Application of Standard Addition to Eliminate Conjugated Linoleic Acid and Other Interferences in the Determination of Total *Trans* Fatty Acids in Selected Food Products by Infrared Spectroscopy

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ABSTRACT: A novel and rapid (5 min) attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopic method AOCS Cd 14d-99 for the determination of total isolated *trans* fatty acids, which absorb at 966 cm^{-1}, was recently developed, collaboratively studied, and applied to food products containing 1–50% *trans* fat (as percentage of total fat). Attempts to apply the ATR-FTIR method to biological matrices of low *trans* fat and/or low total fat content, and to dairy and other products were not satisfactory due to interfering IR absorptions in the *trans* region. One group of interfering compounds with absorption bands near 985 and 948 cm^{-1} was the *cis/trans* positional isomers of conjugated linoleic acid (CLA) found in dairy and meat products from ruminants at levels of <1% (as percentage of total fat). In the present study, we modified the ATR-FTIR method to overcome matrix interferences. This modification, which consisted of applying the standard addition technique to the ATR-FTIR determination, was also applied to several food products, namely, dairy products, infant formula and salad oil dressing, which successfully eliminated interfering absorbances that impacted on accuracy. The presence of <1% CLA in two butter and two cheese products containing 6.8, 7.5, 8.5, and 10.4% *trans* fatty acids (as a percentage of total fat) would have led to errors of –11.6, 10.4, 17.6 and 34.6%, respectively, in *trans* fat measurements had the standard addition technique not been used. The applicability of ATR-FTIR to the quantitation of food products is discussed.

Paper no. J9632 in JAOCS 78, 631–634 (June 2001).

KEY WORDS: Infrared, standard addition, trielaidin, *trans* fats.

Under regulations implementing the Nutrition Labeling and Education Act (NLEA) of 1990 in the United States, total fat for food labeling purposes is defined as total lipid fatty acids expressed as triglycerides (1). Currently, *trans* fats are included in the definition of total fat, but are not included in definitions of mono- or polyunsaturated fatty acids (PUFA). In November 1999, the U.S. Food and Drug Administration (FDA) published proposed rules for labeling the *trans* fatty acid content of food products (2). Efforts to optimize methods for determining *trans* fat in foods were prompted by the continuing interest in *trans* fat labeling, and because the nutritional significance of different *trans* fatty acids can vary depending on their sources.

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Trans fatty acids in the diet are mainly derived from partially hydrogenated vegetable oils (PHVO) and ruminant fats. PHVO have been reported to present a possible risk factor for coronary heart disease (3), whereas some *trans* conjugated fatty acids in ruminant fat have been reported to have several beneficial physiological effects in experimental animals (4). The major *trans* fatty acid present in meat and dairy products from ruminants is *trans*-vaccenic acid (*trans*-11-18:1) (5,6) while one of the many minor ones is the conjugated linoleic acid (CLA) isomer *cis*-9,*trans*-11-18:2 (7–9). *Trans*-vaccenic acid has been shown to be converted to *cis*-9,*trans*-11-18:2 by Δ9 desaturase present in mammalian tissue (10,11) .

The separation and quantitation of total *trans* fatty acids in food products by gas chromatography (GC) as fatty acid methyl esters (FAME) using long polar capillary columns considerably underestimates the C_{18} *trans*-monounsaturated FAME (*trans* 18:1) content by the amount of Δ12- to Δ16-18:1 *trans* FAME positional isomers because these *trans* isomers overlap on the gas chromatogram with the 18:1 *cis* isomers (5,6,12,13). This problem was overcome by using a procedure that combines silver-ion thin-layer chromatography (Ag^+-TLC) and GC (5,6,12,14). This elaborate technique is particularly useful for the analysis of *trans*-monounsaturated fatty acids but becomes less accurate when it is applied to complex mixtures of dienes and PUFA with one or more *trans* double bonds (14). This is because there are varying numbers of minor *trans* 18:2, 18:3, C_{20} and C_{22} FAME components that are not well resolved, easily identified, or accurately quantified.

Alternatively, transmission infrared (IR) spectroscopy has been widely used for the determination of total *trans* fatty acids with isolated (nonconjugated) double bonds (15–19). In 1996, a novel "ratioing" Fourier transform infrared (FTIR) method was adopted for the determination of fatty acids with isolated *trans* double bonds that absorb at 966 cm^{-1} (20). This procedure required the use of an attenuated total reflection (ATR) IR cell in order to decrease the time required for the determination (5 min). With ATR cells, test samples of neat (undiluted) melted fats are neither weighed nor quantitatively diluted in carbon disulfide, a solvent which is both toxic and volatile. This rapid ATR-FTIR procedure was collaboratively studied (21,22), adopted as the American Oil Chemists' Society Official Method AOCS Cd 14d-99 (23), and applied to the determination of *trans* fat in commercial food products (24). The method was also recently used by Sedman *et al.* (25) as a reference method to validate a proposed transmission IR procedure. The ATR-FTIR method was also evaluated for use

with matrices of low *trans* fat and/or low total fat contents such as milk (26) and human adipose tissue (27). Preliminary results indicated that the presence of low levels (<1%) of conjugated *cis/trans* dienes, absorbing near 985 and 947 cm^{-1}, in these matrices interfered with the accurate determination of total isolated *trans* fatty acids (26,27). Attempts to eliminate interfering absorbances by use of spectral subtraction techniques (26,27) were not satisfactory.

To overcome the effect of interferences, the ATR-FTIR method was modified in the present study by inclusion of the standard addition technique. This modification consisted of (i) adding known and increasingly larger amounts of trielaidin (TE) to several *trans* fat test portions; (ii) measuring the resulting relatively intense ATR-FTIR *trans* bands; and (iii) calculating the *trans* content in the parent product using a linear regression equation. The revised procedure was applied to dairy products (butter and cheese) known to contain low levels (<1%) of CLA isomers and to other foods. By using the standard addition technique, the *trans* levels in test portions were thus increased from below 10% (as percentage of total fat) to approximately 17–50%.

MATERIALS AND METHODS

Materials. Lipid standards and trielaidin (TE) were purchased from Nu-Chek-Prep, Inc. (Elysian, MN), Sigma Chemical Co. (St. Louis, MO), and Alltech Associates (Deerfield, IL). All solvents were reagent grade and were obtained from Aldrich Chemical Co. (Milwaukee, WI). Butter, infant formulas, and salad dressings were purchased locally. Two Cheddar cheeses were part of an earlier study carried out at the U.S. Department of Agriculture facility in Wyndmoor, Pennsylvania (28).

FTIR. An FTS-60A Fourier transform infrared spectrometer (Bio-Rad, Digilab Division, Cambridge, MA) was used. The instrument consisted of an SPC 3200 workstation with the IDRIS^{TM} operating system and an optical console. The optical bench included a Michelson interferometer with a quality air bearing, a potassium bromide (KBr) substrate beam splitter, and a DTGS detector. A Spectra-Tech (Shelton, CT) single-reflection ZnSe ATR cell with a capacity of about 50 μL was used for the internal reflection work.

Application of standard addition. Butter was clarified by warming it to 55°C and then filtered. Total fat from cheese, infant formulas, and salad dressings was extracted using a chloroform/methanol/water mixture (29), and the fat was dried under high vacuum. The following steps were performed for each test sample. Each test sample was divided into four portions. To each accurately weighed test portion (neat), known amounts of TE were added to give a final *trans* content between approximately 17 and 50%. The test portions were carefully warmed to about 65°C and mixed to form neat (without solvent) homogeneous solutions. The resulting solutions were measured by ATR-FTIR as described previously (20–24). Specially prepared ultra-degummed bleached cold-pressed soybean oil (Owensboro Grain Co., Owensboro, KY) was used as the reference background material. For each observed spectrum, a baseline was drawn between two fixed points, 990 and 945 cm^{-1}, and the area of the 966 cm^{-1} band was integrated electronically between these same limits. A plot of area of the IR *trans* band vs. the percentage of TE in the total fat was generated for each food product. The resulting linear regression equation was used to calculate the *trans* content (as percentage of total fat) in the original food product.

RESULTS AND DISCUSSION

The ATR-FTIR spectrum (Fig. 1, line A) in the region of the isolated *trans* C-H bending absorption band at 966 cm^{-1} obtained for one of the butter test portions clearly shows interferences including those of conjugated *cis/trans* bands near 985 and 948 cm^{-1}. Following the addition of increasing amounts of TE, symmetric and relatively intense absorption bands were observed with little or no indication of any IR interferences near the base of the band (Fig. 1, lines B–E). This is because the lowest isolated *trans* fat levels measured by ATR-FTIR after addition of TE were >17% (as percentage of total fat). Thus, the standard addition approach obscured other bands near 966 cm^{-1} that may be due to minor potentially interfering components such as CLA.

The regression equations relating the ATR-FTIR area of the 966 cm^{-1} absorbance band to *trans* values and the calculated *trans* values for the dairy products analyzed are given in Table 1. High correlation coefficients were obtained in each case. The calculated *trans* levels for the butter and cheese products were in the range 6.8 to 10.4%, as percentage of total fat. If the standard addition-modified ATR-FTIR procedure were not used, the magnitude of the estimated error in the calculated *trans* values (due to the presence of interferences)

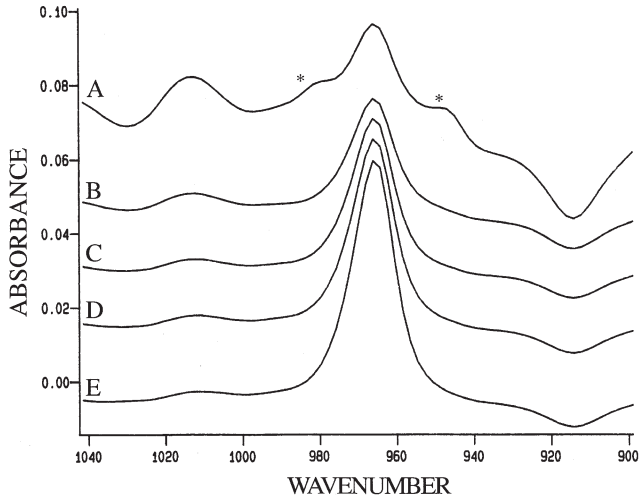

FIG. 1. Comparison of attenuated total reflection-Fourier transform infrared spectra showing the total isolated *trans* fat band at 966 cm^{-1} for a butter product (A) before, and (B–E) after adding trielaidin (TE). Features in the spectra attributed to low levels (<1%) of conjugated linoleic acid isomers (marked with asterisks) were evident before addition of TE (see spectrum A) but were no longer observed after addition of increasing amounts of TE (about 10, 20, 30, and 40%, as percentage of total fat; see spectra B–E, respectively).

TABLE 1
Calculated *trans* Levels in Two Dairy Products Based on the Standard Addition-Modified ATR-FTIR Procedure

Butter 1		Butter 2		Cheese 1		Cheese 2	
TE added[a]	Area (*A*) of *trans* band[b]	TE added[a]	Area (*A*) of *trans* band[b]	TE added[a]	Area (*A*) of *trans* band[b]	TE added[a]	Area (*A*) of *trans* band[b]
0	124.4	0	149.0	0	132.0	0	126.6
10.1	327.2	10.5	340.3	11.7	344.1	10.0	399.2
22.9	557.7	21.3	549.5	22.5	596.2	22.7	626.9
29.2	685.3	30.3	740.0	30.9	732.3	30.1	769.6
41.3	903.2	41.2	942.2	43.1	914.9	40.2	986.2
$A^c = 18.5\ (\%T) + 138.8$		$A^c = 19.7\ (\%T) + 133.5$		$A^c = 18.9\ (\%T) + 160.2$		$A^c = 18.6\ (\%T) + 193.6$	
$R^2 = 0.9996$		$R^2 = 0.9994$		$R^2 = 0.9994$		$R^2 = 0.9976$	
Trans fat[d] = 7.5%[a]		*Trans* fat[d] = 6.8%[a]		*Trans* fat[d] = 8.5%[a]		*Trans* fat[d] = 10.4%[a]	

[a]As percentage of total fat.
[b]Arbitrary units.
[c]Linear regression equation relating area (*A*) of *trans* band to *trans* fat (%*T*, as percentage of total fat).
[d]Intercept /slope. ATR-FTIR, attenuated total reflection = Fourier transform infrared; TE, trielaidin.

would have been significant and equal to 10.4, −11.6, 17.6, and 34.6%, respectively, for the pairs of butter and cheese products listed in Table 1. This error was calculated as follows: [(intercept − area of *trans* band before addition of TE)/intercept] × 100. Calculated *trans* values for two infant formulas and two salad oil dressings are summarized in Table 2.

The total *trans* values obtained for milk fats in this study were consistent with previous reports. Precht (31) reported that in cow's milk the levels of *trans* 18:1 isomers, excluding all other *trans*-diene and -polyene isomers, ranged from 1.91 to 6.34% (30). Emken found the levels of *trans* fat in butter ranged from 2 to 7% (as percentage of total fat). Our values for butter obtained by using the present standard addition ATR-FTIR method, 6.8 and 7.5%, appeared to be near the upper limit (7%) of that reported in one of the cited reports (31). This might suggest that GC methods may still underestimate the total *trans* levels due to peak overlap. Further studies will need to clarify and compare the results of this procedure to GC determinations of total *trans*.

The standard addition modified ATR-FTIR procedure requires only the extraction of fats from the food matrix. It does not require prior chromatographic isolation, cleanup steps, or derivatization of the fat to FAME. It can be applied to food matrices with a *trans* fat content of >1% (as percentage of total fat). The IR absorption represents the total number of *trans* double bonds in the test portions examined, regardless of chain length, and it is only slightly affected by degree of unsaturation. For example, the IR absorptivities of mono-*trans* dienes and PUFA, are slightly lower than those of *trans*-monounsaturated fatty acids, and therefore, the total *trans* content will be somewhat underestimated in food products containing less monounsaturated fatty acids and more PUFA, as found in ruminant and fish fats. However, this low bias is minimized with the standard addition-modified ATR-FTIR procedure because it is based on measuring the absorption of the *trans*-monounsaturated TE added in large excess.

Applicability to foods. The applicability of a method for analyzing *trans* fat in foods is generally determined by two factors: the concentration of *trans* fat (i.e., the percentage of *trans* fat in the total fat of the food), and the lower limit of quantitation of the method. Current FDA regulations state that "saturated fat-free" claims may be used in labeling foods provided that the food contains less than 0.5 g of saturated fat and less than 0.5 g *trans* fatty acid per reference amount customarily consumed and per labeled serving (32). Since 0.5 g *trans* fat per serving is the lowest amount required to be determined for any food under current regulations, the ATR-FTIR method could be used to measure *trans* fat in most food products.

Since total fat per serving is less than 50 g for all the major fat-containing food groups analyzed by the USDA (33), the modified ATR-FTIR procedures (present study) should be applicable to many foods.

Although chromatographic techniques such as Ag-TLC and GC provide information about *trans* fatty acid composition, infrared spectroscopy has been the tool of choice for the determination of total fat with isolated *trans* double bonds. By comparison with the rapid ATR-FTIR method, the standard addi-

TABLE 2
Calculated *trans* Levels for Two Food Products Based on the Standard Addition-Modified ATR-FTIR Procedure

Infant formula 1	Infant formula 2	Salad dressing 1	Salad dressing 2
$A^a = 21.4\ (\%T) + 25.9$	$A^a = 21.2\ (\%T) + 24.8$	$A^a = 20.7\ (\%T) + 58.5$	$A^a = 21.1\ (\%T) + 46.3$
$R^2 = 0.9996$	$R^2 = 0.9998$	$R^2 = 0.9995$	$R^2 = 0.9996$
Trans fat[b] = 1.2%[c]	*Trans* fat[b] = 1.2%[c]	*Trans* fat[b] = 2.8%[c]	*Trans* fat[b] = 2.2%[c]

[a]Linear regression equation relating area (*A*) of *trans* band to *trans* fat (%*T*, as percentage of total fat).
[b]Intercept /slope.
[c]As percentage of total fat. For abbreviations see Table 1.

tion-modified ATR-FTIR procedure required more time, but successfully eliminated any adverse effect on accuracy due to interferences near 966 cm^{-1}. In general, this modified procedure should be applied to products low in total fat (<5%) and when interferences are observed for products with low (<10%) *trans* fat (as percentage of total fat). In particular, the standard addition-modified ATR-FTIR procedure should also be applied to the determination of total isolated *trans* fat in dairy and meat products from ruminants, which are known to contain significant amounts of CLA (nearly 1% of total fat).

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[Received May 22, 2000; accepted March 23, 2001]