BACKGROUND DISCUSSION PAPER ON ASSESSING *TRANS*-FATTY ACIDS IN FOODS:
SUMMARY OF ANALYTICAL METHODS AND EXAMPLES OF THEIR USES

The purpose of this report it to summarize key methods that have been developed and used for the determination of TFA levels in foods and provide examples where those methods have been used by researchers or recommended by government agencies in a number of countries or regions.

1. OVERVIEW OF METHODS USED TO DETERMINE *TRANS*-FATTY ACIDS IN FOODS

The elimination of industrially produced *trans*-fatty acids (TFA) from the food supply has been considered as a priority action and countries are taking various policy measures and regulatory actions to reduce exposure of their populations to TFA. These actions call for the adoption and implementation of standardized methodologies for the quantification, reporting, and monitoring of TFA in fats, oils, and foods in order to assess the efficacy of such undertakings.

Given the diversity of available foods in the current food supply and the complexity of different food matrices, specific analytical techniques may be required for the analysis of particular foods. To address this issue, several analytical methods have been developed and adapted for the analysis of fatty acids from specific food matrices. Therefore, some prior knowledge of both the food to be analyzed and of the scope of the available methods is required to achieve accurate reproducible results.

For instance, in the context of nutrition labelling , capillary gas chromatography (GC) with flame ionization detection (FID) is generally accepted as the routine analytical method of choice for fatty acid analysis, including the determination of TFA content. **Table 1** provides a summary of the current Official Methods for sample preparation for GC-FID analysis of TFA from fats, oils, and foods. Further overview of the available GC-FID and infrared spectroscopy methods is provided in the following section.

Furthermore, **Table 2** highlights some examples where researchers have used these methods or Government agencies have recommended some of the methods.

Table 1. Summary of Official Methods for preparation of FAME for *trans*-fatty acids analysis by GC-FID[1]

Method[2]	Applicable food matrices	Chemical reaction	Methylation catalyst	Internal standard
AOAC 996.06 [1]	Foods excluding dairy products and cheese	Acid hydrolysis/ esterification	BF_3	C11:0 TAG
	Dairy products	Alkaline hydrolysis/ esterification	BF_3	
	Cheese	Acid and alkaline hydrolysis/ esterification		
AOAC 2012.13 [2]	Milk products, infant formula, adult/pediatric nutritional formula	Transesterification	Sodium methoxide	C11:0 FAME/C13:0 TAG
AOCS Ce 2b-11 [3]	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
AOCS Ce 2c-11 [4]	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 [5]	Fatty acids	Esterification	BF_3	Not specified
	Common fats and oils	Saponification/ esterification	$NaOH/BF_3$	Not specified
	Fats and oils with acid value < 2%	Transesterification	KOH	Not specified
ISO 12966-2 [6]	Animal and vegetable fats and oils containing fatty acids down to butanoic acid; free fatty acid content ≤ 0.5%	Transesterification	KOH	C5:0 FAME[3]
	Animal and vegetable fats and oils, milk fats	Transesterification under alkali- and acid-catalysed conditions	Sodium methoxide/ H_2SO_4	Not specified
	Animal and vegetable fats and oils	Saponification/ esterification	$NaOH/BF_3$	Not specified
	Animal and vegetable fats and oils	Transesterification	H_2SO_4	Not specified
ISO 12966-3 [7]	Animal and vegetable fats and oils	Transesterification	Trimethylsulfonium hydroxide	Not specified

[1] Adapted from Srigley and Mossoba [8] with permission.

[2] For further details of the methods, please refer to the official source.

[3] Recommended for quantification of short-chain fatty acids only.

Abbreviations: FAME: fatty acid methyl ester; GC-FID: gas chromatography-flame ionization detection; TAG: triacylglyceride.

Table 2. Summary of country examples that have applied or recommended key methods to determine *trans*-fatty acids in foods

Country/Region[1]	Matrix	Analytical Methods applied or recommended
Argentina [9]	Foods	AOAC Official Method 996.06, method recommended by the Argentinean Ministry of Health.
Australia [10]	Foods	A 2008 Study where the AOCS method Ce 1f-96 was used to determine trans fatty acids in several foods.
Chile [11]	Foods	ISO 15304 (currently withdrawn), is a method listed by the Chilean Institute of Public Health.
Canada [12]	Foods	The Canadian Food Inspection Agency recommends the AOAC Official Method 996.06.
Latin America [13]	Foods	A 2017 study the AOAC Official Method 996.06 was used to monitor trans fats in foods.
Europe [14]	Foods	A modification of the AOAC 996.06 method was used in a 2014 study that monitored trans fats in foods.
Kyrgyzstan [15]	Foods	ISO 12966-1:2014 and ISO 15304:2002 (currently withdrawn) were used to monitor trans fats in foods in a 2017 study.
Portugal [16, 17]	Foods	ISO 12966-1:2014 and ISO 15304:2002 (currently withdrawn) were used to monitor trans fats in foods in two 2016 studies.
Spain [18]	Foods	ISO 5508:1990 (currently withdrawn, the new standard is ISO 12966-1:2014) was the method used to determine trans fats in foods in this 2014 study.
Sweden [19]	Foods	This 2016 study used the AOCS Official Method Ce-1f-96 method to monitor trans fats in foods.
United Kingdom [20]	Foods	ISO 12966-1 method was used to monitor trans fats in foods in 2013 by the UK Department of Health.
Tajikistan [21]	Foods	This 2017 study used the ISO 12966-1:2014 and ISO 15304:2002 (currently withdrawn) methods to monitor trans fats in foods.

[1] Country or region where researchers have use analytical methods to determine the level of trans fatty acids in foods or where such methods have been recommended by Government agencies.

2. SUMMARY OF METHODS FOR *TRANS*-FATTY ACID ANALYSIS IN FOODS

There are various Official Methods endorsed for the analysis of TFA in fats, oils, and food products. Therefore, it is important to be knowledgeable of the advantages and limitations of each in order to choose an appropriate method for the analysis of a specific product or for a particular application.

Currently two routine methods are accepted for the characterization and relative quantification of TFA in fats, oils, and foods: attenuated total reflection-Fourier-transform infrared (ATR-FTIR) spectroscopy and GC-FID. Validated official protocols have been established by the AOCS, AOAC, and ISO for these analytical techniques. The following provides brief summaries of the analytical methodologies required for TFA analysis highlighting some of the important considerations to acknowledge when choosing an appropriate method for either of these applications. It should be noted that there are several comprehensive reviews (8, 22, 23) which provide more detailed information on the analytical techniques for the TFA analysis of fats, oils and food products.

2.1. Attenuated total reflection-fourier-transform infrared spectroscopy (ATR-FTIR)

For rapid determination of total TFA content of fats and oils, a number of ATR-FTIR methods have been developed and validated [24-26]. These methods use the characteristic *trans* bond absorption pattern at 966 cm^{-1} to determine the total TFA levels of a test sample. In general, methods determine TFA levels of a test sample relative to a calibration curve using a TFA free medium, either TFA-free oil or fatty acid methyl ester (FAME) cocktail dissolved in carbon disulfide, that has been spiked with either trielaidin (*t*18:1 triacylglycerol) or methyl elaidate (*t*18:1 FAME) in varying concentrations. Depending on the method, either the area of the absorbance band at 966 cm^{-1} [25, 26], or the height of the negative second derivative of the characteristic absorbance band [24] is used for quantification. Additionally, FTIR may be carried out on derivatized FAME or undiluted pure fats and oils which dramatically reduces time requirements for the analysis of pure oils and fats [22, 24-27]. Furthermore, run times for ATR-FTIR are dramatically shorter than those required for GC-FID.

Although TFA analysis by ATR-FTIR offers significant time savings compared to GC-FID methods, this approach does suffer from several draw backs limiting its widespread application [23, 27]. For instance, high saturated fatty acid content, high levels of conjugated double bonds, and the presence of functional groups may cause interference near the 966 cm^{-1} [23]. For these reasons AOCS Official Method Cd 14d-99 and AOAC Official Method 2000.10 are not recommend for TFA analysis of oils with conjugated linoleic acid (CLA) contents >1%, or oils with appreciable levels of interfering functional groups (e.g. castor oil) [25, 26]. To improve the accuracy of ATR-FTIR, AOCS Official Method Cd 14e-09 uses the negative second derivative of the height of the *trans* absorption band [24]. This approach extends the scope of Official Method Cd 14e-09 to include dairy and ruminant fats, along with fats and oils that contain added CLA [24].

2.2. Capillary gas chromatography-flame ionization detection methods (GC-FID)

GC-FID is the most commonly employed analytical method for the fatty acid compositional analysis of fats and edible oils, and food products containing them. GC-FID analysis tends to be the preferred method for nutrition labelling applications as it permits the determination of total fat, saturated, monounsaturated, polyunsaturated fatty acid, and TFA content in one single GC procedure. However, the main draw back to the use of routine GC-FID analysis is the intensive sample preparation necessary to extract, hydrolyze/saponify, and derivatize test samples prior to analysis. In addition, a level of skill and experience is required to optimize GC methods to achieve the chromatographic resolution necessary for TFA determination in complex samples.

2.2.1. Sample Preparation

For the determination of TFA in foods and oils, samples must be processed to produce more volatile fatty acid derivatives that can then be separated and analyzed by GC-FID. Several official methods have been generated and validated for the analysis of fats, oils, and food products [1-4, 6]. Given the wide variety of food matrices and the varying fatty acids profiles of foods, it is important to choose the appropriate sample preparation methodology to avoid otherwise reporting erroneous results.

For the determination of total fat and saturated, and unsaturated fatty acids in finely ground homogenates of general foods, AOAC Official Method 996.06 provides three separate methods depending on the product being analyzed [1]. For non-dairy products, test samples are first subject to acid digestion which produces free fatty acids. In contrast, it is recommended that dairy products are subject to alkaline digestion. For cheeses and cheese containing products alkaline digestion is first carried out, following which the test sample undergoes acid digestion. After completion of the digestion procedures, test samples are then extracted by a liquid-liquid solvent system and centrifugation. Fatty acid containing extracts are then dried under nitrogen gas and methylated with boron trifluoride (BF_3) in methanol to produce FAME [1]. In addition to Official Method 996.06, the AOAC has developed and validated a method for the determination of TFA in milk products, infant formulas, and adult/pediatric nutritional formula [2, 28]. AOAC Official Method 2012.13 differs from the previous methods in that the test sample does not undergo digestion and extraction prior to methylation. Instead, samples are directly transmethylated with methanolic sodium methoxide [2]. The choice of methylation by sodium methoxide versus BF_3 is preferred for fats containing appreciable amounts of CLA as BF_3 catalysed methylation leads to artificial isomerization of these fatty acid species [22, 29].

The AOCS provides three Official Methods (Ce 2-66, Ce 2b-11, and Ce 2c-11) for the generation of FAME for GC-FID analysis [3-5]. For the analysis of most foods, AOCS Official Method Ce 2b-11 is recommended. In this procedure, test sample homogenates are saponified by alkaline hydrolysis with methanolic sodium hydroxide and then methylated with BF_3 in methanol. FAME are then extracted into hexane following organic and aqueous phase separation by the addition of a saturated salt solution [3]. In some food matrices (e.g. extruded pet foods, encapsulated oils, and oat-based foods) Official Method Ce 2b-11 may not be sufficient to methylate all fatty acids,

in this case Official Method Ce 2c-11 should be used [4]. Official Method Ce 2c-11 differs from Ce 2b-11 in that the test sample is subject to an *in situ* mild acid digestion prior to saponification and methylation [3, 4]. AOCS Official Method Ce 2-66 describes three procedures for the generation of FAME from fatty acids, as well as from fats and oils. Notably, only samples with low free fatty acid content (<2 % w/w) are to be transmethylated by methanolic potassium hydroxide, as this procedure does not produce FAME from free acids [5].

The ISO provides a series of methods for the analysis of TFA from vegetable and non-ruminant animal fats and oils. For food analysis, fats will need to be extracted prior to methylation. ISO 12966-2:2017 (replacing ISO 5509) describes four different methylation procedures for various types of samples, including alkali-catalyzed, sequential alkli- and acid-catalyzed methods, in addition to BF_3 transmethylation protocols [6]. Additionally, ISO 12966-3:2017 describes a method for the production of FAME using tetramethylsulfonium hydroxide in methanol [7]. This procedure produces tetramethylsulfonium salts which will decompose into volatile FAME in the inlet of a GC. This method is not suitable for on-column GC protocols.

2.2.2. Internal Standard Considerations

The quantitative determination of TFA levels in food samples requires the use of an appropriate internal standard. An internal standard serves as a reference analog that is added to the test sample prior to the methylation and isolation of FAME and permits the accurate reporting of fatty acid levels in concentrations (mg/g of sample). For accurate analysis, it is imperative that the internal standard is not native to the test sample and is well resolved from all surrounding chromatographic peaks. For these reasons, the decision of internal standard must be made carefully and may require the use of specific standards for specific applications. For instance, triheneicosanoin (C21:0 TAG) for the analysis of partially hydrogenated vegetable oils is complicated by the presence of CLA isomers in these oils and products containing these oils which may interfere with the quantitation of fatty acids by GC-FID [22]. Additionally, when analyzing samples containing fats originating from ruminant sources, the abundance of CLA isomers are also likely to cause interference. In these cases, a more appropriate internal standard would likely be tritridecanoin (C13:0 TAG), as recommended by Mossoba and Kramer, which is only present in low quantities in ruminant fats, and will likely not interfere with *trans* isomers [22].

Internal standards triundecanoin (C11:0 TAG) and tritricosanoin (C23:0 TAG) are called for in AOAC Official Method 996.06 and ACOC Official Method 2b/c-11, respectively
[1, 3, 4]. Notably, AOAC Official Method AOAC 2012.13 requires the addition of two internal standards methyl undecanoate (C11:0 FAME) and tritridecanoin [2]. In this case, methyl undecanoate is used for the quantitation of fatty acids in the sample, and tritridecanoin is used for the determination of transesterification efficacy.

2.2.3. GC-FID Analysis

GC-FID analysis allows for the separation of a complex mixture of FAME based on their carbon chain length, as well as the number and geometry of double bonds of each FAME. The accurate

determination of TFA from foods requires optimized GC parameters for the adequate resolution of *trans* isomers from surrounding chromatographic peaks. Because of the chromatography requirements for TFA analysis, the use of very long, highly polar, 100% cyanopropyl polysiloxane coated fused silica capillary columns is recommended by all Official Methods [1, 2, 8, 22, 30-32]. Commercially available cyanopropyl polysiloxane columns such as Supleco's SP-2560 and Agilent's CP-Sil 88 are widely available and provide exceptional separation capacity [22, 33]. Although all Official Methods recommend the use of cyanopropyl polysiloxane columns they do offer a variety of GC parameters (e.g. oven temperature programs, pneumatic programs, split ratio, injection volumes etc.) which influences the scope of each method.

For instance, AOCS Official Method Ce 1h-05 calls for an isothermal oven temperature program which is not adequate for the resolution of some CLA isomers therefore this method is not applicable to TFA determination of fats and oils of ruminant origin [30]. For these purpose AOCS Official Method Ce 1j-07 has been adapted. However, due to coelution of *trans* linolenic acid isomers and gondoic acid (C20:1) isomers, Ce 1j-07 is not advised for analysis of foods containing mixtures of dairy and vegetable fats [31]. In such case, it is recommended that both Official Method's Ce 1j-07 and Ce 1h-05 be applied [8]. Furthermore, it is important to note that the parameters outlined in the Official Methods may not achieve baseline resolution of all individual TFA isomers in every application, therefore inhouse optimization may be required.

With GC-FID analysis, FAME are identified by retention time in relation to a known reference standard. Reference standards are comprised of a highly purified mixture of FAME and are to contain all fatty acids of interest. Appropriate reference standards can be prepared in-house; however, there are a number of high quality commercially available comprehensive FAME mixtures on the market for TFA analysis (e.g. Nu Chek Prep GLC-674). The determination of TFA levels in test samples is often calculated based on the sum of peak areas over a specific span of retention times corresponding to where TFA elute [1, 2]. If the determination of specific TFA isomers is required, preparative techniques such as silver ion thin layer chromatography or high-performance liquid chromatography may need to be applied prior to GC-FID analysis. These approaches permit the fractionation of FAME from a test sample into sub-fractions according to the degree of unsaturation and bond geometry [34]. While these methods may greatly improve peak identification, they tend to be laborious.

For nutrition labelling applications, the reporting of total fatty acids is in triacylglycerol equivalents whereas saturated, monounsaturated and polyunsaturated fatty acids, as well as TFA are to be expressed as fatty acid equivalents. Stoichiometric correction factors for the conversion of FAME to triacylglycerol and fatty acids equivalents are provided within some of the Official Methods [1, 2]. Additionally, reference standards can be useful to generate empirical response factors which can be used to correct for differences in FID response factors for different FAME [2, 34]. Alternatively, theoretical corrections factors may be applied to improve the quantitation of reported fatty acid data.

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