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Review

Fatty acid profiles of lipid samples

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Abstract

Most lipids are best characterized by their fatty acids which differ in chain length, the degree of unsaturation, configuration and position of the double bonds, and the presence of other functionalities. The fatty acid profiles are currently most frequently determined by capillary gas chromatography of the methyl esters which are prepared by a variety of methods. These are discussed with an emphasis on more recent developments, along with the stationary phases used for the separations and the methods employed for identification of the fatty acids. HPLC is applied less frequently for ascertaining fatty acid profiles than GC, but a very large number of derivatives for ultraviolet and fluorescence detection have been proposed. This method continues to evoke increasing interest, particularly in conjunction with fluorescence detection. This technique enables attainment of greater sensitivities than with standard GC methods employing flame ionization detection. Extensive applications of it to the analysis of free fatty acids in blood and other biomedical samples are clearly discernible. Other methods, including supercritical fluid chromatography, have found only limited application for fatty acid profiling.

Contents

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List of abbreviations

CZE	Capillary zone electrophoresis
ECD	Electron-capture detector
ECL	Equivalent chain length
FA	Fatty acid
FAME	Fatty acid methyl ester
FFA	Free fatty acid
FID	Flame ionization detector
GC	Gas chromatography
HPLC	High-performance liquid chromatography
MS	Mass spectrometry
ODS	Octadecylsilyl
PUFA	Polyunsaturated fatty acid
RP	Reversed phase
SFC	Supercritical fluid chromatography
TAG	Triacylglycerol
TLC	Thin-layer chromatography
TMS	Trimethylsilyl
WCOT	Wall-coated open tubular

1. Introduction

Fatty acids are a principal component of most lipids. Their diversity in terms of chain length, degree of unsaturation, geometry, and position of the double bonds, as well as the presence of other substituents render their composition the most definitive characteristic of such lipids and their provenient organisms. Such composition is commonly referred to as the fatty acid profile (or pattern or fingerprint) of a lipid. This survey summarizes the chromatographic methods that are available for obtaining fatty acid profiles and the specific applications that have been reported for them, especially in the biomedical field.

Before the fatty acid profile can be determined the lipids must be isolated from the tissue sample and in many cases separated into the various lipid classes. Because these aspects of the overall procedure are outside the purview of this survey, only a brief overview of the essential steps will be presented here.

Even today, the overwhelming majority of papers cite the procedure of Folch et al. [1] or of Bligh and Dyer [2] for extracting the lipids from the tissue matrix. These employ a 2:1 chloroform–methanol mixture to remove the lipids from the sample, with the water derived from the sample actually generating a ternary solvent mixture. The extracted lipids are then washed with aqueous KCl solution to remove contaminants. Less hazardous solvents have been proposed, but have not yet received widespread acceptance. During this procedure precautions should be taken to minimize lipid degradation by enzymes and by oxidation. The details and other important considerations in the isolation of lipids from the matrix have been well summarized [3].

A variety of chromatographic methods have been reported for the separation of lipids into the various classes. The most widespread involve normal-phase systems wherein the solutes are retained (or retarded) on the basis of their relative polarity. This usually takes the form of silica supported in a simple glass column, a TLC plate, a solid-phase extraction column, or preferably, a high-performance liquid chromatographic (HPLC) column. After applying the sample, solvent mixtures of increasing polarity are used to elute lipids off the bed or column in the order of their increasing polarity. Recently, a cyanopropyl stationary phase with evaporative light scattering detection [4] and a reversed-phase (RP) system with low-wavelength UV detection [5] were used in lieu of silica.

For complex lipid samples containing numerous unsaturated species, separation into classes is frequently followed by further fractionation via silver ion chromatography in the thin-layer or column mode, often after conversion to the methyl esters. In such argentation chromatography [6] separation is based on the number, configuration and to some extent the position of the double bonds. The number of double bonds is the governing factor, but *cis* isomers are retained distinctly more strongly than the *trans*. Various strategies have been developed to fractionate samples by double bond number and the

geometric isomers. Although resolution is not always adequate nor clear-cut, such pre-fractionation (often by TLC) can greatly simplify the subsequent resolution of complex mixtures by GC or HPLC.

2. Gas chromatographic methods

2.1. General

Clearly, the overwhelming majority of fatty acid (FA) profiles of lipids are determined by gas chromatography (GC) of the fatty acid methyl esters (FAMEs) employing capillary columns and a flame ionization detector (FID). Most of the FAs in lipids are bound as esters or amides, with (usually) only small amounts occurring as nonesterified or free fatty acids (FFAs). Depending on the information required, the FFAs may be negligibly small, or they may be converted together with the lipid-bound FAs to FAMEs, or measured separately, often after preliminary fractionation into lipid classes. Hence, the procedure adopted for the derivatization of the fatty acids may well be governed by the kind of information sought.

The most common derivatization methods for GC analysis involve transesterification of lipid-bound FAs and esterification of the FFAs to FAMEs. A variety of techniques are in use, including the time-tested acid- and base-catalyzed reactions, the more recently developed on-column or pyrolytic methods, and derivatization with other reagents. Each has its advantages and limitations. Several reviews of these methods have appeared recently [7–13].

2.2. Derivatization

2.2.1. The carboxyl group

The primary advantage of acid catalysis is its general applicability, with both bound and free fatty acids being converted concurrently to FAMEs. BF_3, HCl, and H_2SO_4 are the most extensively used acid catalysts, usually as 14%, 5%, and 2% solutions, respectively. BF_3 is the most commonly used, and FFAs, phospholipids, triacylglycerols (TAGs), and cholesteryl esters were converted to the corresponding FAMEs in 2, 5–10, 25, and 60 min, respectively at 100°C [14]. About twice as long was required with HCl and H_2SO_4 [15]. These can be substituted with similar concentrations of perchloric acid [11,16], which would be shunned by most analysts, however, because of the putative explosion hazard. The higher concentration of BF_3 used, compared to the other acids, may account for its shorter reaction times, but also for the partial degradation of labile fatty acids [17] and the more frequent observance of artifacts [18,19]. This can be mitigated considerably via preliminary saponification with methanolic KOH, followed by re-esterification of the FFAs formed under mild conditions. The methods of the AOAC [20], IUPAC [21], and AOCS [22] are based on this procedure.

Recently, much shorter reaction times have been shown to suffice if conventional heating is replaced by microwave irradiation. Although the wattage, reaction volume, etc. will affect the duration of the irradiation, the reaction times given above for FFAs, phospholipids, and TAGs with 14% BF_3 could be shortened to 20, 30, and 60 s [23,24], respectively, with microwaving. Comparable reductions were reported for 2% H_2SO_4 as catalyst [25]. Moreover, oxidation of unsaturated fatty acids was greatly diminished by irradiation instead of ordinary heating in unsealed tubes [26].

Other Lewis acid catalysts include BCl_3 and $AlCl_3$. The former [27,28] is a milder reagent than its fluorine analog, whereas the latter transesterifies lipids similar to the other reagents, but fails to methylate FFAs at all [29]. Acid catalysis is advantageous for the simultaneous reaction of both bound and free fatty acids, but if no significant amounts of FFAs are present, alkaline reagents should be considered.

Base-catalyzed transesterifications proceed faster than those in acid media and do not degrade labile FAs nor isomerize double bonds. Methanolic solutions of sodium or potassium hydroxide or methoxide are commonly used. With about 1 *M* methanolic $NaOCH_3$, phospholipids, TAGs, and cholesteryl esters require about 1, 5, and 60 min, respectively, at room temperature [10] for complete conversion to FAMEs.

To better understand base-catalyzed transes-

terification it is important to appreciate its mechanism [8,30,31]. Hydroxide ion in methanol is leveled to methoxide and exists predominantly in that form because methanol is more acidic than water [31]. In the presence of both CH_3O^- and OH^-, reversible transmethylation and irreversible saponification proceed simultaneously, but because the former is generally much faster than the latter, a maximum yield of the FAMEs is obtained rapidly, followed by a slow decline as saponification of the FAMEs formed continues. Therefore, the solution should be quenched soon after transmethylation is complete, particularly with hydroxide reagents [8,31–33].

Transesterification can be accelerated by increasing the concentration and the base strength of CH_3O^-. The latter can be achieved by replacing methanol with less acidic or aprotic solvents. This is illustrated by the nearly instantaneous transesterification of milk fat TAGs in an essentially nonalcoholic solution [34] or of other TAGs with methanolic tetramethylammonium hydroxide in diethyl ether [35]. Seed oils were rapidly transmethylated with 3.3 *M* CH_3ONa in methanol [36]. On this basis cholesteryl esters could be transesterified in about 1–2 min at ambient temperature [37].

Strong organic bases (various quaternary ammonium and trimethylsulfonium hydroxides, and tetramethylguanidine) are used to transesterify lipids to FAMEs and covert FFA to their salts, similar to their inorganic analogs. Unlike the latter, however, the FA salts of the organic bases decompose at the high temperatures of a GC injection port to form FAMEs, thus allowing a simple, one-step determination of both esterified and free fatty acids. The specific quaternary bases that have been used for such pyrolytic transesterifications include tetramethylammonium [35,38–42], trimethylphenylammonium [43,44], and (*m*-trifluoromethylphenyl)-trimethylammonium [45,46] hydroxides. Tetramethylguanidine behaves similarly [47].

Trimethylsulfonium hydroxide [48,49] has distinct advantages over the quaternary ammonium hydroxides in terms of the lowest pyrolysis temperature (250°C) and by-products that are innocuous to the column. The reagent is simply added to the sample solution and after mixing is ready for injection. Excellent results have been reported in the FA profiling of lipids from plant and animal sources [50,51] and various bacteria [52–55], although some loss of PUFAs was reported if the solution was not acidified prior to injection [56]. A rapid procedure has been recommended recently for adoption as a standard method [57].

Diazomethane has long been used for the rapid esterification of FFAs. Special reagents, procedures, and apparatus permit relatively safe operation with this compound despite its toxic and explosive nature [58]. Although it has generally been assumed to react selectively with FFAs and was proposed for their determination in plasma [59], extensive transmethylation of phospholipid fatty acids [60] was reported recently.

Lately, there has been a renewal of interest in the alkyl chloroformates as very rapid esterifying reagents for FFAs to the corresponding alkyl esters. The reaction proceeds almost instantaneously in the presence of pyridine or its derivatives and the corresponding alcohol. The reaction can be conducted in a nonaqueous medium such as acetonitrile or even in the presence of considerable water (40%) [61–65]. The tolerance of water by these reagents is illustrated by a procedure developed for organic acid profiling of plasma that is simpler and faster than others currently in use [66].

Other, less extensively used reagents include methyl iodide which alkylates both carboxyl and hydroxyl groups [67] and has been applied to the determination of the sphingolipid fatty acyl composition [68] and FFAs in brain tissue [69]. For a few lipids, notably milk fats that contain fatty acids down to C_4, the preparation of higher esters such as butyl [70,71] or isopropyl [72] has been proposed to mitigate losses due to volatility and appreciable water solubility of the FAMEs. Very small amounts of FFAs can be converted to the pentafluorobenzyl esters and monitored with an ECD [73,74].

2.2.2. Other functional groups

The carboxyl groups are usually derivatized first by one of the methods described above and then other functional groups can also be converted to more volatile forms. For hydroxyl

groups, a general method entails the formation of the trimethylsilyl (TMS) ethers [75], most popularly with trimethylsilylimidazole (TMSIM) and N,O-*bis*-(trimethylsilyl)trifluoroacetamide (BSTFA) [76]. The *tert.*-butyldimethylsilyl (*tert.*-BDMS) ethers are more hydrolytically stable but require longer reaction times [77]. Alternatively, OH groups can be converted to acetates [78,79] or trifluoroacetates [80,81].

Double bonds are derivatized principally for determining the position and geometric configuration of the olefinic linkages. The preparation of epoxides of monoenoic and dienoic acids by reaction with *m*-chloroperbenzoic acid in $CHCl_3$ at room temperature requires 4 h [82] and 12 h [83], respectively, or 3 and 5 min by microwave irradiation [25]. Epoxy derivatives may be analyzed as such or may be converted to the diols (or other derivatives). Diols are prepared directly by oxidizing double bonds with OsO_4 or $KMnO_4$ [84,85], with the *cis* and *trans* isomers yielding the *erythro* and *threo* diols, respectively. A number of mercury adducts, but usually the methoxybromomercuric derivatives, can be prepared from complex mixtures of unsaturated fatty acids and fractionated by TLC according to the degree of unsaturation prior to further analysis by capillary GC [86].

2.3. Resolution

The determination of fatty acid profiles often requires high resolution for the separation of positional and configurational isomers, and for the disengagement of chromatographic zones that overlap by chance [87,88] on a particular stationary phase, because of the sheer multitude of different fatty acids present in lipids. This requirement has made packed columns effectively obsolete for general applications, although they may still be useful for routine samples containing a limited number of fatty acids, or for rapid scouting runs. Therefore, the separations covered here will be largely limited to those effected on fused-silica capillary columns. Because of the high efficiency of such wall-coated open tubular (WCOT) columns, less reliance needs to be placed on their selectivity to achieve high resolution and relatively few different stationary phases will adequately separate the majority of mixtures encountered. Even if high resolution is not required, such capillary columns are superior because their temperature can be programmed or the flow velocity of the carrier gas can be raised to optimize resolution and reduce the analysis time.

The resolution and analysis time of a particular chromatographic system are markedly affected by the carrier gas selected. For a given degree of resolution, the shortest analysis times will be realized with hydrogen gas [89,90], followed by helium, assuming one is operating at flow-rates above the optimum linear velocity, as is usual. Helium is often preferred, however, for reasons of safety.

The purity of the carrier gas is also an important factor to consider, as oxygen and water impurities can degrade stationary phases, which is exacerbated at higher temperatures. This has been observed for the polyethylene glycol (PEG) stationary phases at temperatures substantially below the limits specified by the manufacturer [91]. Thus, the installation of oxygen and water traps in the carrier gas line is usually a worthwhile investment.

The WCOT columns typically used for the analysis of fatty acids are 25–30 m long, with an inner diameter of about 0.25 mm. Columns of 100 m may be essential for the most challenging separations when the highest resolution is required, such as the separation of the positional and geometrical isomers of unsaturated fatty acids.

The high efficiency of such WCOT columns requires less reliance on their selectivity, which is based primarily on the inherent volatility of the fatty acid derivative and the degree of interaction between its polar sites and those of the stationary phase. Except for the moderately polar polyethylene glycols, most stationary phases used for the analysis of FAMEs consist of various proportions of the nonpolar methylpolysiloxanes and the very polar cyanoalkylpolysiloxanes. The methyl groups may be partly replaced by the somewhat more polar phenyl or vinyl groups. A listing and description of the most commonly used stationary phases for capillary GC have appeared recently [12].

On stationary phases composed entirely or preponderantly of methylpolysiloxanes the unsaturated FAMEs are generally eluted prior to their saturated analogs and in relation to the number of double bonds. This results in a clustering of peaks around the even chain lengths, without overlapping into the adjacent even chain length groups [92]. Because separation is based primarily on volatility, however, the unsaturated fatty acids may be inadequately resolved. Polar phases, such as polyethylene glycols, afford resolution by carbon number, and the unsaturated are eluted after the saturated, with minimal overlap of different chain lengths [93,94]. Very polar stationary phases, on the other hand, retain the polar double bonds more strongly and as the degree of unsaturation increases, these components are eluted progressively later than their saturated counterparts (Fig. 1). They are also most suitable for the separation of *cis*, *trans* isomers [95]. Due to the multiplicity of isomers, overlap is inevitable, and two (or more) columns should be used if this is the case [96]. Usually columns of intermediate polarity are used for the separation of the PUFAs, and two authorities [10,93] in the lipids field have expressed their preference for PEG columns for the separation of FAMEs.

The nonpolar stationary phases have advantages in being more thermally stable (exhibiting less column bleed) and permitting operation at lower temperatures; they should be used whenever they provide adequate resolution. For the separation of a wide range of PUFAs, especially when both *cis* and *trans* isomers are present, recourse to polar stationary phases is often necessary.

A large majority of the lipid samples encountered in various fields will be separated satisfactorily by the stationary phases available for the WCOT columns. A notable exception is the partially hydrogenated oils (margarines and shortenings) whose *trans* fatty acid content has generated interest in the health and nutritional areas [97]. Such samples are very complex mixtures of various positional and configurational isomers which cannot be completely resolved even with very long (100 m) highly polar (cyanosilicone) capillary columns. They require preliminary semi-preparative separations by argentation thin-layer chromatography (TLC) according to the number of olefinic linkages and their geometrical isomers [98]. A combination of GC with infrared (IR) methods and preliminary fractionation via the methoxybromomercuric adducts TLC for the degree of unsaturation, followed by $AgNO_3$-TLC for the configurational isomers, was reported in another case [99] of margarine analysis.

2.4. *Identification*

2.4.1. *Retention parameters*

Identification of the abundant, most commonly encountered fatty acids should be based on the retention times of FAMEs measured with authentic standards that are commercially available. This should cover the range, C_4 to C_{24}, and include both saturated and unsaturated compounds. Comparison with chromatograms of well characterized samples also can aid greatly in locating the less stable PUFAs, for example. Cod liver [10] and canola [93] oils have been recommended for such purposes.

For peaks that remain unidentified via the above procedures, retention data in the literature should be consulted. These are presented in a number of ways, commonly as relative retentions [100,101] or, preferably, as equivalent chain length (ECL) values [102]. The latter are akin to the Kováts retention indices and are based on a near linear relationship between the logarithms of the adjusted retention times (log t'_R) of a homologous series of compounds (saturated straight-chain FAMEs) and their carbon chain lengths. Such a correlation is established by plotting the log t'_R of saturated, straight-chain FAMEs against their integer chain lengths. The ECL of an unsaturated (or otherwise substituted) FAME is determined from its log t'_R and corresponds to the non-integer chain length of a hypothetical saturated FAME. Such ECL values are valid for a particular stationary phase when measured isothermally and under the same conditions. Extensive tabulations of such values are available in the literature, the older including

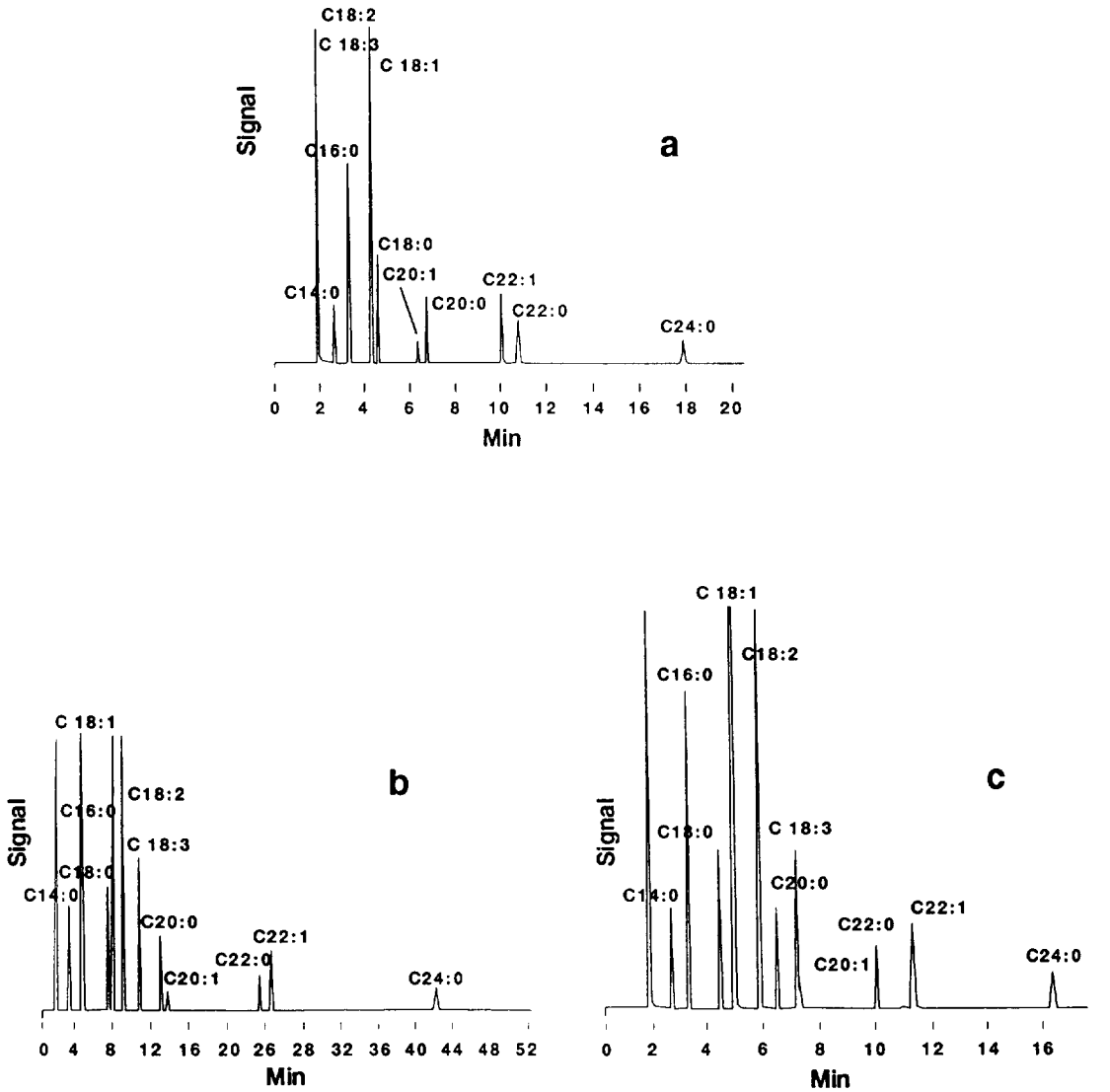

Fig. 1. Selectivities of (a) nonpolar (SPB-1), (b) moderately polar (Omegawax 320), and (c) highly polar (SP-2380) GC stationary phases for the analysis of FAMEs derived from a rapeseed oil standard. The analyses were performed isothermally at column temperatures of (a) 240°C, (b) 200°C, and (c) 180°C. All columns were 30 m long with an internal diameter of 0.32 mm and a stationary phase film thickness of 0.20 μm (SP-2380) or 0.25 μm. Helium carrier gas was used at a linear velocity of 25 cm/s. Detection was by flame ionization. From Ref. [104] with permission.

data both for packed and capillary columns [103], and the more recent concentrating on WCOT [92,94]. The temperature dependence of ECL values obtained on capillary increased with the polarity of the stationary phase and that of the analyte (i.e., the degree of unsaturation) [104]. Improved accuracy in measuring ECL values via an exponential equation has been reported recently [105].

The identification of the fatty acids via authentic standards or literature ECL values is relatively simple and rapid, and will be applicable for the large majority of biological specimens. To confirm the identity of an analyte fatty acid it

is also becoming increasingly routine to utilize gas chromatography–mass spectrometry (GC–MS), in order to compare its mass spectrum with a reference spectrum stored in a computer data bank. If no match is found, however, the problem of identification is magnified considerably. In such a case, the unidentified compound can be isolated and derivatized by chemical degradative procedures or, much more conveniently, by GC–MS following appropriate derivatization. In either case the methods and their variants are numerous and can be highly specialized and quite complicated, so that they fall outside the purview of this survey. Therefore, only a brief overview will be provided. The subject has been extensively reviewed recently [106–111].

2.4.2. Gas chromatography–mass spectrometry

The combination of the retention parameters determined by GC with the structural information provided by MS constitutes one of the most definitive methods for the identification of complex organic compounds. The major application of such methods is to the determination of the position and geometry of double bonds, but cyclopropyl rings, branched chains, or other substituents can be located as well. Two different approaches have been developed, namely, the "on-site" method, wherein chemical modification is used to fix the location of the double bond, or "remote group" derivatization, in which the carboxyl group is reacted to produce a nitrogen-containing substituent that stabilizes the positive charge formed from electron bombardment and thereby reduces double-bond migration. The remote-group method is far more convenient and versatile.

Probably the most useful application of the on-site method involves the conversion of the olefinic linkages to the epoxides [112] and GC separation of the resulting *cis–trans* isomers. The remote-group method does not distinguish between geometrical isomers. Other derivatives can also be prepared from the epoxides. Alternatively, as mentioned, *cis–trans* isomers can be pre-fractionated by argentation chromatography and then further resolved by GC.

Also quite useful is the conversion of polyenoic fatty acids to their polyhydroxy derivatives with OsO_4 and then to their TMS ethers for GC–MS analysis. This method was successfully implemented for acids with up to six double bonds, and was applicable to both isolated and conjugated unsaturated systems [113].

The primary advantage of the remote group approach lies in its generality, being potentially applicable to all types of structures. Methyl esters are usually unsuitable derivatives because they tend to undergo double-bond migration. Appropriate derivatives include amides and esters of nitrogen-containing alcohols such as the pyrrolidides [114,115], picolinyl esters [116,117], oxazolines [118–121], and diethylamide [122]. The first two derivatives require about 50°C higher temperatures than FAMEs for their GC separation and are therefore usually analyzed on nonpolar phases to reduce column bleed. The oxazolines and diethylamides are more volatile and can be chromatographed on polar columns.

For the location of the double bonds, for example, besides the molecular mass, most mass spectra reveal a pattern of fragments that decrease by 14 mass units due to the successive cleavage of methylene groups and by 12 mass units where a double bond is located. This has been somewhat simplified and the picture may be even less clear-cut for the more highly unsaturated fatty acids; this underscores again the complexity and the experience required for the interpretation of such mass spectra.

3. High-performance liquid chromatographic methods

3.1. General

In contrast to GC where fatty acid profiling is based predominantly on a single derivative, the FAMEs, in HPLC a large number of different derivatives have been described. These encompass underivatized fatty acids or their methyl esters and those containing various UV chromophores and fluorophores. These have been reviewed [3,123–125].

Most of the separations by HPLC of free fatty acids or their derivatives are performed on reversed-phase systems which consist of alkyl

chains of various lengths bonded onto a silica base. Both the retention and the selectivity increase as the alkyl chain of the bonded phase is lengthened [126], as was convincingly demonstrated with a synthesized C_{30} RP [127]. Because of their commercial availability, octadecylsilyl (ODS) phases have generally been favored for fatty acid separations. Octylsilyl ones have also found some applications, but shorter ones are seldom used. Since these are nonpolar phases, the order of elution of fatty acids in HPLC corresponds to that of GC with nonpolar phases, but with the unsaturated being eluted considerably ahead of their saturated analogs. In fact, each double bond produces a reduction in retention time approximating that of two fewer methylene groups; this therefore results in close elution of fatty acids such as the 14:0, 16:1, 18:2, etc. Such difficult-to-separate combinations have been referred to as "critical pairs" [128], and also include 16:1 and 20:4, *cis* and *trans* 18:1, 20:0 and 22:1, and 22:0 and 24:1 [125,127]. The extent of their separation provides a reasonable criterion of the resolution capability of a C_{18} column. On the C_{30} RP column the decrease in the retention time per double bond corresponded to distinctly more than two methylene groups and these pairs were well resolved [127]. Thus, the RP chain length may provide some control of selectivity [130], similar to but to a lesser extent than the polarity differences of GC columns.

In contrast to GC where the carrier gas is inert and serves to transport the analytes through the column to the detector, in RP-HPLC the eluent can be modified by varying the proportions of water and the nonaqueous components (usually methanol and/or acetonitrile) to effect changes in retention and therefore in resolution. For fatty acid derivatives, it was found that acetonitrile, relative to methanol, increased retention of carbon chains longer than C_8, whereas methanol was more effective for the shorter chain lengths [130,131]. Acetonitrile yielded poor resolution of certain critical pairs [129] such as 16:0 and 18:1 which are the major fatty acids in animal lipids. In many cases a mixture of acetonitrile and methanol with water is used to achieve optimum resolution. Thus, the ability to modify retention by adding other organic solvents and various buffers to the eluent gives HPLC more flexibility than GC in this respect.

3.2. Derivatization

Virtually all the procedures for the preparation of derivatives for fatty acid profiling by HPLC commence with the saponification of the sample lipids, except FAMEs which can be obtained directly by transesterification, as has been described above. A recommended saponification procedure [3] entails refluxing the specimen with 1 *M* KOH in 95% ethanol for 1 h or leaving the mixture overnight at room temperature. Cholesteryl esters may require a longer reaction time. The mixture is then acidified and the free fatty acids are extracted with ether.

Of the many derivatives proposed for the fatty acid analysis by HPLC, phenacyl esters (and their substituted analogs) are used most frequently. Procedures for their preparation are similar, differing mainly in the catalyst used. Typically, the fatty acids, the phenacyl bromide, and catalyst are heated in a solvent for 15–30 min at 100°C, or for about 2 h at 50°C, or are left overnight at room temperature. The catalysts used predominantly are "18-crown-6" ether and $KHCO_3$ [132], triethylamine [133], and lithium carbonate or N,N-diisopropylethylamine in dimethylformamide [134].

More recently, such reactions have also been conducted in a two-phase system in which the fatty acids are extracted as an ion-pair with tetraalkylammonium ions from an aqueous buffer into an organic solvent where the derivatization is carried out [135]. This general approach has been employed with a number of different derivatizing agents, the required reaction time and temperature varying with the specific reagent.

3.3. Resolution

The desirable characteristics of a separation system are high speed, sensitivity, and efficiency, but these are not always mutually compatible. Speed can be gained at the expense of resolution; sensitivity often increases with larger,

more highly conjugated derivatizing agents, but usually with a concomitant loss in resolution. This occurs because RP selectivity stems from the structural differences in the carbon backbone of the analyte fatty acids, so that enlarging the chromophoric label increases the proportion of the non-selective RP interaction and diminishes the resolution observed for the derivative as a whole. Increased polarity of the chromophoric tag also tends to benefit resolution because it reduces the non-selective interaction. Thus, enhanced sensitivity is frequently accompanied by the loss of some resolution.

3.4. Detection

3.4.1. UV absorption

Underivatized fatty acids and their methyl esters have been separated by HPLC and monitored by refractive index or, more sensitively, by low-wavelength UV detection [136,137]. Not many solvents are transparent in this region; water and acetonitrile are the most suitable eluent components. Although good resolution is observed, the sensitivity is rather modest and a strong function of the degree of unsaturation of the analyte. Unfortunately, the evaporative light scattering detector was not found to be mass-responsive for FAMEs [138]. Such separations are perhaps most useful for preparative rather than analytical purposes.

For analytical applications it is therefore advantageous to derivatize the fatty acids with a reagent possessing a high molar absorptivity at longer UV wavelengths where double bonds do not absorb and where the signal is proportional to the molar amount of analyte. For a given chromatographic system the sensitivity should be primarily a function of the molar absorptivity of the derivative, assuming that it is formed quantitatively.

Of the multitude of different derivatives reported, phenacyl esters or their substituted analogs constitute the large majority. The substituted reagents behave similarly to the parent in most respects, and the primary reason for their selection is generally the greater sensitivity attainable. The parent reagent forms fatty acid derivatives with maximum absorbance at 242 nm and a molar absorptivity of 14 000 [133] or 12 100 [135] l mol^{-1} cm^{-1}, but has a considerably reduced value of 6000 l mol^{-1} cm^{-1} at 254 nm where the analytes were often monitored, especially in the past with fixed wavelength detectors. Borch [133] used step gradients of acetonitrile–water to separate 24 fatty acids from C_{12} to C_{24}, including saturated, unsaturated, and configurational isomers, on a (longer than conventional) 90 cm C_{18} RP column packed with 10-μm particles. The common critical pairs were well resolved; oleic and vaccenic acids were coeluted but were separated from petroselinic acid. However, the separation required about 4 h.

Optimal resolution conditions for phenacyl esters were found to be a 70–100% linear acetonitrile–water gradient on a C_8 RP column [130]. This was applied to the separation of the fatty acids from butter and required only 20 min. Wood and Lee [139,140] studied the elution order of synthesized isomeric octadecenoates and octadecynoates, and found that many of them could be resolved on a 25 cm C_{18} RP containing spherical 5-μm particles.

The free fatty acids in human blood plasma were analyzed as phenacyl esters [141] with good results, but the 3-h analysis time was deemed excessive.

The *cis* and *trans* isomers of unsaturated fatty acids were converted to phenacyl derivatives and separated on an ion-exchange column loaded with silver ions [142]. The main objective was to simplify complex mixtures prior to further analysis by GC–MS.

Phenacyl esters of retinal fatty acids (mainly C_{22} and C_{24} fatty acids with 4 to 6 double bonds) were resolved on a 25 cm RP C_{18} (5-μm particle) column [143]. The "molar absorptivities" were measured for a series of the phenacyl esters of these acids, as well as for the common C_{14} to C_{18} saturated and unsaturated acids, and reported as peak area units/nmol. The results show an approximately three-fold variation in these values. If these data are directly proportional to the actual molar absorptivities, the variations are unexpected and certainly merit further investigation.

Important additional applications of phenacyl esters include the determination of the cellular

fatty acids of microorganisms [144] and of amniotic fluid [145].

The *p*-bromophenacyl esters have also seen wide application. Their absorption maximum occurs at 257 nm in methanol and the molar absorptivity is 18 700 l mol^{-1} cm^{-1} at 254 nm [132]. The higher molar absorptivity and the close proximity of the absorbance maximum to 254 nm make these derivatives almost ideally suited for analysis with a fixed wavelength detector. The molar absorptivity was found to be independent of the fatty acid moiety. Separations of relatively simple mixtures were shown on a C_9 column in both the normal and reversed-phase modes.

More complex mixtures, including saturated as well as unsaturated positional and configurational isomers were separated on a C_{18} column with a water–methanol eluent, with detection limits in the nanogram range [146]. The oleic/eladic and linoleic/lineladic acid pairs were well resolved. Roggero and Coen [129] reported that even though an acetonitrile–water eluent yielded higher capacity ratios, methanol was required to separate the 14:0 and 16:1 and the 16:0 and 18:1 critical pairs. They also formulated five rules for the elution sequence of these esters. Particularly interesting were the results of Takayama et al. [127] who synthesized a C_{30} RP and demonstrated that it separated all pairs that had not been resolved on a C_{18} RP [134], while leaving other (different) pairs unseparated. Thus, the C_{30} phase complemented the C_{18} RP in the separation of fatty acids.

Nearly baseline separation of 12 of the physiologically most abundant fatty acids (C_{12}–C_{22}) was attained on a C_6 RP (3-μm particles) in less than 20 min, with a detection limit of 2 pmol [147]. It was used for the routine determination of FFAs in human serum (Fig. 2). Other applications of *p*-bromophenacyl esters included samples such as mammalian cells and tissues [131] and human uterine decidua vera or human endometrial stromal cells [148], and butter [149]. More recently, a micellar phase transfer method was reported for the determination of fatty acids as the *p*-bromophenacyl esters [150].

Naphthacyl esters were studied with the objective of augmenting the detection sensitivity.

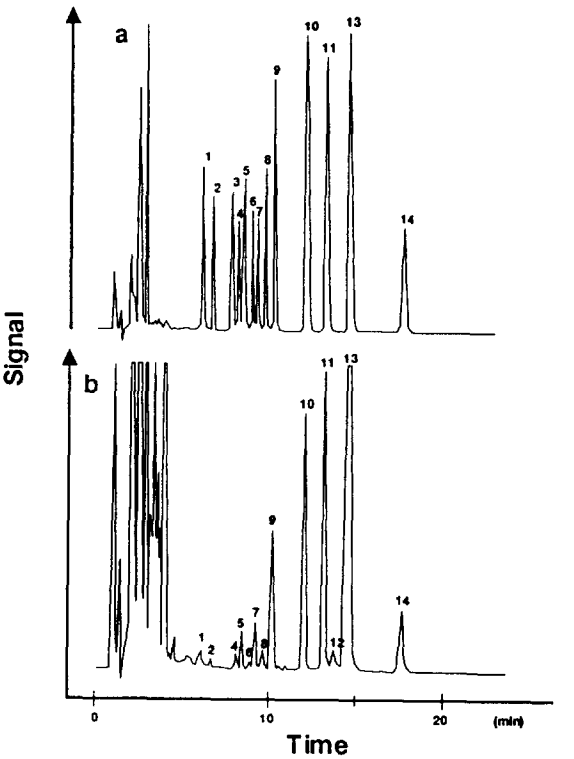

Fig. 2. HPLC of the *p*-bromophenacyl derivatives of (a) a standard fatty acid mixture and (b) FFA from human serum (0.64 mmol/l). Analysis was performed on a Spherisorb C_6 column (250 × 4 mm), packed with 3-μm particles, used with a CN-5μm guard column at a temperature of 30°C. The mobile phase was acetonitrile–water (77:23, v/v), the flow-rate 1.3 ml/min. Absorbance was monitored at 254 nm. Peaks: 1 = lauric (12:0); 2 = myristoleic (14:1ω6); 3 = eicosapentaenoic (20:5ω3); 4 = linolenic (18:3ω3); 5 = myristic (14:0); 6 = docosahexaenoic (22:6ω3); 7 = palmitoleic (16:1ω7); 8 = arachidonic (20:4ω6); 9 = linoleic (18:2ω6); 10 = palmitic (16:0); 11 = oleic (*cis*-18:1ω9); 12 = elaidic (*trans*-18:1ω9); 13 = margaric (17:0, internal standard); and 14 = stearic acid (18:0). In x:$y\omega z$, x = total number of carbon atoms, y = number of ethylenic bonds, ωz = number of carbons from the center of the ethylenic bond furthest removed from the carboxyl group up to and including the terminal methyl group. From Ref. [147] with permission.

Their molar absorptivity was reported to be about $3 \cdot 10^4$ l mol^{-1} cm^{-1} at 247–248 nm [151]. Jordi [134] reported that they gave a large increase in sensitivity compared to several other derivatives studied (*p*-Cl, *p*-Br, *p*-NO_2), permitting 2 pg to be detected. However, others [139] preferred the phenacyl esters for routine applications, such as the analysis of various seed

oils, because of their higher solubility and better resolution.

p-Methoxyphenacyl esters were employed in the analysis of a wide range of bacterial fatty acids [152–154] containing hydroxyl groups and cyclopropane rings.

Substituted hydrazines have been used to derivatize fatty acids. The most important of these have been the 2-nitrophenylhydrazines which were employed by Miwa and co-workers [155–157] in the determination of FFA in serum. Good resolution was observed and these derivatives can be monitored at 230 nm with 100–200 fmol detection limits or at 400 nm with about a four-fold lower sensitivity but also less interference (Fig. 3). A 25-μl serum sample could be analyzed in 15 min. A simplified procedure was described [158] for the direct conversion of saponified platelet phospholipids to the corresponding fatty acid hydrazides without prior isolation.

p-Methoxyanilide derivatives have a high molar absorptivity ($2.43 \cdot 10^4$ l mol^{-1} cm^{-1} at 254 nm) but several FA pairs were poorly resolved and derivatization required well over an hour [159]. Other derivatives that have been reported are nitrobenzyl esters [160–162].

Triacylglycerides can also be converted, directly and rapidly (1 min reaction time at room temperature), into their hydroxamic acids and monitored with low wavelength UV detection (molar absorptivity 2450 l mol^{-1} cm^{-1} at 206 nm). It is essential, however, that the RP packing be based on iron-free silica [163].

3.4.2. Fluorescence

Compared to UV detection, fluorometric detection via suitable derivatives of fatty acids amplifies the sensitivity by one to two orders of magnitude. The selectivity is also greatly enhanced but the resolution is generally diminished somewhat. Most of the proposed derivatives are based either on anthracene or an other polynuclear aromatic system, or on coumarin. Fluorescence detection in HPLC has been reviewed [164].

Fluorescent derivatives have found broad application in the biomedical field, particularly for the determination of FFAs in blood samples. Their eminent suitability stems from their very high sensitivity that enables minute sample vol-

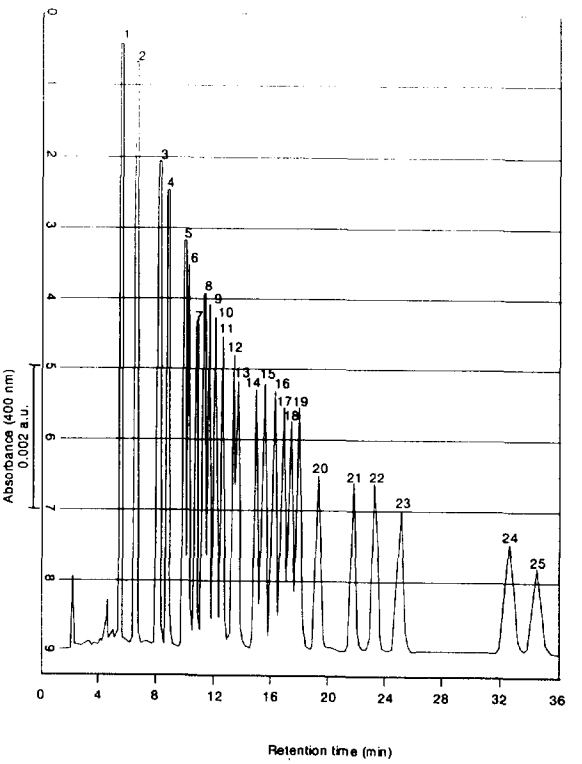

Fig. 3. Chromatogram of the 2-nitrophenylhydrazides of a standard mixture of 25 fatty acids obtained with visible detection. HPLC analysis was carried out isocratically with acetonitrile–methanol–water (75:11:14, v/v/v) as the eluent at a flow-rate of 1.2 ml/min on a YMC–FA (C_8) column (250 mm × 6 mm I.D.) packed with 5 μm octyl-bonded silica. Peaks: 1 = octanoic acid ($C_{8:0}$); 2 = decanoic ($C_{10:0}$); 3 = dodecanoic ($C_{12:0}$); 4 = *cis*-9-tetradecenoic ($C_{14:1}$); 5 = *cis*-5,8,11,14,17-eicosapentaenoic ($C_{20:5}$); 6 = *cis*-9,12,15-octadecatrienoic ($C_{18:3}$); 7 = tetradecanoic ($C_{14:0}$); 8 = *cis*-4,7,10,13,16,19-docosahexaenoic ($C_{22:6}$); 9 = *cis*-9-hexadecenoic ($C_{16:1}$); 10 = *cis*-5,8,11,14-eicosatetraenoic ($C_{20:0}$); 11 = *cis*-9,12-octadecadienoic ($C_{18:2,cis,cis}$); 12 = *trans*-9,12-octadecadienoic ($C_{18:0,trans,trans}$); 13 = *cis*-8,11,14-eicosatrienoic ($C_{20:3}$); 14 = hexadecanoic ($C_{16:0}$); 15 = *cis*-7,10,13,16-docosatetraenoic ($C_{22:4}$); 16 = *cis*-9-octadecenoic ($C_{18:1,cis}$); 17 = *trans*-9-octadecenoic ($C_{18:1,trans}$); 18 = *cis*-11,14-eicosadienoic ($C_{20:2}$); 19 = heptadecanoic ($C_{17:0}$) (I.S.); 20 = *cis*-13,16,19-docosatrienoic ($C_{20:3}$); 21 = octadecanoic ($C_{18:0}$); 22 = *cis*-11-eicosaenoic ($C_{20:1}$); 23 = *cis*-13,16-docosadienoic ($C_{22:2}$); 24 = eicosanoic ($C_{20:0}$); 25 = *cis*-13-docosaenoic ($C_{22:1}$) acid hydrazide. Each peak corresponds to 150 pmol. From Ref. [158] with permission.

umes of low FFA concentrations to be analyzed by simple derivatization procedures without prior extraction, and their resolution that is satisfactory for the levels of compositional complexity encountered in plasma samples.

9-Diazomethylanthracene was the first reagent proposed for the formation of anthrylmethyl esters of fatty acids by reaction in an inert solvent [165,166]. Excitation was performed at 360 nm and the emission was measured at 440 nm. The detection limit of 15 pg/μl was ten-fold lower than that determined by UV absorption of the same derivative at 254 nm. The use of 9-(chloromethyl)anthracene was advocated [167] as an alternative because the reaction proceeds under milder conditions, similar to those employed for the preparation of the phenacyl esters. 9-(Hydroxymethyl)anthracene [168,169] can also be utilized in conjunction with a substituted pyridinium iodide coupling agent to produce these esters. Because of their high sensitivity, the anthrylmethyl esters have been extensively used for the determination of free fatty acids in sera and plasma, and related samples [170–174]. In a comparison of such a fluorometric RP–HPLC method and a GC method for the analysis of the FFAs commonly found in plasma, Baty et al. [169] concluded that GC was able to somewhat better separate the fatty acids of interest, even though the precision and sensitivity of both methods were similar.

The substituted reagent, panacyl bromide (*p*-9-anthroyloxy)phenacyl bromide, has been suggested as a relatively more selective labeling agent for carboxyl groups [175] than the other commonly used derivatives.

Fatty acids have also been derivatized as the 9-aminophenanthryl esters [176], with excitation and emission wavelengths at 303 and 376 nm, respectively. They were also used in the analysis of human serum with detection limits of 10–15 pmol.

N-(9-acridinyl)-bromoacetamide presents distinct advantages over other fluorophoric reagents not only in terms of sensitivity but also because the acridinyl substituent, when protonated in an aqueous acetonitrile–0.2% phosphoric acid eluent, does not contribute substantially to the overall retention on an RP column. The capacity factors are determined primarily by the structure of the analyte. The detection limit was about 10 fmol [177]. A similar reagent, 9-bromomethylacridine, was used with micellar phase-transfer catalysis for the automated determination of FFAs [178].

One of the most sensitive reagent for FFAs is 3-bromomethyl-6,7-dimethoxy-1-methyl-2(H)-quinoxalinone [179–181], which has detection limits of 0.3–1 fmol and gives good resolution on account of its relatively small size. The 3-propionylcarboxylic acid hydrazide of this reagent was found to be more stable and to enable derivatization of FFAs in an aqueous medium, without prior extraction. FFAs could be determined in 5 μl of serum with a detection limit of 2.5–5 fmol [182,183].

Another of the newer sensitive and specific reagents is 2-(2,3-naphthalimido)ethyl trifluoromethanesulfonate [184]. The derivatization reaction required only 10 min at room temperature and the detection limit was 4 fmol; this compares with 100 fmol for UV detection. It was applied to the determination of fatty acids in mouse brain. In an attempt to gain even greater sensitivity, the anthracene analog was synthesized and tested [185]. Its detection limit of 0.8–2.7 fmol places it among the most sensitive reagents reported. Fig. 4 shows a chromatogram of a comprehensive mixture of fatty acids derivatized with this reagent.

Some other reagents include dansylsemipiperazide [186], monodansyl cadeverine [187], and various 4-substituted-7-aminoalkylamino-2,1,3-benzoxadiazoles [188]. The latter yielded detection limits of about 4 fmol.

Alternatively, various substituted coumarins have been suggested for the derivatization of fatty acids. Their preparation is similar to that of the phenacyl esters, and their smaller size than that of the polynuclear aromatics affords somewhat higher resolution. The most extensively used of these has been 4-bromomethyl-7-methoxycoumarin, which was introduced by Dünges [189]. A detection limit of 7 pmol has been reported, with excitation at 328 nm and emission at 380 nm [190]. McGuffin and Zare

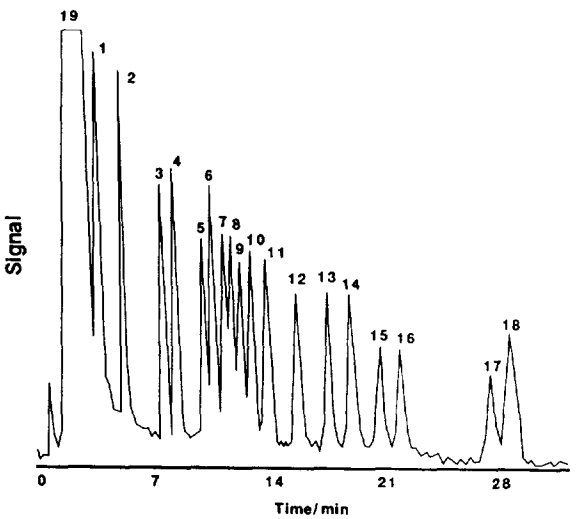

Fig. 4. Chromatogram of 2-(2,3-anthracenedicarboximido)ethyl fatty acid derivatives. HPLC separation was performed on a Develosil-ODS-K3 column with methanol–acetonitrile–water (135:45:20, v/v/v) as eluent at a flow-rate of 0.8 ml min^{-1}. The column temperature was 55°C. The fluorescence intensity was monitored at 456 nm (excitation at 298 nm). Peaks: 1 = AE caprylate (23 fmol); 2 = caprate (31 fmol); 3 = laurate (26 fmol); 4 = myristoleate (30 fmol); 5 = *cis*-5,8,11,14,17-eicosapentaenoate (24 fmol); 6 = linolenate (31 fmol); 7 = myristate (26 fmol); 8 = *cis*-4,7,10,13,16,19-docosahexaenoate (27 fmol); 9 = palmitoleate (25 fmol); 10 = arachidonate (27 fmol); 11 = linoleate (27 fmol); 12 = *cis*-8,11,14-eicosatrienoate (26 fmol); 13 = palmitate (27 fmol); 14 = oleate (29 fmol); 15 = *cis*-eicosadienoate (21 fmol); 16 = heptadecanoate (22 fmol); 17 = stearate (22 fmol); 18 = gondoate (33 fmol). Peak 19 is from the reagent. From Ref. [195] with permission.

[191] used this derivative in conjunction with laser-induced fluorescence and a microcolumn (0.20 mm I.D., 1.2 m long) to demonstrate a high-resolution system for the trace analysis of FFAs. Limitations include analysis time and the need for custom preparation of the column. More recently [192], a similar system that generated 275 000 theoretical plates and exhibited excellent baseline stability, was used to separate 14 standard fatty acids at the 5–10 fmol level (Fig. 5) and was applied to the analysis of fish oils.

The acetoxycoumarins [193] behave similarly but exhibit somewhat higher sensitivities than their methoxy analogs; they, too, have been used for the analysis of sera and plasma samples [194,195]. Other recently reported coumarin reagents include 4-bromomethyl-6,7-methylenedioxycoumarin [196] and its acetoxy analog [197]. The reaction of the latter could be catalyzed via an anion exchange resin (in lieu of 18-crown-6 ether and $KHCO_3$) and was complete at room temperature in 20 min [198].

The determination of monohydroxy fatty acids by fluorescence labeling of the hydroxy group with 1-anthroylnitrile was reported by Metori et al. [199]. The method was applied to natural tissue samples and was sensitive down to the pmol level.

3.4.3. Other methods

The C_{12}–C_{18} FFA have been separated by RP ion-pair HPLC with conductivity detection [200] on an ODS column with a methanol–5 m*M* tetrabutylammonium eluent. The detection limit was 2 ng of margaric acid.

Also noteworthy is the separation of FA hydroperoxides by HPLC with post-column luminescence detection [201,202]. The separation of hydroperoxides has been reviewed [203].

4. Other separation methods

Supercritical fluid chromatography (SFC) in many respects is a hybrid between GC and HPLC, especially in terms of the ability to use both capillary GC and packed HPLC columns and FID and UV detectors. Carbon dioxide, alone or with modifiers, is usually used as the mobile phase. SFC has been used in lipid analysis, but only to a limited extent for fatty acids and their derivatives. The subject has been reviewed recently [204].

FAMEs and FFA have been separated by SFC on capillary [205–210] and packed columns, in both reversed-phase [211,212] and normal-phase [213–216] modes. The separation of the saturated C_{12}–C_{20} acids on a cyano phase was greatly

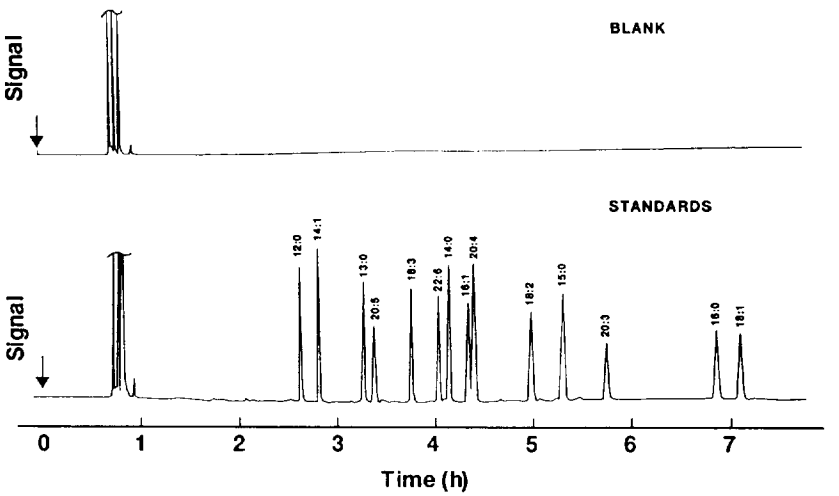

Fig. 5. High-efficiency HPLC separation of 14 standard fatty acid derivatives at the 5–10 fmol level (bottom) and blank (top). Column 152 cm × 200 μm I.D. fused-silica capillary, 3-μm Micro-Pak SP-18 stationary phase, (240 000 plates). Mobile phase: methanol–acetonitrile–water (90:2:8), 0.75 μl/min, 30°C. Solutes: fatty acids as indicated. Detector: laser-induced fluorescence, 325 nm excitation wavelength, 420 nm emission wavelength. From Ref. [192] with permission.

improved by the addition of water to the CO_2 mobile phase [211].

A noteworthy application of SFC was the separation of thermally labile hydroperoxides [217], with prior extraction of the sample with the mobile phase (supercritical CO_2-ethanol). Other applications have been made to samples of fish [213,215], fungal [212], and vegetable oils [206,209,213] and to butter [206]. The evaporative light scattering detector can be used with SFC [214]. Fatty acid esters and triacylglycerols were separated from the same sample [210]. These separations demonstrate the feasibility of the SFC separation of fatty acids and their esters, but the resolution obtainable with capillary GC is so superior at present that SFC cannot compete effectively with it for the determination of fatty acid profiles.

Capillary zone electrophoresis (CZE) has been used to obtain partial fatty acid profiles of butter and palm oil [218]. It should be particularly useful for the rapid and convenient determination the short-chain fatty acids in such samples.

Although TLC is still applied very extensively for preliminary separations and estimations of the FA present, the instrumental methods are generally used to obtain the quantitative profiles.

5. Conclusions

The characterization of lipids via their fatty acid profiles is a widely accepted practice, particularly in the biomedical field. Capillary gas chromatography is currently the most frequently used approach, but other techniques are receiving increased attention. The primary objectives of developing new methods or improving established ones are enhanced speed, sensitivity, and simplicity of analysis. Speed can be gained by more facile sample preparation, perhaps in conjunction with microwave irradiation instead of conventional heating, or by optimizing programmable separation variables (via computer-controlled instruments). The trend toward attaining greater sensitivity is reflected most clearly in the proliferation of new or modified established fluorogenic reagents. Direct methods with simpler or no lengthy isolation steps are also being

emphasized. The amenability of a method to automation has also been an important consideration. Recent advances mirror these trends, which are likely to continue into the near future.

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