenoic acid or dihomo-γ-linolenic acid [6,7]. The relative proportions of arachidonic acid and eicosapentaenoic acid in biological membranes influence the types of eicosanoids produced. Eicosanoids that derive from eicosapentaenoic acid have functions that are in many respects very different from those derived from arachidonic acid [130]. Specific functions of different eicosanoids are particularly of great significance in the development of coronary heart disease [131]. Therefore, the fatty acid composition of tissues, in particular of membranes, is related to metabolic regulation, and analysis of fatty acid composition may be the key to recognizing changes, abnormalities and pathogenesis of diseases.

Fatty acids in foods have wide-ranging implications for human health. The type of dietary fat consumed (especially its percentages of saturated, mono-unsaturated and polyunsaturated fatty acids) influences concentrations of cholesterol in low-density (LDL) and high-density lipoproteins, and therefore has important implications for the development of atherosclerosis [2–4]. Recently, the implications of *trans* fatty acids, which occur mainly in partially hydrogenated oils and in ruminant fats on serum lipoproteins, have been recognized [5]. Newer studies also demonstrate that oxidized fatty acids as components of LDL promote the development of atherosclerosis [4,132,133]. Since the fatty acid composition of lipoproteins is one major determinant of its oxidizability [134,135],

in future the fatty acid composition of lipoproteins, especially LDL will be given increasing attention. Dietary fatty acids also influence immunity [136] and development of different types of cancer [137].

It is envisaged that analysis of the fatty acids from tissues will contribute to the understanding of the relationship between the intake of fatty acids and the diseases to which they can be linked.

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