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Rapid determination of *trans*-fatty acids in human adipose tissue Comparison of attenuated total reflection infrared spectroscopy and gas chromatography

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Abstract

A rapid attenuated total reflection (ATR) infrared (IR) spectroscopy procedure was used for quantitating the levels of total *trans*-fatty acid methyl ester (FAME) derivatives in neat (without solvent) test samples isolated from human adipose tissue. This procedure requires no weighing of the laboratory sample. The single-beam spectrum of the *trans*-containing FAMEs was 'ratioed' against that of a reference material having only *cis* double bonds in order to obtain a symmetric absorption band at 966 cm^{-1} on a horizontal background. A single-reflection ATR diamond cell that requires only about 1 μl of neat FAMEs was used. The average level of *trans*-fatty acids in human adipose tissue found by ATR (3.07±0.27%) was generally higher than that obtained by gas chromatography (2.59±0.20%). Reasons for such a difference are discussed. © 1998 Elsevier Science B.V.

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1. Introduction

Recent interest in *trans*-fatty acids (TFAs) was sparked by epidemiological evidence linking TFAs to higher plasma total cholesterol and low-density-lipoprotein (LDL) cholesterol and increased incidence of coronary heart disease (CHD) [1,2]. By contrast, analysis of human adipose tissue TFAs indicated no significant correlation between dietary TFA intake and acute myocardial infarction [3], cardiovascular risk factors [4], and sudden cardiac death [5]. The major constituents of TFAs found in human adipose tissue are *trans* C18:1 positional isomers, while the minor ones included C18:2 with isolated as well as conjugated double bonds. Recently, the identities of the conjugated C18:2 geometric isomers c9t11, t9c11, c9c11, and t9t11 found in human adipose tissue were confirmed by using gas chromatography (GC)–infrared (IR) spectroscopy and GC–mass spectrometry [6].

The discussion of the nutritional significance of TFAs has led to increased interest in rapid analytical methods for quantifying their levels. The quantitation of fatty acids with isolated *trans* double bonds by IR spectroscopy has been widely used. Standard IR methods [7,8] are based on measuring the unique C–H out-of-plane deformation absorption at 966 cm^{-1} for analytes that contain isolated (non-conju-

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gated) *trans* double bonds. Usually, the *trans* band overlaps with broad features in the IR spectrum and leads to a strongly sloping background [9] that reduces the accuracy of the quantitation, especially at the *trans* levels of interest for biological applications (below 5%). In order to obtain a symmetric *trans* band on a horizontal background, an IR procedure was recently proposed for hydrogenated fats [10,11] and applied to commercial food products [12], in which the *trans* analyte single-beam spectrum was 'ratioed' [13] against that of a reference background material that is *trans* free. By using an attenuated total reflection (ATR) cell [14] the infrared measurement became rapid because it could then be carried out on neat (without solvent) analytes. Hence, the requirement to weigh and quantitatively dilute *trans* analytes in carbon disulfide was eliminated.

In the present study, a novel single-reflection diamond ATR cell was used because it required only about a single microliter of neat fatty acid methyl esters (FAMEs) isolated from human adipose tissue. Quantitative GC data are also reported.

2. Materials and methods

2.1. Materials

The biological samples were human subcutaneous adipose tissues. They were a gift from Professor Dr. med. H.J. Boehles (Johann-Wolfgang-Goethe University Clinic, Frankfurt, Germany) and were obtained from a random group of male and female children with inguinal hernia aged 1–13 years. Lipid standards and reagents were supplied by Nu Check Prep, Inc. (Elysian, MN, USA), Sigma Chemical Co. (St. Louis, MO, USA), and Alltech Associates (Deerfield, IL, USA). The TFA–FAME references, myristelaidic acid methyl ester (C14:1, t9), palmitelaidic acid methyl ester (C16:1, t9), petroselaidic acid methyl ester (C18:1, t6), elaidic acid methyl ester (C18:1, t9), *trans*-vaccenic acid methyl ester (C18:1, t11), octadecadienoic acid methyl ester mixture (containing C18:2 isomers: t9t12, c9t12 and t9c12), and octadecatrienoic acid methyl ester mixture (containing eight geometrical C18:3 isomers: t9t12t15, c9t12t15, t9c12t15, t9t12c15, c9c12t15, c9t12c15, t9c12c15, and c9c12c15) were purchased from Sigma (Deisenhofen, Germany). Conjugated C18:2 isomers, c9t11 and t9t11, were purchased from Matreya, Inc. (Pleasant Gap, PA, USA). All solvents were reagent grade and were supplied by Aldrich Chemical Co. (Milwaukee, WI, USA).

2.2. Methods

2.2.1. Lipid extraction and fatty acid methyl ester preparation

Lipids were extracted according to Folch et al. [15]. Methyl esters were prepared according to Fritsche and Steinhart [16] by transesterification with potassium methoxide; 0.5 ml potassium methoxide (5%, w/w, in methanol) was added to the extracted fat solution in a Teflon screw-capped Pyrex tube (100×18 mm). The tube was tightly capped, vortexed, and heated at 60°C for 15 min. After cooling the solution down, 1.5 ml sulfuric acid (2%, w/w) was added and the tube was vortexed again. One μl taken from the clear organic phase was injected into the gas chromatograph.

2.2.2. ATR-IR spectroscopy instrumentation

An FTS-60A Fourier transform infrared spectrometer (Bio-Rad, Digilab Division, Cambridge, MA, USA), consisting of an SPC 3200 workstation with the IDRIS TM operating system was used. A Graseby Specac (Fairfield, CT, USA) Golden Gate Single Reflection Diamond ATR cell that requires about 1-μl test samples was used. This accessory contains a 2×2 mm diamond with an active sampling area of 0.6 mm in diameter.

2.2.3. ATR procedure

Neat FAME test samples (1 μl) were placed without weighing on the horizontal (face-up) diamond sampling surface of the cell. Sixty-four scans were collected at 4 cm^{-1} resolution. After a measurement was made, the surface of the diamond was cleaned by wiping it with low-lint paper.

2.2.4. ATR calibration and quantitation

ATR quantitation was based on the measurement of the integrated area under the 966-cm^{-1}. A calibration plot of 'area' vs. 'percentage' *trans*-FAMEs was generated for reference mixtures in the range of 0.41–7.59% methyl elaidate (ME) in methyl oleate

(MO). The regression line parameters for the *trans* standards were 0.001077 (intercept) and 0.01779 (slope). The correlation coefficient was 0.9997. A Matreya commercial oil reference mixture, RM-3, was used as the IR reference background material. It reportedly consists of the methyl esters of myristic (1.0%), palmitic (4.0%), stearic (3.0%), oleic (45.0%), linoleic (15.0%) linolenic (3.0%), arachidic (3.0%), behenic (3.0%), erucic (20.0%) and lignoceric (3.0%) fatty acids. For test samples, the percentage *trans* was calculated from the area of the observed absorption band at 966 cm^{-1} and the linear regression equation that describes the calibration plot.

2.2.5. GC instrumentation

Analysis of FAMEs by GC was carried out on a HP 5890 Series II plus gas chromatograph fitted with an automatic sampler (HP 7673) for a split-type injection and a flame ionisation detector (FID). A fused-silica capillary column coated with 100% cyanopropyl polysiloxane (CP™Sil 88, 50 m, 0.25 mm I.D., 0.20 μm film thickness, Chrompack, Middelburg, The Netherlands) was used to separate the *cis/trans*-fatty acid isomers. The GC conditions were previously reported [16].

2.2.6. FAMEs identification and quantification

The identification and quantification of the FAMEs, including the individual TFA isomers C16:1, t9; C18:1, t6–t11; C18:2, t9t12, c9t12, and t9c12; and C18:3, t9t12t15, c9t12t15, t9c12t15, t9t12c15, c9c12t15, c9t12c15, t9c12c15, and c9c12c15; as well as conjugated C18:2, c9t11 and t9t11 were performed with an external standard. The contributions from all *trans* C18:3 isomers to the total TFA levels in adipose tissue was usually $<$0.01%. The TFA amounts were calculated as relative percentage of all identified FAMEs. Quantitative analyses were carried out with a HP Chem Station version A.03.34-1994.

3. Results and discussion

The C–H out-of-plane deformation *trans* IR band observed at 966 cm^{-1} is uniquely characteristic of isolated double bonds with *trans* configuration [9]. These double bonds are found mostly in *trans*-monoenes, and at much lower levels in methylene-interrupted and non-methylene-interrupted *trans*, *trans*-dienes, mono-*trans*-dienes, and other *trans*-polyenes. The newly adopted 1994 AOAC and 1995 AOCS Official Methods [7,8] are not fully satisfactory because they assume that the band at 966 cm^{-1} is isolated. However, this band overlaps with broad features in the spectrum [9] and reduces the accuracy of the IR quantitation at *trans* levels below 5%. This disadvantage was eliminated by using the 'ratioing' and ATR procedure [10–12]. The accuracy, repeatability and reproducibility of this procedure were satisfactorily tested on hydrogenated vegetable oils in a limited (five-laboratory) collaborative study [17] with ATR cells having a 50-μl capacity; for instance, for three pairs of triacylglycerol blind duplicates spiked with 0.52, 1.1, and 4.15% trielaidin, the means for %trielaidin (as percent of total triacylglycerols) were determined to be 0.53, 1.00, and 4.08%, respectively. The corresponding relative standard deviations for these test samples were 17.33, 15.58, and 5.99, respectively.

The performance in quantitation of the diamond ATR cell was documented. A high correlation coefficient (0.9997) was obtained over the range of interest (below 8%, Fig. 1), which indicated that the variables were sufficiently linear for the determination of *trans* FAMEs isolated from human adipose tissue (Table 1). The accuracy of the ATR determination depended on finding the most appropriate background reference material suitable for the analysis of human adipose tissue. Ideally, this material should be *trans* free and should have a fatty acid composition that is as close as possible to that of human adipose tissue. The RM-3 reference material used (see Section 2) consisted of a mixture of FAMEs that ranged from 14 to 24 carbons long. Other materials, such as MO or FAME mixtures used for hydrogenated vegetable oils or for commercial food products [6–8], did not exhibit spectra with a horizontal background for human adipose tissue, and were not used. This is because they were less successful than the RM-3 mixture at ratioing out spectral components due to the biological matrix under investigation.

The TFA averages found in the present study, 3.07±0.27% (IR) and 2.59±0.20% (GC, see chro-

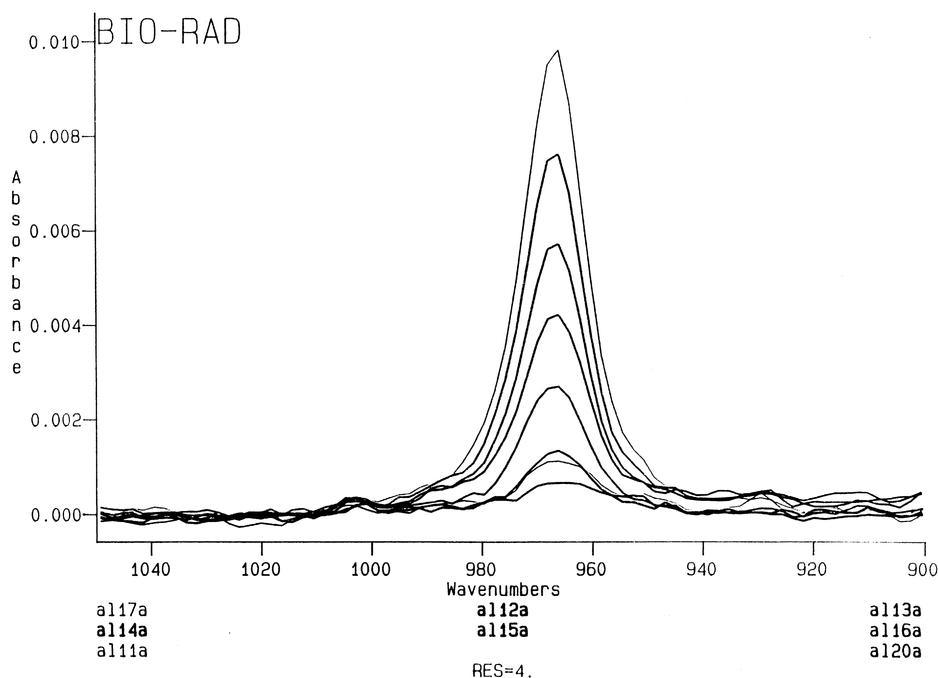

Fig. 1. Spectra showing the 966 cm^{-1} *trans* double bond infrared band for different concentrations, namely 0.410, 0.805, 0.998, 2.00, 2.97, 4.12, 5.62, and 7.59% methyl elaidate (ME) in methyl oleate (MO) used to generate a linear calibration plot.

Table 1
Quantitation of fatty acid methyl esters (FAMEs) with isolated *trans* double bonds (as percent of total FAMEs) from human adipose tissue

Test sample no.	% *trans* by ATR	% *trans* by GC
1	3.84	2.77 (0.41)
2	1.87	1.82 (0.62)
3	1.44	1.16 (0.39)
4	5.33	3.98 (0.56)
5	5.19	4.71 (0.48)
6	2.75	2.62 (0.38)
7	3.91	2.97 (0.37)
8	2.03	1.85 (0.45)
9	4.59	3.64 (0.53)
10	2.33	2.52 (0.34)
11	2.56	1.99 (0.64)
12	2.70	2.67 (0.69)
13	2.73	2.46 (0.46)
14	2.55	2.10 (0.28)
15	2.33	2.03 (0.53)
16	2.22	2.21 (0.47)
17	4.35	3.36 (0.44)
18	2.61	2.07 (0.44)

The levels of the conjugated C18:2, c9t11 and t9t11 determined by GC are given in parentheses.

matogram in Fig. 2), fell within the range reported in the literature; these %TFA values (as percent of total fatty acids) averaged 1.61 [3], 2.66 [5], 4.14 [4], 4.5 [18] and 6.3% [19]; these levels of TFAs in adipose tissue presumably reflect differences in the intake of TFAs by various populations [19]. As shown in Table 1, the *trans* levels found by ATR were usually higher than those determined by GC. One important reason for this difference is that the GC method underestimates total *trans* C18:1 isomers in favor of the *cis* isomers. This is because a satisfactory separation of the *trans* C18:1 from the *cis* C18:1 positional isomers in adipose tissue is not feasible on any capillary GC column. Under optimum conditions, GC peaks for the *trans* C18:1 FAME isomers with double bonds from C-13 to C-16 overlapped with those attributed to *cis* C18:1 FAME isomers [20]. Another reason for such a difference may be due to the fact that the absorptivity of ME, used as IR calibration standard, is different from those of di- and polyunsaturated FAMEs with one or more isolated *trans* double bonds. Moreover, weak absorbances near 985 and 948 cm^{-1} (Fig. 3) due to

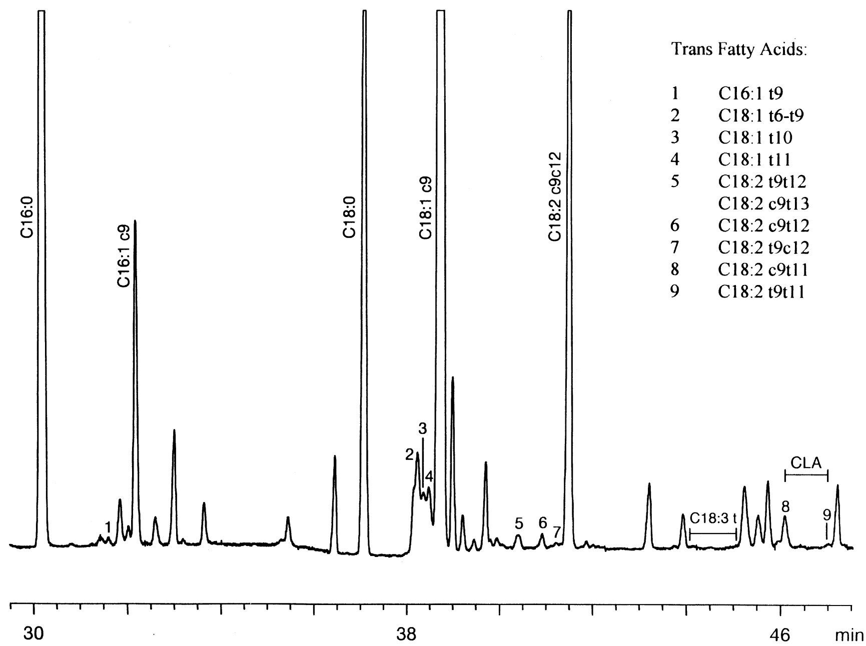

Fig. 2. Capillary gas chromatographic profile for fatty acid methyl esters (FAMEs) from human adipose tissue. The labeled GC peaks for *trans* FAMEs were quantified. The major component of peak 5 is the non-methylene-interrupted C18:2 c9t13 [22].

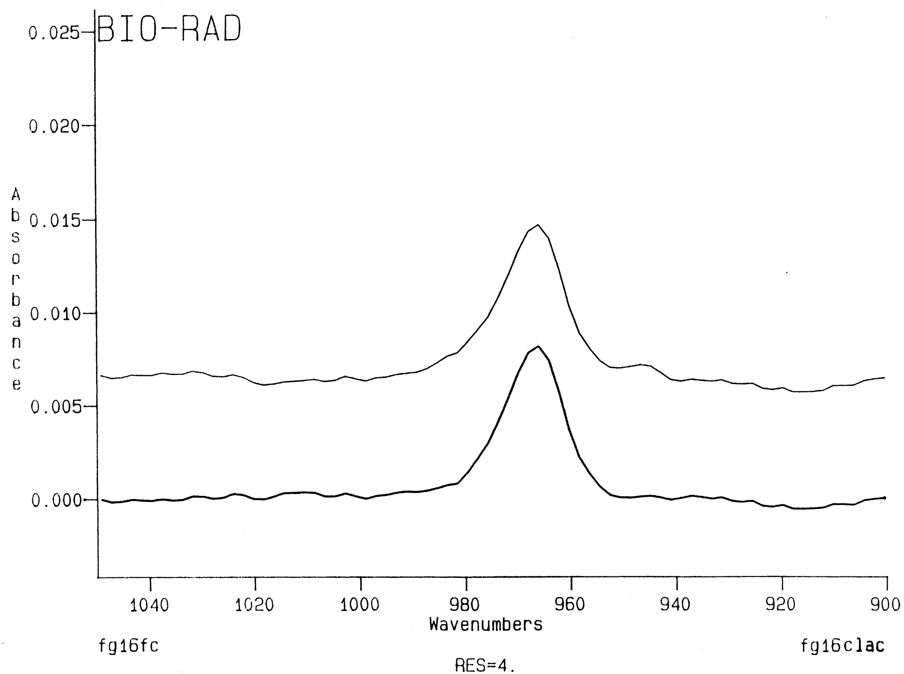

Fig. 3. (a) Infrared spectrum observed for fatty acid methyl esters with isolated *trans* double bonds (966 cm^{-1}). The overlapping conjugated diene bands (near 985 and 948 cm^{-1}) were digitally subtracted in (b) before quantitation.

conjugated dienes [6,21] were also found to overlap with the 966-cm^{-1} *trans* band (Fig. 3). In order to minimize such interferences, the contribution of these conjugated diene bands was spectrally subtracted. Inspection of Table 1 also reveals that differences between IR and GC values would generally be small, if the GC values for conjugated TFAs are taken into account. This observation indicates that the generally higher IR values found for fatty acids with isolated *trans* double bonds may, in part, be due to contributions from conjugated *trans* isomers.

4. Conclusion

The adverse impact of a highly sloping spectral background on accuracy was minimized. The use of a 1-μl ATR cell added speed and convenience to the procedure because weighing test portions and quantitatively diluting them with carbon disulfide were no longer required. The levels of TFAs in human adipose tissue found in the present work are consistent with those reported in the literature. The *trans* levels found by ATR were usually higher than those determined by GC, partly because the GC method underestimates *trans* C18:1 isomers in favor of *cis* C18:1 isomers. The effect of the interference of conjugated dienes on the accuracy of the ATR determination of total *trans* FAMEs in human adipose tissue should be investigated further.

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