Methyl Esters from a Partially Hydrogenated Vegetable Oil Is a Better Infrared External Standard Than Methyl Elaidate for the Measurement of Total *trans* Content

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ABSTRACT: An infrared spectrophotometric procedure, based on the fatty acid methyl ester mixture derived from a partially hydrogenated vegetable oil as the calibration standard, has been developed for accurate analysis of the total *trans* content of hydrogenated fats. This procedure produces more accurate results than the current official methods of Association of Official Analytical Chemists and American Oil Chemists' Society, both of which use methyl elaidate as the external standard. The results obtained with this procedure were in close agreement to those determined by the combined procedure of silver-nitrate thin-layer chromatography and capillary gas–liquid chromatography. The improved results, obtained with the partially hydrogenated vegetable oil methyl esters as the calibration standard, may be attributable to its assortment of *trans* isomers, which may have different absorptivities relative to methyl elaidate.
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KEY WORDS: Gas chromatography, hydrogenated vegetable oils, infrared spectrophotometry, silver nitrate-thin layer chromatography, *trans* fatty acids

Current infrared (IR) spectrophotometric methods generally use methyl elaidate as the external standard for the measurement of total *trans* fatty acids (TFA) in hydrogenated fats (1–3). However, partially hydrogenated vegetable oils (PHVO) contain a variety of *trans* isomers of oleic, linoleic acid, and α-linolenic acids (4–6). Although *trans* isomers of oleic are always the most prevalent *trans* isomer group in PHVO, within this isomer group, often elaidic acid (9*t*-18:1) is not the major isomer. The *t*-18:1 isomers in PHVO form a Gaussian distribution that centers around 10*t*- and 11*t*-18:1 (6). Furthermore, the various *trans* isomers may not absorb to the same extent on IR (7). For example, the absorptivity of methylene-interrupted *cis,trans/trans,cis* isomers of linoleic acid is about 84% of the absorbance of methyl elaidate. The absorptivity of methylene-interrupted *trans,trans*-linoleic acid isomers, despite having two *trans* bonds, is about 174% of the absorptivity of methyl elaidate. Therefore, elaidic acid and its ester forms are not the ideal IR external standards for the measurement of total *trans* content in PHVO. It is expected that a fatty acid methyl ester (FAME) mixture of known *trans* content and well-characterized fatty acid composition, derived from a PHVO, might be a more suitable external standard than methyl elaidate. We have tested this hypothesis with a partially hydrogenated canola oil (PHCO) as the external standard.

EXPERIMENTAL PROCEDURES

Materials and reagents. PHCO of iodine value 65 was donated by Caravelle Foods (Brampton, Ontario, Canada). The total *trans* content of the PHCO sample was determined as 50.5% by the combined procedure of silver nitrate thin-layer chromatography ($AgNO_3$ TLC) and gas chromatography (GC) (see below). Methyl elaidate and methyl oleate were purchased from Sigma Chemical Company (St. Louis, MO). Margarine samples were purchased from a supermarket in Ottawa. All solvents and reagents were of reagent grade.

Preparation of FAME. About 100–150 mg of fat was placed in a 50-mL centrifuge tube, and 4 mL of 0.5N NaOH in methanol was added. The tube was flushed with nitrogen, capped with a Teflon-lined screw cap, and heated at 100°C for 5 min in a heating block. The tube was removed from the heating block and allowed to cool briefly, and 5-mL of 14% BF_3 in methanol was added. After nitrogen flushing and screw capping, the heating continued in the heating block at 100°C for 30 min. The tube was removed and cooled to room temperature. Distilled water (5 mL) and hexane (5 mL) were added, and the FAME were extracted by vigorous shaking for about one minute. The top layer was removed, briefly dried over sodium sulfate, and the hexane was evaporated. When large amounts of methyl esters were required, as for obtaining 2 g PHCO–FAME for the preparation of IR calibration solutions (see below), the methylation was performed in several tubes with about 100–150 mL of fat in each tube, and the methyl esters were combined.

Fourier-transform infrared (FTIR) spectrophotometry. IR measurements were made with a Perkin Elmer 1600 series FTIR spectrometer (Perkin Elmer Corp., Analytical Instruments, Norwalk, CT) in a sealed liquid FTIR cell of thickness 0.1 mm with KBr windows.

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Initially, a stock solution was made by dissolving 2 g of PHCO–FAME in 50 mL carbon disulphide (CS_2). This solution was equivalent to 0.0202 g TFA/mL CS_2. The stock solution was diluted with various volumes of CS_2 to form a series of calibration solutions. The amount of TFA in the calibration solutions ranged from 0.00202 to 0.1010 g/10 mL CS_2 (Fig. 1). These solutions were scanned on FTIR, and a calibration curve of *trans* absorbance versus amount in g of TFA/10 mL solution was constructed (Fig. 1).

Fat from margarine was extracted with hexane and converted to FAME by the procedure described above. Accurately, 0.1 ± 0.0001 g of FAME was weighed and dissolved in 10 mL CS_2. The sample solutions were immediately scanned on the FTIR.

The FTIR spectrometer was set up to collect IR spectra at 4 cm^{-1} resolution in the range from 1110 to 910 cm^{-1}. Conditions employed were identical for all calibration solutions and samples. A clean IR liquid cell was carefully filled with CS_2, avoiding any air bubble entrapment, and scanned from 1110 to 910 cm^{-1}. This single beam spectrum of CS_2 was used as the reference background spectrum for spectra of samples and calibration standards. An aliquot of the calibration solution or sample solution was transferred into the same IR cell and scanned on the IR, covering the same spectral range as that used for pure CS_2. The spectrum obtained was ratioed against the CS_2 reference background spectrum. Before every analysis, the cell was washed three times with pure CS_2 and then three times with the solution to be analyzed. On the spectrum, a baseline tangent to the peak minima adjacent to the analytical peak at 967 cm^{-1}, which is the characteristic absorbance of *trans* double bonds, was constructed. Then, the baseline-corrected absorbance was obtained as the difference in the absorbance at the peak maximum of the analytical peak and the absorbance of the baseline tangent at the same wave number as the peak maximum. This was repeated for each sample. The position of absorption minima varies according to the amount of TFA in the sample (8,9). Therefore, to obtain accurate results, the baselines of the samples were drawn exactly as the baseline in the spectrum of one of the calibration standards that had approximately the same intensity of absorption at 967 cm^{-1}.

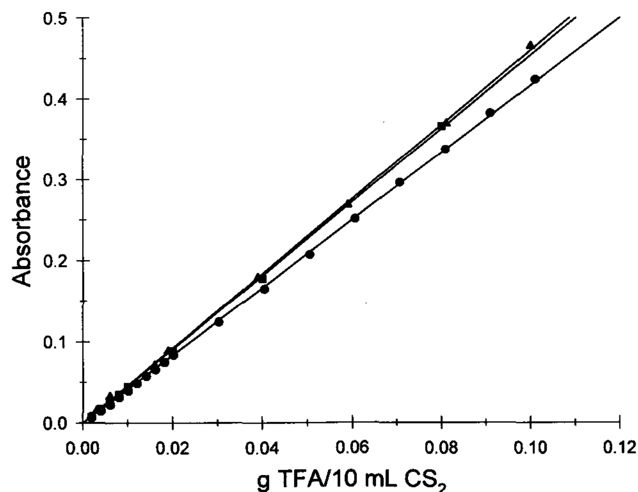

FIG. 1. Calibration curve of *trans* absorbance vs. g total fatty acids/10 mL CS2, developed with calibration solutions prepared from ○ partially hydrogenated canola oil–fatty acid methyl esters (regression equation, $Y = 4.219X - 0.004$), ■ mixtures of methyl elaidate and methyl oleate (regression equation, $Y = 4.541X - 0.001$), and ▲ pure methyl elaidate (regression equation $Y = 4.615X - 0.003$). Each point on a curve is a mean of three analyses.

The baseline-corrected absorbance (A) at 967 cm^{-1} for each calibration solution was plotted against the amount of TFA (in grams) per 10 mL CS_2 solution. By means of first-order regression analysis, the slope and intercept of the line that best fits the above plot were determined.

From the baseline-corrected absorbance (A_s) for each sample, the weight in grams (W_T) of *trans* fatty acids per 10 mL CS_2 solution was determined as shown in Equation 1 by using the values for intercept and slope established for the calibration curve regressions:

$$W_T = (A_S - \text{intercept})/\text{slope} \quad [1]$$

Then, the TFA content, as weight percentage of total FAME, was determined from:

$$\%\ \text{TFA} = (W_T/W_S) \times 100 \quad [2]$$

where W_S is the weight in grams of the FAME of the sample dissolved in 10 mL CS_2.

$AgNO_3$ TLC/GC. The $AgNO_3$ TLC plates were prepared and developed as described previously (4,5). The bands were carefully isolated and analyzed by GC. The GC analyses were performed on a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA), equipped with a split/splitless injector and a fused-silica capillary column (100 m × 0.25 mm i.d.; film thickness 0.20 μm) coated with SP-2560 (Supelco, Inc., Bellefonte, PA). The oven temperature was programmed from 160 to 210°C at a rate of 1.5°C min^{-1} and held at the final temperature for 55 min. Hydrogen was the carrier gas. FAME were identified by reference to a well-characterized FAME mixture derived from a partially hydrogenated canola oil (5).

RESULTS AND DISCUSSION

PHCO calibration standard. The PHCO, used in the present study, was chosen as the calibration standard because it contained approximately 50% TFA, which is the maximum value that can be achieved during commercial partial hydrogenation of vegetable oils with conventional metal catalysts (10). This sample allowed us to prepare a set of calibration solutions whose TFA content ranged from about 1 to 50% which is the range normally encountered in common dietary fats of retail foods (5,11,12). Also, the PHCO used here contained an assortment of TFA (Table 1), including *trans* isomers of oleic and linoleic acids, which are representative of those nor-

TABLE 1
Fatty Acid Composition of the Partially Hydrogenated Canola Oil Used as the Infrared Calibration Standard[a]

Fatty acid	%Total fatty acids
Sum saturated	26.8
Sum *cis*-18:1	21.9
Linoleic	0.2
9*c*,15*c*-18:2	0.1
18:2 conjugated	0.2
7*t* + 8*t* + 9*t* + 10*t* + 11*t*-18:1	33.2
12*t*–18:1	5.7
13*t* + 14*t*-18:1	8.1
15*t*-18:1	1.9
16*t*-18:1	0.7
Sum *trans*-18:1	49.5
tt-18:2	0.1
8*t*,12*c* + 9*c*,13*t*-18:2	0.6
9*c*,12*t*-18:2	0.1
9*t*,12*c*-18:2	0.2
10*t*,15*c* + 9*t*,15*c*-18:2	0.2
Sum *trans* fatty acids	50.5

[a]*c*, *cis*; *t*, trans.

mally present in dietary fats prepared from hydrogenated vegetable oils (4–6).

The calibration curve of IR absorbance vs. the amount of TFA in the PHCO–FAME calibration solutions is shown in Figure 1. For comparison purposes, calibration curves for a series of CS_2 solutions of pure methyl elaidate and mixtures of methyl elaidate and methyl oleate, developed under identical IR settings as that for the PHCO–FAME, are also given in Figure 1. The calibration curves show that *trans* isomers in the hydrogenated canola oil do not absorb to the same extent as that of methyl elaidate or mixtures of methyl elaidate and methyl oleate. These results, therefore, suggest that the actual content of TFA in a hydrogenated oil would be greater than that determined with methyl elaidate as the external standard. The PHCO sample contained 0.1% *trans,trans*- and 1.1% *cis,trans*-isomers of linoleic acid (Table 1), and these isomers may have contributed to a larger extent to the observed lower absorbance of PHCO. This is because *trans* double bonds in linoleic acid geometric isomers have lower absorptivities compared to methyl elaidate (7). The PHCO sample contained a number of *t*-18:1 isomers (Table 1). It is generally presumed that the various *t*-18:1 isomers absorb to the same extent on IR, but there are no actual experimental data to support this assumption. Therefore, it is not known to what extent the individual *t*-18:1 isomers influenced the *trans* absorbance of PHCO.

Effectiveness of PHCO calibration standard. To demonstrate the effectiveness of the PHCO methyl ester calibration standard, margarine samples were analyzed for total TFA content by the method proposed here, and the results were compared with those determined by the AOAC Method 994.14 (1) and AOCS Method Cd 14-61 (2), both of which use methyl elaidate as the external standard. Although tedious, a more accurate and reliable means of quantitation of the *trans* components involves initial fractionation of FAME by $AgNO_3$ TLC according to the degree and geometry of unsaturation and subsequent analysis of the isolated *t*-18:1 fraction by capillary GC. This combined procedure was used as the reference method for assessing the performances of the three IR methods. GC alone cannot be used to measure TFA content, primarily due to the incomplete resolution between the *trans* and *cis* isomers of 18:1 as a result of the overlap of high-delta *t*-18:1 isomers (12*t*-18:1 to 16*t*-18:1) with *c*-18:1 isomers (4,5). Therefore, the primary purpose of the $AgNO_3$ TLC step in the combined $AgNO_3$ TLC/GC procedure is to separate the *t*-18:1 isomers as a group from that of the *c*-18:1 isomers. The proportion of the *t*-18:1 isomers that overlapped with the *c*-18:1 isomers was calculated by comparing the 18:1 region of the GC chromatogram of isolated *t*-18:1 fraction with that of the unfractionated FAME sample. For this purpose, the *t*-18:1 isomers (7*t*-18:1 to 11*t*-18:1) that were well separated from the *c*-18:1 isomers served as the internal standard. The total *t*-18:1 content was then calculated by summing the proportion of the *t*-18:1 isomers (12*t*-18:1 to 16*t*-18:1) that overlapped with the *c*-18:1 isomers with the well-separated *t*-18:1 isomers. Finally, the total TFA content was calculated by summing the above calculated *t*-18:1 with the *trans* isomers of linoleic and linolenic acids. The percentages of the *trans* isomers of linoleic and linolenic acid were directly obtained by GC because these are resolved on GC with almost no interference (4,5). The results obtained for the margarine samples by the combined $AgNO_3$ TLC/GC and the three IR methods are shown in Table 2. The IR determinations were performed in triplicate, but the $AgNO_3$ TLC/GC determination was not repeated, because of the length of time required to analyze a single sample by $AgNO_3$ TLC. However, this is unlikely to undermine the confidence on the accuracy of the $AgNO_3$ TLC/GC results because, unlike the IR methods, there is no weighing involved with the $AgNO_3$ TLC/GC method of this study. In most of the analytical methods, precise weighing of small samples and standards is difficult and could impart a substantial error to the final results. The data in Table 2 show a good agreement between the PHCO methyl ester method and the $AgNO_3$ TLC/GC method; the average difference in the values between the two methods was only about 3%. Whereas, the values determined by the AOCS method were about 16% lower than the actual value, and those determined by the AOAC were 12% lower. The assortment of *trans* isomers present in PHCO may have accounted for the improved results obtained with the PHCO methyl ester external standard.

Limit of quantitation. Although the limit of quantitation of *trans* levels per se was not investigated in the present study, the reproducibility of the analytical results for all margarine samples, including the sample that had the lowest *trans* content (2.3%), was satisfactory (Table 2). These data suggest that the minimum *trans* content that can be measured with good accuracy would be at least 2%. Several other IR procedures, including some of the official methods, claim mini-

TABLE 2
Total Isolated *trans* Content (% of total fatty acids) in Several Margarines: Comparison of Results of Three Infrared Spectrophotometric Procedures Compared with the Actual Values Determined by $AgNO_3$ TLC/GC

Margarine	Actual value $AgNO_3$ TLC/GC	AOCS[a] 14-61	AOAC[a] 994.14	PHCO–FAME–[a] external std.
1	3.0	2.1 ± 0.01	2.3 ± 0.02	2.8 ± 0.02
2	5.8	4.9 ± 0.07	5.1 ± 0.01	5.8 ± 0.01
3	8.5	7.3 ± 0.01	7.7 ± 0.01	8.6 ± 0.01
4	8.9	7.0 ± 0.03	7.3 ± 0.02	8.1 ± 0.02
5	11.4	9.1 ± 0.04	9.6 ± 0.02	10.5 ± 0.0
6	14.1	11.9 ± 0.07	12.4 ± 0.03	13.7 ± 0.03
7	16.2	14.7 ± 0.07	15.3 ± 0.09	16.7 ± 0.12
8	19.4	16.5 ± 0.07	17.1 ± 0.06	18.6 ± 0.06
9	23.6	19.8 ± 0.04	20.6 ± 0.12	22.4 ± 0.13
10	28.1	23.9 ± 0.14	24.9 ± 0.09	27.0 ± 0.10
11	33.1	27.3 ± 0.21	28.3 ± 0.21	30.6 ± 0.22
12	36.6	33.3 ± 0.07	34.5 ± 0.05	37.3 ± 0.06
13	43.5	37.5 ± 0.07	38.9 ± 0.09	42.0 ± 0.12
14	48.6	44.6 ± 0.50	46.3 ± 0.48	49.9 ± 0.47

[a]Values are mean ± SD of three analyses; $AgNO_3$ TLC/GC, silver nitrate thin-layer chromatography/gas chromatography; PHCO–FAME, partially hydrogenated canola oil–fatty acid methyl esters; AOCS, American Oil Chemists' Society; AOAC Association of Official Analytical Chemists.

mum detection limits of 1% (3,9) or 0.5% (8,13). However, although individual laboratories may be able to detect such low levels, satisfactory reproducible results for samples with lower levels of *trans* fatty acids have never been reported when IR methods were subjected to interlaboratory evaluations (13–15). Because of this difficulty, AOAC Official IR method 994.14 (1,15) was not recommended for samples that contain less than 5% *trans* fatty acids. Single-step direct GC analysis on very polar capillary columns may be a better option for determination of low levels of *trans* content (15,16).

This study demonstrates that methyl esters of PHVO of known TFA content are better external standards than methyl elaidate for IR measurement of total TFA content in hydrogenated vegetable oils. It is proposed that a well-characterized product (detailed fatty acid composition and total *trans* content) of partially hydrogenated oil FAME be made available from a laboratory chemical supplier for use as IR external standard. A procedure such as the combined $AgNO_3$ TLC/GC described here could be used for establishing the fatty acid composition and actual TFA content of the hydrogenated oil FAME external standard.

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