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Abstract

The analysis of food lipids presents significant challenges due to the wide variety of sample matrices, large range of total fat contents, and complex compositions of fatty acids. This chapter reviews conventional analytical techniques for the quantification of total fat and fatty acids in foods and food ingredients, including the gravimetric determination of total fat, the calculation of fat and fatty acids using gas chromatography (GC), and the analysis of proximates content (i.e., fat, protein, carbohydrate, moisture, and ash) by Fourier transform infrared (FTIR) spectroscopy. Current official methods of analysis are evaluated and the use of certified reference materials and spike-recovery experiments for verifying method performance is discussed. Recent advances in automated and semi-automated sample preparation systems and rapid and portable spectroscopic devices are highlighted for their potential to significantly improve the speed by which accurate determinations of total fat and fatty acids may be achieved.

***Keywords*:** Fat, fatty acid, lipid analysis, official method of analysis, portable analyzers, standard reference material

3.1 Introduction

Lipids are a diverse class of compounds that contribute to the organoleptic, physiochemical, and nutritional aspects of foods and food ingredients. Food lipids provide a major source of energy in the diet. They also contribute

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essential fatty acids and nutrients and serve as carriers for lipid-soluble vitamins. Food lipids are broadly divided into categories of fats and oils based on origin of the lipid substance and its physical state at room temperature. Fats are animal-based solids, such as lard and tallow, which remain solid at room temperature due to their high concentration of saturated fatty acids that allow for the close packing of triglycerides. Several vegetable-based oils, including palm and coconut oils and partially and fully hydrogenated oils, contain high concentrations of saturated and/or *trans* fatty acids that also produce a solid lipid matrix at room temperature. Most vegetable and seed oils, however, are found as liquids at room temperature owing to their high concentrations of monounsaturated and polyunsaturated fatty acids. From the consumer's perspective, information related to differences in the content and composition of total fat and fatty acids in foods and food ingredients is important for making educated, healthful food choices.

The Nutrition Labeling and Education Act (NLEA) of 1990 amended the Federal Food, Drug and Cosmetic Act (FD&C Act) to require mandatory nutrition labeling for packaged foods regulated by the FDA and the US Department of Agriculture (USDA) [1, 2]. The NLEA also gave the FDA authority to regulate health claims on food labels and in food labeling [3]. Under provisions of the NLEA, declarations for the content of total fat are to be expressed in triacylglycerol (TAG) equivalents, whereas those for saturated fat are expressed as free fatty acid equivalents [1, 2]. The contents of *cis*-monounsaturated and *cis*-polyunsaturated fatty acids are also permitted as voluntary declarations on product labels, except under certain conditions when a claim about fatty acids or cholesterol is made on the label or in the labeling of a food [1, 2]. More recently, the content of total *trans* fatty acids was added to the nutrition label of conventional foods and dietary supplements [1, 4]. FDA compliance programs help to ensure that the labels of foods and dietary supplements available in the US market contain accurate declarations of product composition and that they are truthful and not misleading.

A wide range of analytical techniques are currently available for the analysis of total fat and fatty acids in foods and food ingredients. Conventional analytical methods for the determination of total fat include the gravimetric determination of solvent-extracted lipids and the calculation of total fat based on the analyzed content of individual fatty acids in a test sample. Fourier transform infrared (FTIR) spectroscopic procedures are also available for the determination of total fat and other proximates (i.e., ash, protein, moisture, carbohydrate) in food commodities. FDA regulations do not specify particular methods of analysis, but the Agency accepts those that yield accurate results with satisfactory precision and are considered

appropriate for the analysis of specified nutrients and other food components. These methods, collectively referred to as official methods of analysis, are rigorously evaluated for method performance and validated in national or international collaborative studies by method-endorsing organizations such as AOAC INTERNATIONAL, the American Oil Chemists' Society (AOCS), and the International Organization for Standardization (ISO). Such methods are routinely applied to the analysis of foods and food ingredients in FDA field laboratories and independent contract laboratories that provide data for manufacturers for nutrition labeling purposes. FDA labeling regulations indicate that manufacturers may use any analytical method for determining nutrient contents, including the use of historical or nutrient database data. However, for compliance purposes, a product and its label are subject to analytical methods the agency considers appropriate (i.e., official methods) for verifying nutrient contents.

This chapter reviews conventional analytical techniques for the quantification of total fat and fatty acids in foods and food ingredients, as stated above. A schematic overview of these techniques is presented in Figure 3.1. Current official methods of analysis are presented, and the importance of method validation procedures, such as collaborative study testing and the use of certified reference materials and spike-recovery experiments, is critically discussed. Recent advances in the application of automated and semi-automated sample preparation systems and rapid

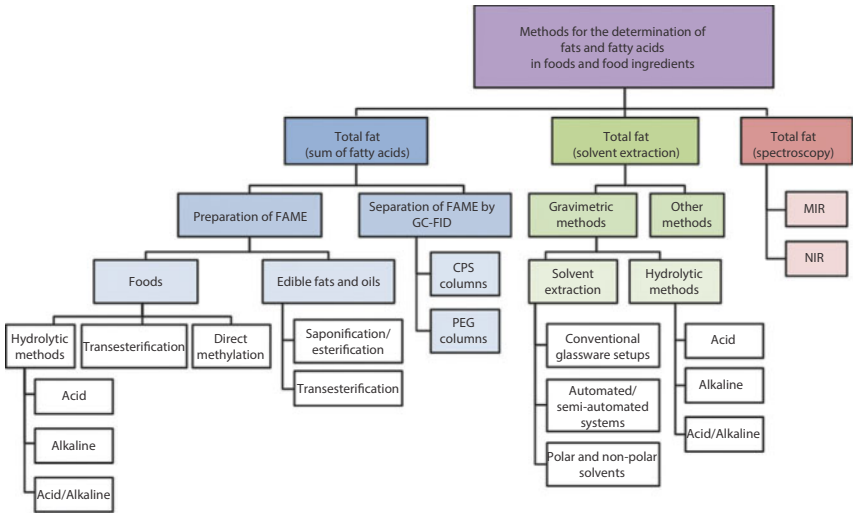

Figure 3.1 Schematic overview of conventional analytical methods for the determination of total fat and fatty acids in foods and food ingredients.

and portable spectroscopic devices are highlighted for their potential to significantly improve the speed by which accurate determinations of total fat and fatty acids may be achieved.

3.2 Official Methods for the Analysis of Fat in Foods

3.2.1 Importance of Official Methods of Analysis

Official methods of analysis are those which have been systematically evaluated and subsequently approved by a method-endorsing organization for routine use in regulatory and contract laboratories, among others. These methods are characterized by their scope, intended use, and applicable sample matrices. The approval of new official methods often includes the successful completion of a multi-laboratory validation (MLV) study to further investigate the performance of a candidate method. MLV studies are designed to test the clarity of the written protocol and the interlaboratory precision (repeatability and reproducibility), taking into account variability introduced from different analysts, laboratory environments, and analytical instruments [5]. These studies require a significant commitment of time and resources by study leaders and participants. As such, only qualified methods which have been previously investigated at the level of a single laboratory validation study are considered as appropriate candidate methods for an MLV study. These methods are defined for their performance specifications with regard to accuracy, precision, sensitivity, linearity, limits of detection and quantification, and robustness/ruggedness for determining one or more analytes in a specified matrix or matrices [5, 6]. Minimum criteria for successful completion of a quantitative MLV study include the analysis of five test materials, participation from a minimum of eight laboratories reporting valid data for each of the test materials, and the measurement of a minimum of one or two replicate samples, provided as blind replicates or split levels (Youden pairs) [5]. Acceptable ranges of analyte concentration are determined from the precision data, expressed as the reproducibility relative standard deviations (RSD_R) and/or the Horwitz ratio (Horrat) [7].

3.2.2 Official Methods for the Gravimetric Determination of Total Fat

The gravimetric approach provides a crude estimation of total fat content based on the mass of lipid extracted from a test sample. This approach tends to underestimate the caloric content of total fat by including in the

gravimetric determination the mass of non-fatty acid constituents, namely lipid-soluble vitamins, unsaponifiable material, and certain nonfat macromolecules that are also extracted [8]. The gravimetric determination of total fat content may be achieved by extraction of total lipids with nonpolar solvent under reflux conditions or by use of a combination of nonpolar and polar organic solvents to overcome interactions between lipids and the sample matrix, such as chloroform and methanol, which are recommended in the method of Bligh and Dyer [9]. Alternatively, the test sample may be subjected to a two-step hydrolytic procedure in which the matrix is first hydrolyzed and then extracted for total lipids with nonpolar solvent. Both approaches have been approved as official methods of analysis. A wide range of methods are now available for the crude determination of total fat content in foods and food ingredients. These protocols, which are listed in Table 3.1 (solvent extraction) and Table 3.2 (hydrolytic procedures), vary in applicable matrix and sample preparation procedure.

3.2.2.1 Solvent Extraction Procedures

Solvent extraction methods, such as the conventional Soxhlet procedure [10], involve the semi-continuous washing, or percolation, of dried and homogenized samples with organic solvent under reflux conditions using specific glassware. Ether (ethyl and/or petroleum) and hexanes are common solvents although they are reportedly inefficient for extracting polar lipids [11]. Solvent extraction methods tend to be straightforward and require minimal specialized training [10]. In addition, the organic solvent used to extract the test material requires no filtration prior to evaporation. However, these methods require the use of large volumes of organic solvent, which are costly to dispose of and hazardous to the environment. AOAC Official Method 948.22 [12] describes a solvent extraction procedure for the determination of crude total fat in nuts and nut products. Samples are extracted with ether in a Soxhlet-type extractor for 16 hours, then the lipid extract is evaporated to dryness at 95–100 °C and weighed. The Randall/Soxtec modification of the Soxhlet solvent extraction procedure, as recommended in AOAC 2003.05 and 2003.06 [13, 14], allows for a shorter duration extraction because the test portion is submerged in boiling solvent.

Many instrument manufacturers now offer automated or semi-automated systems, such as the Soxtec systems from FOSS (Hillerød, Denmark), the CEM Discover SP-X (Matthews, NC, USA), and the Buchi B-811 Extraction System (New Castle, DE, USA), for the extraction of food lipids with organic solvent. These instruments offer numerous advantages over

Table 3.1 Solvent extraction (SE) official methods for the gravimetric determination of crude total fat in foods and food ingredients.

Method	Applicable matrices	SE protocol	Organic solvent
920.39 [102] (also referred to as AOAC 945.38F)	Animal feed ingredients and mixed feeds	Soxhlet	Anhydrous EE
938.06 [103]	Butter	SE, indirect method	EE or PE
948.22 [12]	Nuts and nut products	Soxhlet	EE
960.39 [104]	Meat	Soxhlet	Anhydrous EE or PE
985.15 [15]	Meat and poultry products	Automated SE with microwave moisture analyzer	Methylene chloride
991.36 [105]	Meat and meat food products	Randall/Soxtec	PE
2003.05 [13]	Animal feeds, forages, and cereal grains	Automated/Semi-automated SE (Randall/Soxtec)	Anhydrous EE
2003.06 [14]	Animal feeds, forages, and cereal grains	Automated/Semi-automated SE (Randall/Soxtec)	Hexanes
AOCS Am 5-04 [16]	Oilseeds, meats, feeds, and foods	Automated/Semi-automated SE	PE

Abbreviations: EE, ethyl ether; PE, petroleum ether; SE, solvent extraction.

conventional glassware setups, including accelerated extraction durations and higher extraction efficiencies. Automated systems have also found their way into the official methods of analysis. AOAC Official Method 985.15 [15] describes a procedure for the determination of crude total fat in meat and poultry products. With this method, homogenized test samples are dried using a microwave moisture analyzer, extracted with methylene chloride in an automated solvent extractor, and then returned to the microwave moisture analyzer for drying, removal of residual solvent, and weighing. AOCS Official Method Am 5-04 [16] was approved for the rapid determination

Table 3.2 AOAC official methods for the hydrolytic determination of crude total fat in foods and food ingredients.

Method	Applicable matrices	Hydrolytic protocol	Organic solvent
983.23 [19]	Composite foods	Enzymatic digestion	Chloroform-methanol
922.06 [23]	Flour	Acid	EE/PE
925.12 [24]	Macaroni products	Acid	EE/PE
925.32 [26]	Eggs	Acid	EE/PE
935.38 [25]	Bread	Acid	EE/PE
945.44 [106]	Fig bars and raisin-filled crackers	Acid	EE/PE
948.15 [27]	Seafood	Acid	EE/PE
948.16 [107]	Fish meal	Acid	Acetone
950.54 [108]	Food dressings	Acid	PE
954.02 [109]	Pet food	Acid	EE
963.15 [110]	Cacao products	Acid	PE
920.111 [29]	Cream	Alkaline	EE/PE
920.115 [30]	Sweetened condensed milk	Alkaline	EE/PE
922.09 [31]	Malted milk	Alkaline	EE/PE
932.06 [32]	Milk powder	Alkaline	EE/PE
933.05 [33]	Cheese	Acid and alkaline	EE/PE
945.48 [34]	Evaporated milk (unsweetened)	Alkaline	EE/PE
952.06 [35]	Ice cream and frozen desserts	Alkaline	EE/PE
974.09 [36]	Whey cheese	Alkaline	EE/PE
986.25 [37]	Milk-based infant formula	Alkaline	EE/PE
989.05 [28]	Milk	Alkaline	EE/PE
995.19 [111]	Cream	Alkaline	EE/PE

Abbreviations: EE, ethyl ether; PE, petroleum ether.

of total fat content in oilseeds, meats, feeds, and foods. This method recommends the use of an automated or semi-automated extraction system, such as the XT10 and XT15 extractors (Ankom Technology, Macedon, NY, USA), with filter bag technology designed to reduce errors due to sample loss. The determination of crude total fat content using AOAC 985.15 [15] or AOCS Am 5-04 [16] is considered an indirect method of analysis in that quantification is based on the difference in sample weight before and after extraction [17]. In contrast, with direct methods the weight of the extracted lipid is measured directly and the content of total fat is calculated as the mass of the extracted lipid taken as a proportion of the test portion weight.

3.2.2.2 Hydrolytic Procedures

As an alternative to the solvent extraction methods, hydrolytic procedures involve a two-step process by which the sample is first treated with acid and/or alkaline reagents or an enzyme in order to breakdown the matrix prior to extraction with solvent. Hydrolytic procedures enable the disruption of lipid-carbohydrate bonds, proteins, polysaccharides, and plant cell walls. Such sample pretreatment is particularly necessary for dairy products in order to facilitate extraction of neutral lipids contained within the milk fat globule membranes [18]. A complete digestion or hydrolysis of the test material enables the extraction solvent to come in contact with all lipids contained within the test material. Thus, an exhaustive and quantitative extraction of total lipids is expected.

An enzymatic hydrolytic procedure for the determination of fat in foods is decribed in AOAC Official Method 983.23 [19]. It was developed by Daugherty and Lento [20] as a modification of the Bligh and Dyer method [9] and is applicable to the analysis of composite food samples. AOAC 983.23 [19] involves the enzymatic digestion of food samples using 1% amylase enzyme in 0.5 M sodium acetate solution, placed in a shaking water bath set at 45–50 °C for 60 min. Total lipids are then extracted by addition of chloroform, methanol, and water to cause separation of the aqueous and organic phases. The chloroform layer is transferred to a tared 100-mL beaker and evaporated to dryness. A modification of AOAC 983.23 by Phillips *et al.* [21] was proposed to simplify the established standard procedure and eliminate the requirement for enzymatic digestion. The authors found that the simplified method was less labor-intensive and permitted a higher rate of sample throughput than the standard procedure [21]. More recently, Phillips *et al.* [22] extended the application of their

simplified method to the quantitative determination of total fat in a variety of complex food matrices, including those in which the fat component was a constituent of low-fat foods (e.g., baked goods, salad dressing, and snack foods) and those in which the matrix was finely ground or homogenized (e.g., baby food, finely ground nuts and seeds, peanut butter, flour, and lyophilized oyster tissue).

A procedure for the determination of total fat in flour by acid hydrolysis is described in AOAC Official Method 922.06 [23]. Test samples are placed with ethanol and 8 M HCl in a 50-mL beaker and heated at 70–80 °C for 30–40 min with stirring. After cooling, more ethanol is added and the sample transferred to a Mojonnier fat-extraction apparatus for repeated extraction of total lipids with ethyl and petroleum ethers. The ether extracts are then combined and evaporated on a steam bath. The lipid residue is dried in a 100 °C oven to constant weight. Modifications of AOAC 922.06 [23] are available as AOAC official methods for the analysis of macaroni products [24], bread [25], eggs [26], and seafood [27] (Table 3.2).

A procedure for the determination of total fat in milk is described in AOAC Official Method 989.05 [28]. Test samples are weighed and mixed with concentrated ammonium hydroxide solution in order to neutralize any acid present, precipitate protein, and disrupt lipid-protein bonds of the milk fat globule membrane [18]. Three sequential extractions with ethanol and ethyl/petroleum ethers are performed. The ether extracts are decanted into weighing dishes and evaporated in a chemical fume hood on a hot plate set at 100 °C. The lipid residue is then dried to constant weight and cooled in a desiccator overnight. This procedure, commonly referred to as the Roese-Gottlieb method, has been adapted and approved as an AOAC official method for the analysis of cream [29], sweetened condensed milk [30], malted milk [31], milk powder [32], cheese [33], evaporated milk [34], ice cream and frozen desserts [35], whey cheese [36], and milk-based infant formula [37] (Table 3.2). Versions of the Roese-Gottlieb procedure are also available as ISO/IDF (International Dairy Federation) methods, including those specific for cream [38], evaporated milk [39], and milk-based infant foods [40], among others.

Several alternative methods have also been approved for the analysis of fat in milk, including the Babcock method (AOAC 989.04 [41]), the Gerber method (AOAC 2000.18 [42]), the rapid detergent method (AOAC 960.26 [43]), the automated turbidimetric methods (AOAC 969.16 [44] and 973.22 [45]), and the mid-infrared spectroscopic method (AOAC 972.16 [46]). These and other methods for the analysis of milk fat have been discussed elsewhere [47–50].

3.2.3 Official Methods for the Determination of Total Fat by GC

Interest in the content and composition of fatty acids in foods and food ingredients prompted the development and validation of novel analytical methods for the quantification of fatty acids by GC with flame ionization detection (FID) [51, 52]. As such, analytical methods for the gravimetric determination of total fat were modified to include protocols for the preparation and separation of fatty acid methyl esters (FAME) by GC-FID. The novel GC methods were found to yield comparable determinations of total fat content to those achieved using conventional gravimetric methods [52, 53].

The determination of total fat by summation of individual fatty acids allows for the accurate quantification of the nutritional content of total fat in a test sample. With this approach, fatty acids are derivatized to FAME and quantified by GC-FID. Preparation of other derivatives may be useful in specific applications, such as the use of benzyl esters for quantitative recovery of short-chain fatty acids from milk fat [54]. An internal standard is added during sample preparation to facilitate calculation of FAME on a mg/g basis. Conversion factors are applied to express the analyzed contents of FAME as TAG or free fatty acid equivalents for nutrition labeling purposes [55]. Several sample preparation procedures are available, including the two-step protocol involving total fat extraction followed by preparation of FAME, the direct transesterification procedure for analysis of liquid and reconstituted dairy products and infant formulas, and the direct methylation approach involving the *in-situ* digestion of test materials followed by derivatization. FAME derived from edible fats and oils may be prepared by transesterification or by using consecutive saponification and esterification reactions. Such approaches have been approved as official methods of analysis (Table 3.3).

3.2.3.1 Sample Preparation Procedures

A procedure for the determination of fat (total, saturated, and *cis*-unsaturated) in cereal products containing 0.5–13% total fat is decribed in AOAC Official Method 996.01 [56]. Test samples are heated in glass extraction tubes with ethanol, 8 M HCl, and C13:0 TAG internal standard solution (5 mg/mL in chloroform) in a shaking water bath set at 80 °C for 40 min. Samples are subsequently cooled to room temperature and transferred with ethanol to Monjonnier fat extraction flasks for liquid-liquid extraction with ethyl and petroleum ethers. The aqueous phase is re-extracted twice with mixed ether. The ether extracts are then combined

Table 3.3 Official methods for the preparation of FAME from foods and food ingredients.

Method	Applicable sample matrices	Chemical reaction	Methylation catalyst	Internal standard
AOAC 969.33 [66]	Animal and vegetable fats and oils	Saponification/esterification	BF_3	Not specified
	Fatty acids	Esterification	BF_3	
AOAC 996.01 [56]	Cereal products containing 0.5–13% total fat	Acid hydrolysis/esterification	$NaOH/BF_3$	C13:0 TAG
AOAC 996.06 [58]	Foods excluding dairy products and cheese	Acid hydrolysis/esterification	BF_3	C11:0 TAG
	Dairy products	Alkaline hydrolysis/ esterification		
	Cheese	Acid and alkaline hydrolysis/ esterification		
AOAC 2012.13 [60]	Milk products, infant formula, adult/ pediatric nutritional formula	Transesterification	Sodium methoxide	C11:0 FAME/ C13:0 TAG
AOCS Ce 2b-11 [62]	Most fat-containing matrices that do not require acid pretreatment (e.g., food stuffs, beverages, tissues, oils)	Saponification/ esterification	$NaOH/BF_3$	C23:0 TAG

(*Continued*)

Table 3.3 Cont.

Method	Applicable sample matrices	Chemical reaction	Methylation catalyst	Internal standard
AOCS Ce 2c-11 [63]	Fat-containing matrices that require acid pretreatment (e.g., extruded pet foods, oat-based foods, some encapsulated oils)	Acid hydrolysis/ saponification/ esterification	$NaOH/BF_3$	C23:0 TAG
AOCS Ce 2-66 [67]	Fatty acids	Esterification	BF_3	Not specified
	Common fats and oils	Saponification/ esterification	$NaOH/BF_3$	
	Fats and oils with acid value < 2%	Transesterification	KOH	
ISO 5509 [64]	Animal and vegetable fats and oils, fatty acids, soaps	Saponification/ esterification	$NaOH/BF_3$	Not specified
	Animal and vegetable fats and oils, milk fats	Transesterification	Trimethylsulfonium hydroxide	
	Animal and vegetable fats and oils with free fatty acid content < 2%	Transesterification	KOH	

Abbreviations: BF_3, boron trifluoride; KOH, potassium hydroxide; NaOH, sodium hydroxide; TAG, triacylglycerol.

and evaporated to near dryness on a steam bath under a stream of nitrogen gas. Methylation of the extracted lipid is performed by saponification with 0.5 M sodium hydroxide (NaOH) in methanol, followed by esterification with boron trifluoride (BF_3, 14%) in methanol. An automated acid hydrolysis extraction procedure based on AOAC 996.01 [56] using the SoxCap 2047 Hydrolysis Unit from Foss (Hillerød, Denmark) was recently validated for the quantification of total fat, lipid classes, and *trans* fat in cereal products [57]. Sample matrices included corn chips, snack mix, crackers, oatmeal cookies, pie crust mix, all-purpose baking mix, and toaster pastries, with total fat contents varying from 5.0–38% (w/w). Method performance with the automated system was comparable to that observed for AOAC 996.01 [56] with similar degrees of analytical variability. The automated system also showed advantages of decreased potential for operator error and reduced requirement for solvents, which was reportedly safer for the analyst and greener for the environment [57].

Three procedures for the determination of fat and fatty acids in finely ground and homogenized foods are recommended in AOAC Official Method 996.06 [58]. For foods excluding dairy products and cheese, the test portion is digested using 8.3 M HCl and ethanol in a shaking water bath set at 70–80 °C for 40 minutes. For dairy products, including milk, cream cheese and yogurt, the test portion is treated with ethanol and ammonium hydroxide solution (19% w/v) in order to weaken the lipid-protein bonds and break up fat emulsions. For cheeses and samples prepared with cheese (e.g., pizza), a combination of acid and alkaline hydrolysis procedures is necessary to fully disrupt the lipid-protein and lipid-carbohydrate interactions. Test portions are digested with ammonium hydroxide solution (19% w/v) and ethanol in a shaking water bath set at 70–80 °C for 20 min, then 12 M HCl is added and the sample placed in a boiling steam bath for an additional 20 min. Following digestion (acid and/or alkaline), total lipids are extracted with ethyl and petroleum ethers and converted to FAME with BF_3 (7%) in methanol. AOAC 996.06 [58] presents collaborative study data for the determination of total fat and saturated and *cis*-monounsaturated fatty acids in eight food matrices (wheat-based cereal, peanut butter, fish sticks, parmesan cheese, chocolate cake, fruit snack, ground beef, and yogurt) with analyzed total fat contents varying from 1.5–46% (w/w).

Recently AOAC 2012.13 [59] was approved for the determination of fat and fatty acids, including the contents of saturated, monounsaturated, polyunsaturated, and *trans* fatty acids in milk products, infant formula, and adult/pediatric nutritional formulas [60]. Products containing milk fat and/or vegetable oils with or without supplementation with long-chain polyunsaturated fatty acids (e.g., arachidonic acid; eicosapentaenoic acid,

EPA; and docosahexaenoic acid, DHA) are within the scope. The protocol involves the direct transesterification of liquid or reconstituted powdered test samples using methanolic sodium methoxide. A neutralization solution consisting of disodium hydrogen citrate and sodium chloride is added following derivatization to prevent further hydrolysis of FAME. Quantification on a g/100 g basis is achieved by the addition of C11:0 FAME internal standard, whereas transesterification efficiency may be evaluated by inclusion of C13:0 TAG. Results from the collaborative study for AOAC 2012.13, which were based on data from 18 participating laboratories, showed transesterification yields that ranged from 98.9% to 100.0% [60]. Interlaboratory reproducibility relative standard deviation (RSD_R) values for individual fatty acids were found to vary by analyte concentration. Individual fatty acids present at concentrations of at least 3 g/100 g showed satisfactory RSD_R values of ≤4% [60].

Direct methylation procedures involve the simultaneous hydrolysis and derivatization of lipids from food matrices in a single reaction vessel. These methods offer advantages of improved sample throughput, decreased potential for sample loss during transfer, and reduced volumes of solvents and reagents when compared with the conventional two-step extraction/derivation procedures [61]. AOCS Official Methods Ce 2b-11 [62] and Ce 2c-11 [63] have been approved for the preparation of FAME from food matrices using the direct methylation approach. AOCS Ce 2b-11 [62] is applicable to the analysis of most food matrices. It involves the saponification of homogenous samples in 0.5 M NaOH in methanol under reflux conditions (100 °C for 15 min), followed by addition of 14% BF_3 in methanol to methylate free fatty acids. Subsequent addition of saturated sodium chloride solution, followed by mixing, causes the organic and aqueous phases to separate readily, leaving a diluted FAME preparation that is ready for chromatographic separation. AOCS Ce 2c-11 [63] is used for the analysis of food matrices which require acid pretreatment, including extruded pet foods, oat-based foods, and some encapsulated oils. This method involves the *in-situ* acid digestion of homogeneous samples, followed by saponification with 0.5 M NaOH in methanol and methylation with 14% BF_3 in methanol.

Several official methods are also available for the preparation of FAME from edible fats and oils. ISO 5509 [64] is appropriate for the preparation of FAME from animal and vegetable fats and oils, fatty acids, and soaps. Test samples are first prepared according to ISO 661 [65] for composite formation and removal of insoluble substances. Samples are then treated with one of three protocols depending on the content of acid-labile constituents, namely keto, epoxy, hydroxyl, hydroperoxy,

cyclopropyl and cyclopropenyl groups, or acetylenic fatty acids [55]. The main protocol, which is appropriate for the analysis of most oils, fats and fat derivatives (fatty acids, soaps) with the exception of milk fats and fats containing acid-labile groups, involves the preparation of FAME using 0.5 M NaOH in methanol for saponification and BF_3 (12–15%) in methanol for methylation [64]. Alternate protocols for the preparation of FAME by transmethylation recommend the use of base-catalyzed reagents, namely trimethylsulfonium hydroxide in methanol (for milk samples) and potassium hydroxide in methanol for fats and oils with free fatty acid contents of < 2% (w/w) [64]. ISO 5509 [64] has been validated for the analysis of FAME from refined coconut, soybean, and vegetable oils, crude vegetable and fish oils, an oil/fatty acid blend, lard, and a cacao butter/milk fat blend. Variations of the main protocol (NaOH, BF_3 method) were approved as AOAC 969.33 [66] and AOCS Ce 2-66 [67] official methods (Table 3.3).

3.2.3.2 Analysis of FAME by GC-FID

The technique of GC-FID has been widely applied to the quantitative determination of fatty acid derivatives from foods and food ingredients. The reader is referred elsewhere for detailed discussions of the theory and practical aspects of GC-FID [11, 54, 68–70]. Fundamental rules for the GC separation of FAME are based on physical properties of chain length, degree of unsaturation (i.e., number of double bonds), and double bond geometry (i.e., *cis* and *trans* configurations) [55]. Chromatographic separations are achieved by optimizing conditions for oven temperature (i.e., isothermal or ramped programs), carrier gas (flow rate and chemical composition), and selection of GC column. A wide range of capillary GC columns are now commercially available, varying in length and internal diameter and the nature and thickness of the stationary phase [11]. Columns with cyanopropyl polysiloxane (CPS) stationary phases, such as the SP-2560 (Supelco, Bellefonte, PA, USA) and the CP-Sil 88 (Agilent J&W, Santa Clara, CA, USA), are frequently used in the analysis of food lipids because they allow for a comprehensive separation of most positional and geometric FAME isomers. This section focuses on the evaluation of official methods for the GC analysis of FAME derived from foods and food ingredients (Table 3.4). In addition, the AOCS Lipid Library, which serves as an open access online source of information for topics related to lipid science and technology, provides an invaluable wealth of literature pertaining to the analysis of fats and fatty acids by GC-FID [71]. The Cyberlipid Center is another online resource for this topic [72].

Table 3.4 Official methods for the separation of FAME by GC-FID.

Method	Applicable matrices	GC column	Temperature program	Carrier gas
AOAC 996.01 [56]	Cereal and cereal products	Fused column, 30 m × 0.25 mm ID; Rtx-2330	120 °C for 4 min, ramp at 5 °C/min to 230 °C, hold for 5 min	He
AOAC 996.06 [58]	Foods	Fused silica CPS column, 100 m × 0.25 mm ID, 0.2 μm film; SP-2560	100 °C for 4 min, ramp at 3 °C/min to 240 °C, hold for 15 min	He
AOAC 2012.13 [60]	Milk products, infant formula, adult/ pediatric nutritional formula	Fused silica CPS column, 100 m × 0.25 mm ID, 0.2 μm film; SP-2560, CP-Sil 88	60 °C for 5 min, ramp at 15 °C/min to 165 °C, hold for 1 min, ramp at 2 °C/min to 225 °C, hold for 20 min	He or H_2
AOCS Ce 1h-05 [73]	Edible fats and oils from vegetable and non-ruminant sources	Fused silica CPS column, 100 m × 0.25 mm ID, 0.2 μm film; SP-2560, CP-Sil 88	Isothermal at 180 °C for 65 min	He or H_2
AOCS Ce 1i-07 [77]	Marine and other oils containing long-chain PUFA	Fused silica PEG column, 30 m × 0.25 mm ID; Suplecowax-10, FAMEWAX, HP-INNOwax, CP-WAX, Carbowax-20M, Omegawax 320	170 °C, ramp at 1 °C/min to 225 °C, a final hold at 225 °C is used for very long chain FAME (>C25:0)	He or H_2
AOCS Ce 1j-07 [76]	Extracted fats	Fused silica CPS column, 100 m × 0.25 mm ID, 0.2 μm film; SP-2560, CP-Sil 88	Isothermal at 180 °C for 32 min, ramp at 20 °C/min to 215 °C, hold for 31.25 min	He or H_2

Several official methods are offered by AOAC for the analysis of FAME from foods and food ingredients by capillary column GC-FID (Table 3.4). AOAC 996.01 [56] was validated for the GC separation of FAME derived from cereal and cereal products. Chromatographic conditions involve the use of a 30-m fused silica CPS column (e.g., Rtx-2330; Restek, Bellefonte, PA, USA) and a ramped temperature program with helium as the carrier gas. Results from the collaborative study for AOAC 996.01 [56], which were based on data from 15 participating laboratories, indicated that this method [56] was most appropriate for the determination of total fat and saturated fatty acids, whereas determinations of unsaturated fatty acids showed greater variability due to the partial co-elution of C18:0, C18:1, and C18:2 isomers.

Included in AOAC Official Method 996.06 [58] are the chromatographic conditions for the separation of FAME by GC-FID using a 100 m CPS column with a ramped temperature program and helium as the carrier gas. This method has been validated for the quantification of total, saturated, and *cis*-unsaturated fatty acids in a variety of food matrices. AOAC 996.06 [58] may be applied to the determination of total *trans* fatty acids by integrating the total area for peaks eluting between C18:1 (*cis*) and C18:2 (*cis*). Quantification of individual *trans* FAME is not possible due to co-elutions of individual positional isomers (with *trans* double bonds) in this region of the chromatogram. Modifications of AOAC 996.06 [58], which were based on chromatographic conditions of AOCS Official Method Ce 1h-05 [73], were proposed by Rozema *et al.* [74] to improve the accurate determination of *trans* fat in food samples. The authors noted that at least 15 *trans* fatty acid isomers could be specifically identified and quantified using the modified method [74].

A procedure for the GC separation of FAME derived from milk products, infant formula, and adult/pediatric nutritional formulas is decribed in AOAC 2012.13 [59]. Chromatographic conditions include the use of a 100 m CPS column, a ramped temperature program, and helium or hydrogen as the carrier gas. The oven is initially maintained at a relatively low temperature (60 °C for 5 min) in order to achieve peak resolution of short-chain FAME [11]. The subsequent ramps in temperature were designed to optimize resolution of the C18:1 *cis* and *trans* isomers and to allow for a complete elution of FAME, including the long-chain polyunsaturated fatty acids, EPA and DHA [60]. Quantification of the mono-*trans* isomers of C18:2 and C18:3 was also possible for samples containing refined, bleached, and deodorized vegetable oils [60]. Empirical response factors may be determined by analysis of a FAME reference standard (GLC-Nestle-36 from Nu-Chek Prep, Elysian, MN, USA), specifically prepared for AOAC 2012.13 [59], containing known concentrations of individual FAME.

The AOCS offers three official methods for the separation of FAME derived from foods, food ingredients, and dietary supplements. These protocols are specifically GC-based and the analyst is referred elsewhere (e.g., ISO 5509 [64], AOCS Ce 2b-11 [62]/Ce 2c-11 [63]) for sample preparation procedures. Theoretical correction factors are applied for individual FAME to better reflect the FID response of active carbon atoms [75]. AOCS Ce 1h-05 [73] was validated for the determination of saturated and unsaturated fatty acids (*cis* and *trans* isomers) in edible fats and oils from vegetable and non-ruminant sources, including crude, refined, partially hydrogenated, and fully hydrogenated oils. Chromatographic conditions involve the use of a 100-m CPS column, an isothermal oven temperature maintained at 180 °C, and helium or hydrogen as the carrier gas. This method [73] is not appropriate for the analysis of marine oils or products containing conjugated linoleic acid isomers due to the requirement for a prolonged GC run at 180 °C in order to elute long-chain, highly unsaturated FAME. A related method was approved as AOCS Ce 1j-07 [76] for the analysis of extracted fats, including those derived from dairy and ruminant products, but not products containing mixtures of dairy and vegetable fats, due to the co-elution of *trans* C18:3 isomers with C20:1 FAME. AOCS Ce 1j-07 [76] involves the use of a 100-m CPS column, a ramped temperature program, and helium or hydrogen as the carrier gas. Thus together, AOCS official methods Ce 1h-05 [73] and Ce 1j-07 [76] allow for the comprehensive analysis of fat and fatty acids in most food matrices using a single GC column with differences in oven temperature program, carrier gas flow rate, and inlet and detector temperatures. AOCS Ce 1j-07 [76] was updated in 2013 to include collaborative study data for 22 food and dietary supplement matrices with total fat contents varying from 0.2% to 99% (w/w). Interlaboratory reproducibility (RSD_R) values, which were based on data from nine participating laboratories, were less than 10% for 17 of the test samples. The remaining five test samples, namely frozen cheese pizza, canned cat food, DHA/EPA fortified orange juice, dry cereal fortified with flax, and gamebird feed, showed higher variability (RSD_R values <20%).

The AOCS Ce 1i-07 method [77] is appropriate for the analysis of FAME from marine oils, including fish oils, fish oil concentrates (ethyl esters), and algal oils. Chromatographic conditions involve the use of a 30-m column with a polyethylene glycol (PEG) stationary phase, a ramped temperature program, and helium or hydrogen as carrier gas. AOCS Ce 1i-07 [77] is not appropriate for quantification of individual *cis* and *trans* FAME due to the limited selectivity of the PEG stationary phase for resolving geometric isomers. Thus, an overestimation in the quantification of all-*cis* EPA and DHA may occur with refined, bleached, and deodorized marine oil

samples [78, 79] due to the co-elution of the *trans* isomers of EPA and DHA with the corresponding all-*cis* isomers [80]. In their evaluation of the GC columns for the separation of FAME derived from fish sources, Santercole *et al.* [80] noted the importance of the PEG column for identifying unsaturated fatty acids, but recommended that a complementary analysis be performed in order to achieve resolution of geometric FAME isomers, especially in products containing mixed sources of oils. In addition to AOCS Ce 1i-07 [77], other methods, such as AOAC 991.39 [81], AOCS Ce 1b-89 [82], and those reported in the Voluntary Monographs of the Global Organization for EPA and DHA Omega-3s (GOED) [83] have also been approved for the analysis of FAME from marine oils using 30 m PEG columns.

3.2.4 FTIR Spectroscopic Methods

Foods and food ingredients are largely composed of five major constituents, namely fat, protein, carbohydrate, moisture, and ash. The analytical determination of these compounds, collectively referred to as proximates, is essential for evaluating the quality and composition of raw food materials and foods and food ingredients during and after processing [84]. Conventional analytical methods for measuring food proximates, such as the wet chemistry methods recommended by AOAC INTERNATIONAL and AOCS, are often laborious, time consuming, and require specialized training. Alternative approaches which allow for the simple, rapid, and simultaneous determination of proximates offer the food industry the potential to significantly improve the cost and time-savings efficiency of analytical determinations, especially when used in real-time processing applications [85].

FTIR spectroscopic analyzers are particularly amenable to the analysis of proximate constituents of foods and food ingredients due to the potential for the simultaneous determination of multiple analytes from a single test sample [84]. In addition, FTIR spectroscopic procedures offer the advantage of being rapid and nondestructive, and they require little or no sample preparation or use of solvents or reagents. Over the last few decades, advances in FTIR spectroscopic analyzers, including the introduction of attenuated total reflection (ATR) accessories and sophisticated data handling routines, have significantly increased the breadth of applications for FTIR spectroscopy for quantitative analyses [84]. Quantification by IR spectroscopy is based on the additive nature of the Beer-Lambert law in which spectral features of major functional groups are proportional to the concentrations of corresponding constituents in a

test sample. As such, contents of fat, protein, carbohydrate, and moisture are readily quantified due to strong spectral features in the mid-IR (MIR) region of the electromagnetic spectrum (4,000–400 cm^{-1}). In contrast, due to the broad, overlapping spectral features observed in the near-IR (NIR) region (14,000–4,000 cm^{-1}), the use of powerful multivariate chemometric techniques, such as partial least-squares regression (PLSR), principal components analysis (PCA), or artificial neural networks (ANN), is required in order to extract quantitative information from this region of the spectrum [86]. Both MIR and NIR spectroscopic procedures have been validated as official methods for quantitative proximates analysis, among others.

A procedure for the determination of fat, lactose, protein, and solids in milk by MIR spectroscopy is described in AOAC Official Method 972.16 [46]. The analysis is based on the absorption of MIR energy at specific wave numbers, namely those corresponding to the CH groups of fatty acid chains and the carbonyl groups of ester linkages for fat, the peptide linkages between amino acids for protein, and the OH groups of lactose molecules [46]. Rigid performance specifications for the determination of individual constituents allow for the transferability of the method to different instrument models. AOAC 972.16 functions as a secondary method of analysis requiring instrument calibration for the concentration ranges of interest. The calibration for fat is achieved by preparing mixtures of water and homogenized cream and accurately measuring total fat content in those calibration samples according to a standardized method such as AOAC 989.05 [28]. A linear regression equation is then used to determine the concentration of fat in unknown test samples. The performance of AOAC 972.16 was later validated in collaborative study testing for use with FTIR spectrophotometers [87]. AOAC Official Method 972.16 is frequently used for proximates determination in milk by the dairy industry, as well as in contract and academic laboratories. A similar protocol is available as ISO 9622:2013 [88].

A procedure for the determination of fat, protein, and moisture in meat and meat products by NIR spectroscopy is described in AOAC Official Method 2007.04 [89]. This method was validated for use with the FoodScan NIR spectrophotometer from FOSS (Hillerød, Denmark) and the FOSS ANN calibration model and associated database [90]. The procedure is applicable to the analysis of fresh meat (beef, pork, and poultry), emulsions, and finished products with constituent ranges of 1–43% fat, 27–74% moisture, and 14–25% protein. The protocol recommends the preparation of test samples by grinding or homogenization using a standardized procedure such as AOAC 983.18 [91]. The test portion is then placed in a sample cup in the sample chamber and rotated during analysis in order to collect

spectral subscans from 16 different regions of the sample matrix. The ANN calibration model, which relies on a database of sample spectra and chemical analyses of proximates composition, is then used to interpret results for fat, protein, and moisture based on spectral readings. Collaborative study results for AOAC 2007.04, which were based on data from 16 participating laboratories, showed excellent precision performance with interlaboratory RSD_R values for fat that varied from 0.5–6.9% [90].

The FTIR spectroscopy technique has also been widely applied as a secondary method of analysis for the quantification of total *trans* fatty acids, or *trans* fat, in edible fats and oils and extracted fats [55, 70, 92]. The determination of total *trans* fat by FTIR spectroscopy is based on the C–H out-of-plane deformation band observed at 966 cm^{-1} that is quantitatively characteristic of the absorbance of fatty acids with isolated *trans* double bonds [93]. The current method, AOCS Official Method Cd 14e-09 [94], describes a procedure for the determination of total isolated *trans* fat in edible fats and oils by FTIR spectroscopy using a heated ATR accessory. A linear regression equation for total *trans* fat is generated based on the spectra of mixtures of neat TAG standards with *trans* fat contents spanning the concentration range of interest. Spectra are transformed to the negative second derivative and the height of the absorbance band at 966 cm^{-1} is used as the dependent variable in the calibration equation. AOCS Official Method Cd 14e-09 [94] has recently been validated for use in two novel, portable ATR-FTIR devices [92, 95, 96].

Recently, AOCS Standard Procedure Cd 14f-14 [97] was approved for the rapid determination of the content of total saturated, monounsaturated, polyunsaturated, and *trans* fatty acids in edible fats and oils by FT-NIR spectroscopy. This procedure, which is based on the method of Azizian and Kramer [98], employs the use of pre-calibrated FT-NIR models for the classification and quantification of fatty acids in edible fats and oils. The FT-NIR PLSR calibration models were developed based on the spectral readings of neat commercial vegetable oils, margarines, shortenings, lard, and partially hydrogenated oils, and mixtures thereof. Primary reference data for fatty acids were determined by GC-FID using a 100-m CPS capillary column. Accuracy of the GC determinations was achieved by prior fractionation with thin layer chromatography and high performance liquid chromatography in order to separate, identify, and quantify all individual FAME. Method performance was evaluated in a three-laboratory round robin collaborative study using five different FT-NIR spectrometers acquired from a single manufacturer [99]. The authors found that the predicted fatty acid concentrations were within acceptable limits of agreement compared with data obtained by GC-FID. Moreover, the precision data for

contents of total saturated, monounsaturated, polyunsaturated, and *trans* fatty acids were comparable to those reported for the GC-based method AOCS Ce 1h-05 [73].

3.2.5 Method Validation for Novel Sample Matrices

The primary justification for using an official method is the certainty by which accurate determinations of sample composition are made. A reasonable level of accuracy is expected if the protocol of an official method is followed properly and the method is applied to samples which fall within the scope. However, additional validation procedures are required for samples which fall outside the scope in order to verify method performance with the novel matrix. This process is referred to as the matrix extension of a method [6] and it includes the use of certified reference materials and spike-recovery experiments to provide an additional level of certainty in the analytical determination of target analytes. Matrix extension experiments are included during regulatory analyses when dealing with issues of safety or noncompliance. They also serve key roles in quality assurance programs and during the single laboratory method development and validation process [100, 101].

Regulatory and contract laboratories routinely include in their sample sets certified reference materials made available by organizations such as the National Institute of Standards and Technology (NIST, Gaithersburg, MD) and the US Pharmacopeial Convention. Certified materials from NIST are referred to as Standard Reference Materials (SRM) and they are provided with a certificate of analysis that documents the intended use of the SRM, appropriate storage and handling conditions, and expiration date [100]. The SRM matrices contain highly characterized amounts of analytes which are determined by using two independent analytical approaches in combination with data provided by external laboratories [100]. Quantitative mass fraction values presented in the certificates of analysis are assigned to one of three categories, namely certified, reference, or information values based on the level of confidence in accuracy, taking into consideration potential sources of analytical bias. The NIST offers a wide range of certified reference materials to be used in the analysis of total fat and fatty acids (Table 3.5).

Certified reference materials are used to verify method performance in validation experiments. For instance, accuracy in the quantification of an analyte may be assessed by comparing its analytical determination with the corresponding mass fraction value reported in the certificate of analysis.

Table 3.5 Standard reference materials (SRM) for the determination of fat and fatty acids currently available from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA).

SRM	Sample matrix	Quantitative mass fraction values
1544	Frozen diet composite	Total fat, FA
1546a	Meat homogenate	Sum of FA
1548a	Typical diet	Total fat
1549a	Whole milk powder	Total fat, sum of FA
1845a	Whole egg powder	Sum of FA
1849a	Infant/adult nutritional formula	Fat (extracted), sum of FA
1946	Lake Superior fish tissue	Fat (extracted), sum of FA
2384	Baking chocolate	Fat (extracted)
2387	Peanut butter	Fat (extracted), sum of FA
3233	Fortified breakfast cereal	Sum of FA
3243	Soy flour	Sum of FA
3251	*Serenoa repens* Extract	Sum of FA
3274	Botanical oils	Sum of FA
3275	Fish oils	Sum of FA

Abbreviations: FA, fatty acids.

Precision may also be evaluated by the replicate analysis of a certified reference material for specific analytes over a period of one or more days. The extended validation of the simplified gravimetric method of Phillips *et al.* [21] for the determination of total fat in foods involved the analysis of several certified reference materials from the NIST, the AOCS, and the American Association of Cereal Chemists [22]. Slight modifications to the method were applied for samples of meat, fish, shrimp, cheese, and fried plantains in order to improve total fat recovery and reduce analytical variability [22].

Spike-recovery experiments are also used to evaluate the performance of an analytical method in a novel, specified matrix. For regulatory analyses, spike-recovery experiments are performed by adding to the test material, or a similar certified reference material, a known amount of analyte

that is comparable in structure and concentration to the incurred analyte of interest. The sample is then analyzed and the recovery of the spiked analyte is calculated as the experimental determination of spiked amount taken as a percentage of the theoretical spiked amount. Acceptable recovery limits are based on the concentration of the spiked analyte in the test material. The range of acceptable recovery limits is expected to increase with decreasing spike concentration in the test material, especially for analytes near their limits of quantification [6].

3.3 Conclusions

A wide range of analytical techniques, including wet chemistry and spectroscopic methods, are currently available for the determination of total fat and fatty acids in foods and food ingredients. These methods vary in sample preparation and analysis protocols and also in the types of information they provide. Gravimetric or spectroscopic methods are suitable for routine analysis in the determination of total fat content. However, in situations in which the complete fatty acid profile is required for nutrition labeling purposes, the analysis by GC-FID continues to be the analytical approach of choice. Many methods have been validated as official methods of analysis for the determination of total fat and fatty acids in foods and food ingredients. A reasonable level of accuracy is expected when official methods are applied to the analysis of samples that fall within the scope. For novel sample matrices, additional method validation procedures, such as the analysis of certified reference materials and spike-recovery experiments, are essential for ensuring confidence in the accuracy of analytical determinations.

The development of novel analytical methods in the field of food analysis is driven by the objective to improve the speed, sensitivity, and/or simplicity by which accurate determinations of product composition are made. When considering the analysis of fats in foods, recent advances in methods which show the greatest potential for becoming future trends are those which improve the rates of sample throughput or reduce or eliminate the use of large volumes of hazardous reagents and solvents. Techniques which take the analysis beyond the confines of the laboratory setting for use in real-time process applications are also desirable. Such advances in technology include the automated or semi-automated sample preparation systems and the rapid and portable spectroscopic devices. Such technologies offer the food industry potential for significant improvements in cost and time-savings efficiency.

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