Rapid Determination of the Total *trans* Content of Neat Hydrogenated Oils by Attenuated Total Reflection Spectroscopy

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ABSTRACT: A Fourier transform infrared spectroscopy procedure is described for quantitating the levels of total *trans* triglycerides or their fatty acid methyl ester derivatives in neat fats and oils. It requires either warming or no preparation of the laboratory sample, and about 5 min for spectroscopic measurement, band area integration, and calculation of the *trans* content from a linear regression equation. To eliminate the strongly sloping background of the 966-cm^{-1} *trans* band, the single-beam spectrum of the *trans*-containing fat is "ratioed" against that of an unhydrogenated oil or a reference material that contains only *cis* double bonds. Thus, a symmetric absorption band on a horizontal background is obtained. The area under the *trans* band can then be accurately integrated between the same limits, 990 and 945 cm^{-1}, for all *trans* levels investigated. To speed up the analysis, an attenuated total reflection liquid cell was used, into which oils, melted fats or their methyl esters were poured without weighing or quantitative dilution with the toxic and volatile carbon disulfide solvent. The *trans* levels determined by attenuated total reflection were closer to those found by capillary gas chromatography when the hydrogenated fat was measured against the corresponding unhydrogenated oil than when it was measured against a *cis* reference material. Small differences were found between *trans* levels in hydrogenated fat test samples and the corresponding methyl ester derivatives (9.3 and 2.2% at about 2 and 41% *trans*, respectively). The lower limits of identification and quantitation were 0.2 and 1%, respectively.
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KEY WORDS: Attenuated total reflection, infrared, *trans* fatty acids.

Regulations to implement the U.S. Nutrition Labeling and Education Act of 1990 require that the amounts of *trans* fatty acids be included in the amount of total fat declared on the label and not in the amount of mono- or polyunsaturated fatty acids (1). The cholesterol-raising potential of hydrogenated fat due to the presence of *trans* fatty acids (2–4) prompted Grundy (5) to suggest that labeling requirements be modified to designate *trans* monounsaturated as well as palmitic, myristic, and probably lauric fatty acids as cholesterol-raising species. In 1994, the U.S. Food and Drug Administration (FDA) was petitioned to include *trans* fatty acids as part of nutrition labeling for saturated fatty acid content (6); the FDA is reviewing this labeling issue. The discussion of the nutritional significance of *trans* fatty acids has led to increased interest in accurate and rapid analytical methods for quantifying *trans* fatty acids.

The determination of total *trans* fatty acids by infrared (IR) spectroscopy is a widely used procedure (7) that has been standardized (8–11). Its importance stems from the fact that the C-H out-of-plane deformation band, observed at 966 cm^{-1}, is uniquely characteristic of isolated double bonds with *trans* configuration. These double bonds are found mostly in *trans*-monoenes, and at much lower levels in minor hydrogenation products, such as methylene-interrupted and nonmethylene-interrupted *trans,trans*-dienes, mono-*trans*-dienes, and other *trans*-polyenes. This procedure is extremely useful, even though it is limited to products that contain less than 5% conjugated unsaturation (7) because conjugated *trans,trans* (near 990 cm^{-1}) or *cis/trans* (near 990 and 950 cm^{-1}) double bonds exhibit absorption bands that are sufficiently close to interfere with the isolated *trans* double bond absorption. A band near 935 cm^{-1}, due to the O-H out-of-plane deformation in carboxylic acids, also interferes with the isolated *trans* band at 966 cm^{-1}, particularly at low *trans* levels (less than 15%) (7). However, this interference could be easily eliminated by esterifying the long-chain fatty acids.

The overlap of the *trans* absorption at 966 cm^{-1} (Fig. 1A) by other broad bands in the spectrum produces a strongly sloping background that converts the *trans* band into a shoulder at levels below 2% and reduces the accuracy of the determination. In 1965, Firestone and LaBouliere (12) reported that the IR spectroscopy determination of *trans* unsaturation yields a high bias for triacylglycerols and a low bias for fatty acid methyl esters. Since then, many procedures in the literature have proposed changes that range from minor refinements to major modifications. These include applying arithmetic compensation to eliminate biases (12); using dual-beam differential spectrophotometry to eliminate background inter-

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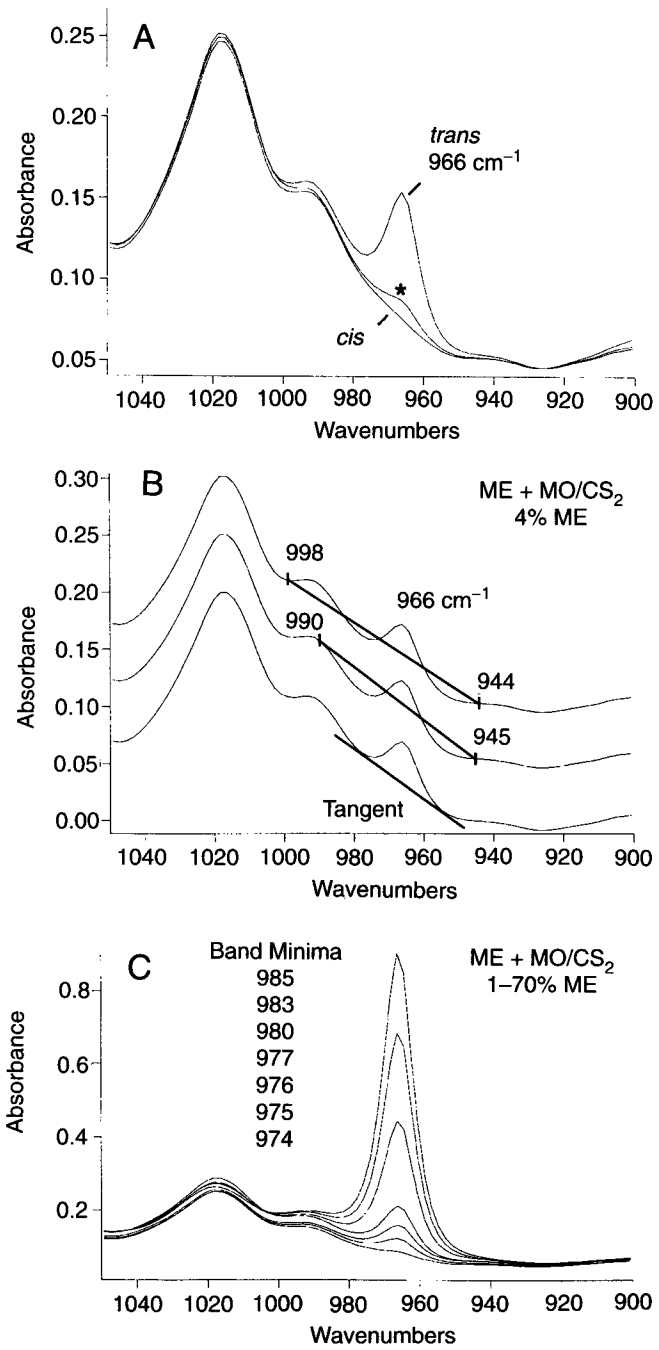

FIG. 1. A, Fourier transform infrared (FTIR) spectroscopy absorption spectra obtained for CS_2 solutions of 7% (top line) and 1% (middle line) methyl elaidate (ME) in methyl oleate (MO). Because the background is strongly sloping, the band at 966 cm^{-1}, characteristic of isolated *trans* double bonds, is not symmetric (top) and becomes a shoulder (*, middle line) at low levels (below 2% ME). The bottom spectrum was obtained for MO in CS_2. B, FTIR spectroscopy absorption spectra obtained for the same CS_2 solution of 4% ME in MO, showing different base-lines drawn according to the official AOCS method (Ref. 9) (top), and two other procedures described in References 17 (middle) and 19 (bottom); the base-line is drawn between two fixed points (top and middle) and as a tangent (bottom). C, FTIR spectroscopy absorption spectra for CS_2 solutions of 1, 4, 7, 10, 30, 50, and 70% ME in MO. The band minimum shifted from about 974 cm^{-1} for the weakest *trans* band to about 985 cm^{-1} for the strongest one, which means that if a tangent is drawn the integration limits will vary with concentration.

ferences (13); applying the internal absorption band ratioing procedure (14,15); eliminating the volatile carbon disulfide (CS_2) solvent by using attenuated total reflection (ATR) (15,16) or 0.1-mm transmission (17,18) cells; analyzing fats as methyl esters to eliminate triacylglycerol high bias (17–22); using the advantages offered by Fourier transform infrared (FTIR) spectroscopy instrumentation (15,17,18, 20–22); modifying the calibration procedure for two-component (14,15,17,19-22) or multiple-component (15,16,18) mixtures that contain a *trans* reference material by generating one (14–21) or two (22) calibration plots (one for *trans* levels above 10% and the other for those below 10%) instead of calculating the absorptivity of a standard (9,10,12,13); using regression analysis (15,17,18,20,22); using the band height (8–16,18,19,22) or area (17,18,20,21) as the independent variable; drawing the base line between two fixed points (9,10,12–18,20,21) or two concentration-dependent tangent points (8,11,19,22); and applying partial least-squares chemometric procedures (18) or post-measurement spectral subtraction manipulations (18,21). This last procedure (18,21) corrected the highly sloping background in the absorption spectrum. However, it entailed the additional IR spectroscopy measurement of an appropriate reference material and the digital subtraction of this reference absorption spectrum from that of the test portion.

In the present study, a procedure is proposed that measures the 966-cm^{-1} *trans* band as a symmetric feature on a horizontal background. This was achieved by "ratioing" the *trans* analyte single-beam spectrum against that of a reference material, such as triolein (TO), methyl oleate (MO), or the corresponding unhydrogenated oil or its methyl esters. The ATR technique (23) was also used to avoid the weighing of test portions and their quantitative dilution with the volatile CS_2 solvent.

MATERIALS AND METHODS

Lipid standards and reagents were supplied by Nu-Chek-Prep, Inc. (Elysian, MN), Sigma Chemical Co. (St. Louis, MO), and Alltech Associates (Deerfield, IL). All solvents were reagent-grade and were supplied by Aldrich Chemical Co. (Milwaukee, WI). Partially hydrogenated fat test samples were donated by Dr. Wayne E. Emmons of SGS Control Services, Inc. (Deer Park, TX). Methyl esters were prepared according to AOAC Official Method 969.33 (24).

An FTS-60A FTIR spectrometer (Bio-Rad, Digilab Division, Cambridge, MA) was used, which consisted of an SPC 3200 workstation with the IDRIS™ 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. Initial experiments were conducted with a sealed precision path-length (1 mm) transmission liquid cell (NaCl windows), acquired from Spectra-Tech (Shelton, CT). For the internal reflection work, the Bio-Rad 45° rectangular ZnSe (50 × 5 mm × 5 mm) liquid ATR cell was used. The ZnSe optical element

was enclosed in a stainless-steel cuvette with a capacity of about 1.5 mL.

For transmission work, mixtures of methyl elaidate (ME) and MO reference materials were dissolved in CS_2 (about 20 mg/mL). Caution: CS_2 is a flammable liquid, an acute fire and explosion hazard, and a volatile (boiling point 46.3°C) and toxic solvent that is readily absorbed through skin. For the internal reflection study, the ATR liquid cell was filled with neat test sample without weighing. The ATR cell was first warmed to about 50°C in an oven whenever melted fats were analyzed. At 4 cm^{-1} resolution, 64 or 256 scans were collected. The larger scan number was necessary for low (below 1%) *trans* levels. For each observed spectrum, a base line was drawn between two fixed points, 990 and 945 cm^{-1}, and the area of the 966 cm^{-1} band (integrated between these same limits) was calculated (electronically). Calibration plots (area vs. percentage *trans*) were generated by ATR for 0.91–45.87% ME in the ME and MO [or 0.40–44.18% trielaidin (TE) in the TE and TO] neat reference mixture.

RESULTS AND DISCUSSION

The adverse impact of a highly sloping background on accuracy can be appreciated by considering its effect on the measurement of the absorption band height or area. In either case, a linear base-line must be defined as shown in Figure 1B, which illustrates three different base lines drawn for identical spectra (4% *trans*) according to three published procedures (9,17,19). When a line is drawn between two fixed points, the base-line intersects the spectrum and leads to negative areas. If a line is drawn such that it is tangent to the spectral curve, the two tangent points will vary for test samples with different *trans* concentrations (Fig. 1C). Consequently, none of these procedures is fully satisfactory.

With single-beam FTIR spectroscopy instruments, an absorption spectrum is obtained by "ratioing" the single-beam spectrum of a *trans* analyte dissolved in CS_2 against that of the solvent (Fig. 2A). To observe a symmetric 966-cm^{-1} band on a horizontal background for a CS_2 solution of *trans* fatty acid methyl esters (FAME), a CS_2 solution of MO was used (instead of CS_2) for measuring the reference background single-beam spectrum (Fig. 2B). The resulting absorption spectra (Fig. 2C) demonstrate that this method can almost completely ratio out the broad features that caused the highly sloping background, and eliminate the disadvantages of fixing the base-line points (at 990 and 945 cm^{-1}), particularly at low *trans* levels. With this approach, one can also ratio stored single-beam spectra, which were previously measured for different test samples, against the spectrum of another reference background material, such as the methyl esters of the fatty acids in an unhydrogenated oil.

Use of the ATR liquid cell added speed and convenience to the method because weighing test portions and diluting them with CS_2 were no longer required. ATR quantitation was based on measurement of the integrated area under the 966-cm^{-1} absorption band between 990 and 945 cm^{-1} (Fig. 3A).

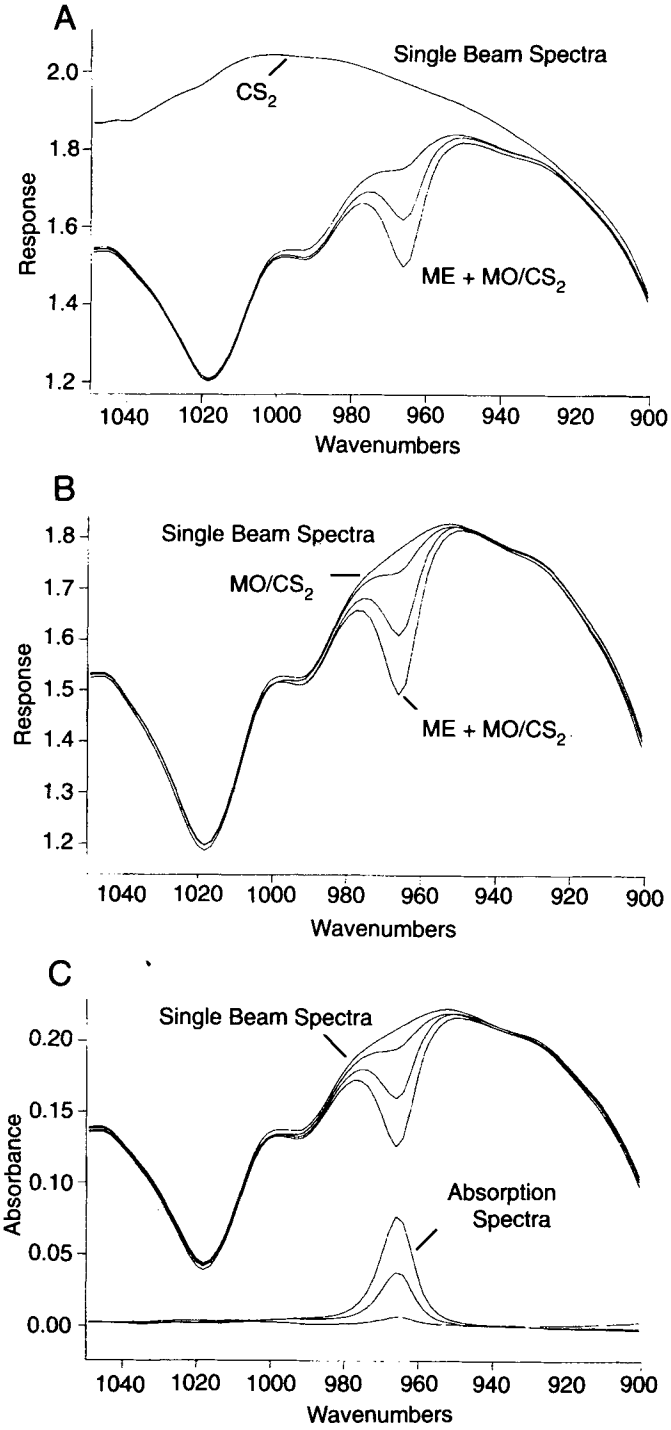

FIG. 2. A, Single-beam spectra for the reference background material CS_2 (top spectrum), and for CS_2 solutions of 1, 4, and 7% ME in MO (three bottom spectra). B, same as A after the addition of standard MO to the CS_2 reference background material. C, By "ratioing" the single-beam spectrum for each of these CS_2 solutions of 1, 4, and 7% ME in MO against that of MO in CS_2, the resulting FTIR spectroscopy absorption spectra exhibited symmetric 966-cm^{-1} bands on a horizontal background. See Figure 1 for abbreviations.

Calibration plots (Fig. 3B and 3C) of area vs. percentage ME or TE were generated for reference mixtures in the range of 0.91–45.87% ME in MO, or 0.40–44.18% TE in TO, with

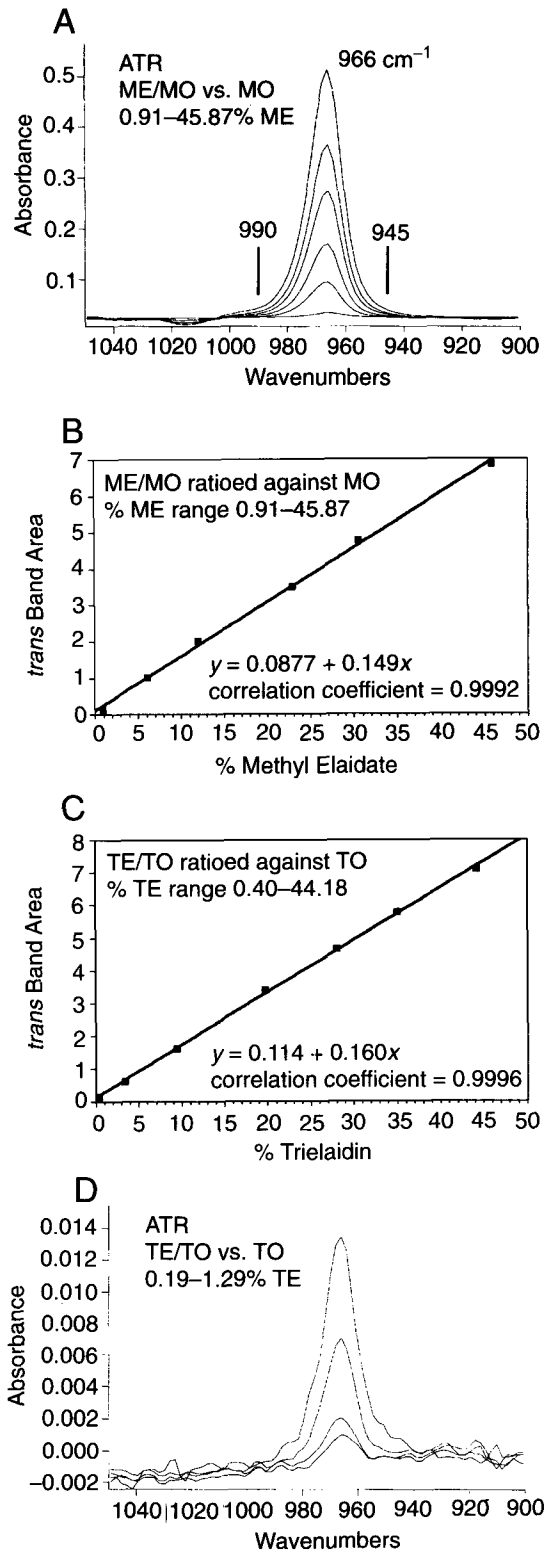

FIG. 3. A, When an attenuated total reflection (ATR) liquid cell was used, CS_2 was no longer needed. Symmetric bands on a horizontal background were also obtained when neat mixtures of ME in MO were "ratioed" against neat MO. Calibration plots of the area of the 966-cm^{-1} band (integrated between 990 and 945 cm^{-1}) against the percentage of B, ME in MO, and C, trielaidin (TE) in triolein (TO). D, Absorption bands for low *trans* levels ranging from 0.19 to 1.29% TE in TO. See Figure 1 for abbreviations.

TABLE 1
Attenuated Total Reflection Quantitation of Trielaidin (TE) in Corn Oil vs. Corn Oil[a]

Amount (g)		TE (%)		Infrared spectroscopy as % of true
TE	Corn oil	True	Found	
0.0059	2.2017	0.27	0.11	39.7
0.0219	2.1577	1.01	0.84	83.7
0.1130	1.8327	5.81	5.89	101.3
0.2560	2.0969	10.88	11.12	102.0
0.4274	1.6072	21.01	21.08	100.3
0.5931	1.4113	29.59	29.52	99.8

[a]n = 4; average = 100.8; relative SD, % = 0.7.

MO or TO as the reference background material, respectively. The regression line parameters for the *trans* standards are given in Figure 3 (B and C). The high correlation coefficient in each case indicates that the variables are sufficiently linear for routine analysis over the given ranges. For test samples, the percentage *trans* was calculated from the area of the observed absorption band at 966 cm^{-1} and the corresponding linear regression equation describing the calibration plot.

To determine the limit of quantitation, *trans* levels in the range 0.27–29.59% TE were measured in reference materials that were prepared by adding known amounts of TE to corn oil. The resulting single-beam spectra were ratioed against the spectrum of corn oil. The analytical results (Table 1) indicate that at levels near or below 1% TE, the values determined by ATR were lower than expected. In contrast, accuracy was satisfactory in the range 5.81–29.59% TE. These findings were verified with another set of reference materials (0.27–39.50% TE) in which TO was substituted for corn oil (Table 2). Once again, accuracy was satisfactory for nine determinations ranging from 1.29 to 39.50% TE. These data suggest that the limit of quantitation is probably 1% TE, even though the identification limit is close to 0.2% TE (Fig. 3D).

TABLE 2
Attenuated Total Reflection Quantitation of Trielaidin (TE) in Triolein (TO) vs. TO

Amount (g)		TE (%)		Infrared spectroscopy as % of true
TE	TO	True	Found	
0.0046	1.7011	0.27	0.12	42.7
0.0118	1.6852	0.70	0.57	81.2
0.0223	1.7088	1.29	1.19	92.2
0.0569	2.2686	2.45	2.42	98.8
0.0861	1.5476	5.27	5.56	105.5
0.2710	2.3267	10.43	10.17	97.5
0.4630	2.6417	14.91	15.60	104.7
0.6431	2.2588	22.16	22.28	100.5
0.8675	1.8238	32.23	32.26	100.1
0.8231	2.3066	26.30	26.40	100.4
0.8884	1.3609	39.50	39.13	99.6

[a]n = 9; average = 99.9; relative SD, % = 3.9.

At levels below 1%, the *trans* band may no longer be symmetric and the signal-to-noise ratio was clearly reduced. Satisfactory repeatability was demonstrated by measuring a reference material that consisted of 1.5% TE in TO (vs. TO) four times. The relative standard deviation was 1.8%.

Quantitative ATR results for partially hydrogenated soybean oils are summarized in Table 3. The percentage TE values were lower when the reference background material was refined, bleached, and deodorized (RBD) soybean oil than when it was TO. The difference was a constant equal to about 2.6 percentage points (Fig. 4A). Similar results were found for the corresponding methyl esters, which are also listed for comparison in Table 3. Test sample 2 was the RBD soybean oil selected as a reference background material. When its single-beam spectrum was ratioed against that of TO, a weak feature was observed that was not symmetric, which is consistent with a *trans* level below the 1% TE limit of quantitation. A similar result was obtained for the methyl esters of test sample 2 when they were measured against MO. The differences between the percentage *trans* values found for the partially hydrogenated fat test samples and those found for the corresponding methyl esters (Table 3) are small (9.3 and 2.2% at about 2 and 41% *trans*, respectively); these data suggest that the ATR determination is nearly independent of whether the partially hydrogenated fat is esterified. As mentioned above, in those cases in which the reference background material was TO or MO, the percentage *trans* determination was higher by about 2.6 percentage points than when it was the RBD soybean oil or its methyl esters. One small factor contributing to this difference may be the level of *trans* isomers, generally about 0.5%, found in RBD oils. The nature of the reference background material may account for this discrepancy. The major constituents of the reference background RBD oil are also found in the partially hydrogenated fat. Because the composition of the unhydrogenated RBD oil is closer than that of TO (a single compound) to the composition of the partially hydrogenated fat, background effects are probably ratioed out less accurately by TO than by unhydrogenated RBD oil, which should be the preferred reference background material. This argument is also true for the corresponding methyl esters and MO.

TABLE 3
Attenuated Total Reflection Quantitation of Percentage *Trans*

Test sample	Fat		Esterified fat	
	vs. Oil	vs. Triolein	vs. Esterified oil	vs. Methyl oleate
1	16.11	18.73	16.33	18.97
2	—[a]	—[b]	—[a]	—[b]
3	1.86	4.48	2.05	4.72
4	7.99	10.65	8.09	10.75
5	11.37	14.00	12.08	14.74
6	22.81	25.42	23.77	26.43
7	37.53	40.13	38.35	41.04

[a]Used as reference background material.
[b]Percentage *trans* value was below the limit of quantitation.

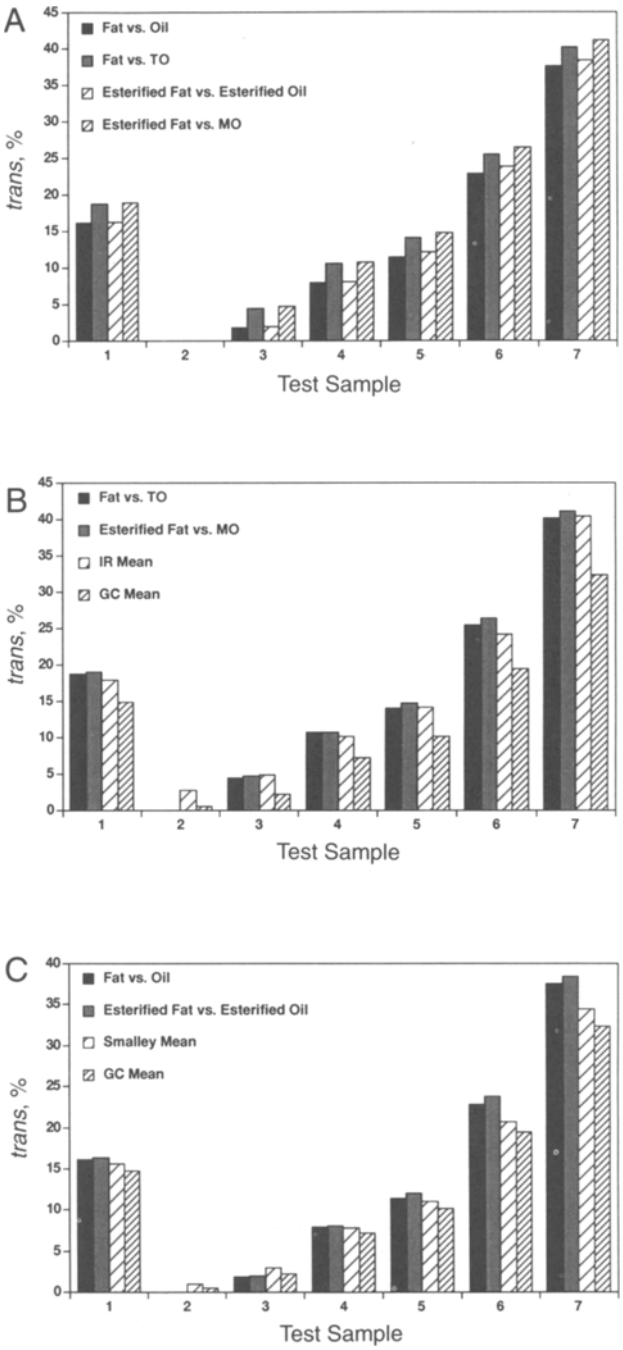

FIG. 4. A, Quantitative ATR data given as percentage TE or ME for seven test samples. Small differences in *trans* levels were found between hydrogenated fats and the corresponding methyl esters. The determination was strongly dependent on the nature of the reference background material. Quantitative comparison between the Smalley collaborative study results and B, the high ATR values (reference background: TO or MO, and C, the low ATR values (reference background: unhydrogenated refined, bleached, and deodorized soybean oil or its corresponding methyl esters). See Figures 1 and 3 for abbreviations.

These ATR data were compared with those found for the same test samples in an interlaboratory study (1994 AOCS *trans* Series Smalley Check Sample Program) that involved 40 laboratories. These test samples were analyzed by using

standard IR spectroscopy (9) and capillary gas chromatography (GC) (25) methods in 10 and 30 laboratories, respectively (Table 4). The Smalley IR spectroscopy mean values (26) were in good agreement with the ATR values that were obtained with the TO or MO reference background material (Fig. 4B). When the unhydrogenated oil or its corresponding methyl esters were used as reference background material, the resulting ATR data were closer to the overall Smalley mean values (26) and, as expected, higher than the Smalley GC mean values (26) (Fig. 4C). This is understandable because (i) only the *trans* monoenes are determined by GC (25), whereas all isolated *trans* double bonds give rise to the 966-cm^{-1} band (7), and (ii) some minor *trans* monounsaturated positional isomers are unaccounted for in the GC determination because they overlap the more intense GC peaks observed for monoenes with *cis* double bond configuration (27). However, these reasons cannot explain the large differences found at low *trans* levels. For the two test samples with the lowest *trans* content (test samples 2 and 3 in Table 4), the Smalley IR spectroscopy mean values (2.85 and 4.83%, respectively) were more than five- and twofold higher than the corresponding values found by GC (0.555 and 2.24%, respectively). Because the *trans* content of RBD soybean oil is more likely to be 0.555 than 2.85%, the accuracy of the GC determination (25) at low levels is probably better than that of the IR spectroscopy method (9) used in the collaborative study. The *trans* values found by ATR were closer to the Smalley GC mean values when the single-beam spectrum of the partially hydrogenated fat (or its methyl esters) was ratioed against the single beam spectrum of the unhydrogenated RBD soybean oil rather than against that of TO (or MO).

In the ATR procedure, it is desirable to use a reference background material that is as close as possible in composition to the hydrogenated oil being measured, namely, the corresponding unhydrogenated oil. Ideally, it is equally important that such reference materials contain no *trans* double bonds. Bleaching and deodorization of polyunsaturated vegetable oil unavoidably produce low, yet measurable levels of *trans* fatty acids. In practice, the use of appropriate reference materials that contain the lowest possible *trans* levels is recommended. For example, oils with relatively low levels of polyunsaturation, such as palm or olive oil, may meet this criterion. However, their fatty acid compositions are different from soybean or corn oil, which are more commonly used in hydrogenated products.

The strongly sloping background of the 966-cm^{-1} band, observed for hydrogenated soybean oils, was successfully ratioed out by using unhydrogenated RBD soybean oil or reference background materials with only *cis* double bonds. The band area could then be integrated between the same fixed limits at all *trans* levels. By using an ATR liquid cell, the need for the volatile and toxic CS_2 solvent was eliminated, and the determination was almost independent of whether the analyte was a fat or the corresponding FAME. The limit of quantitation of the *trans* band was 1%. The ATR determination was strongly dependent on the nature of the reference background material used; with RBD soybean oil (or its methyl esters), the *trans* levels were lower than with TO (or MO), and probably more accurate because they were closer to the Smalley GC mean values.

TABLE 4
1995 Smalley Series, Percentage *trans*

Test sample	Infrared spectroscopy mean	Gas chromatography mean	Smalley mean
1	17.87	14.81	15.59
2	2.85	0.555	1.05
3	4.83	2.24	2.95
4	10.16	7.20	7.85
5	14.23	10.16	11.04
6	24.22	19.40	20.71
7	40.42	32.32	34.44

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