Determination of *trans* fatty acids in dietary fats

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Introduction

Several unusual *cis* and *trans* isomers of naturally occurring unsaturated fatty acids is found in many dietary fats. With partially hydrogenated vegetable oils (PHVO), *cis* and *trans* isomers of oleic acid are the main components with double bond positions located from Δ5 to Δ16 (1). In addition, PHVO contain various positional and geometric isomers of linoleic acid with *trans* or non-methylene interrupted double bonds (2). The isomers of linoleic acid are generally prevalent in mildly hydrogenated vegetable oils. Levels up to 7% have been found in some margarines (2,3). Hydrogenated fish oils contain numerous *cis* and *trans* isomers of mono- and poly-unsaturated fatty acids with a wider range of chain lengths (4). *Trans* fatty acids also occur naturally in dairy products, especially those from ruminant animals. Rumen microorganisms biohydrogenate dietary polyunsaturated fatty acids to *trans* fatty acids with 18:1Δ11*t* being the most prevalent isomer.

The widespread use of PHVO, mainly as a substitute for saturated fats of tropical origin, has raised questions concerning the health consequences of intake of *trans* fatty acids. Recent reports indicate that *trans* fatty acids are hypercholesterolemic as that of saturated fatty acids and adversely affect the LDL/HDL cholesterol ratio (5-7). Furthermore, *trans* fatty acids as compared to oleic and linoleic acids increase serum levels of lipoprotein (a) (8). High Lp(a) is an independent and greater risk factor than is high serum cholesterol for coronary heart disease (9).

Because of these adverse health effects, accurate determination of the *trans* fatty acid content is important. In Canada and some European countries, the voluntary nutritional labeling regulations of foods require that monounsaturates only of the *cis* configuration be declared on the label. Furthermore, according to Canadian regulations, polyunsaturates are restricted to *cis,cis* methylene-interrupted structures. These labeling regulations necessitate, not only the determination of the total *trans* content, but also accurate determination of *cis* and *trans*-monounsaturated fatty acids and the general fatty acid composition of food fats.

Determination of total *trans* content

A number of methods are described in the literature for the determination of total *trans* content, including infrared (IR), Raman and nuclear magnetic spectroscopy (NMR), gas chromatography (GC), GC coupled to Fourier transform (FT) IR spectroscopy (GC/FTIR), reversed-phase and silver ion high-performance liquid chromatography (HPLC), and silver nitrate thin-layer chromatography ($AgNO_3$-TLC) in conjunction with GC (10). Of the various methods, IR spectroscopy has been the method of choice for determination of total *trans* content in food fats. The methods based on TLC and HPLC are generally used for isolation of *trans* fatty acids for subsequent structural identification, while Raman and NMR spectroscopy and, GC/FTIR are more suited for structural elucidation of pure *trans* fatty acids.

An isolated *trans* double bond absorbs in the IR region at a wave number of approximately 967 cm^{-1}, equivalent to a wavelength of 10.3 μm, as a result of the deformation of the C-H bonds adjacent to the *trans* double bond. The measurement of the intensity of this absorption under controlled conditions is the basis of the official methods of AOCS (11), AOAC (12) and IUPAC (13) for the determination of total *trans* unsaturation in fats. The AOCS method is exactly same as that of AOAC and can be used for either triglycerides, methyl esters or unesterified fatty acids. The

absorbance or transmittance is recorded by scanning a carbon disulphide solution of the fat sample from 1110 (9 μm) to 910 cm^{-1} (11 μm) against a carbon disulphide blank. A baseline is drawn from 990 (10.10 μm) to 939 cm^{-1} (10.65 μm) for unesterified acids, from 998 (10.02 μm) to 944 cm^{-1} (10.59 μm) for methyl esters or from 995 (10.05 μm) to 937 cm^{-1} (10.67 μm) for triglycerides and the absorptivity is calculated. The *trans* content is calculated by comparing this absorptivity to that of a standard solution of either elaidic acid, methyl elaidate or trielaidin in carbon disulphide. Because, conjugated *trans* bonds absorb near the 10.3 μm band of isolated double, the method is limited to samples containing less than 5% conjugated fatty acids. Further, because of the low intensity of absorption, the accuracy of the AOCS IR method is poor for samples containing less than 5% isolated *trans* unsaturation. Capillary GLC may be the ideal technique for accurate determination of low levels of *trans* unsaturation (discussed below).

The AOCS method gives higher values when fat samples are analyzed as triglycerides, particularly for samples containing less than 15% *trans* unsaturation. Accurate determination of *trans* content in triglycerides require their conversion to methyl esters prior to IR analysis.

The IUPAC (13) method specifies the use of methyl esters and measures the absorption against a blank containing methyl stearate at the same concentration as the sample. The *trans* content is calculated using a calibration curve of absorption versus % isolated *trans* unsaturation developed using a series of carbon disulphide solutions containing different ratios of methyl elaidate and methyl stearate. The use of methyl stearate removes the interference from methyl ester absorption and thus gives greater accuracy down to 1% *trans* content.

Madison *et al.* (14) proposed a 2-component calibration procedure similar to that of IUPAC (13). However, they suggested standard mixtures of methyl elaidate and methyl linoleate for the development of the calibration curve. Calibration and test solutions are scanned from 900 to 1050 cm^{-1} against a carbon disulphide blank. A baseline is drawn between peak minima at about 935 and 1020 cm^{-1}, and the baseline-corrected absorbance of the *trans* peak (967 cm^{-1}) is obtained. The baseline for the test sample spectrum is drawn exactly as the baseline in the standard spectrum, by overlaying the two spectra. This method allows analysis of *trans* contents in the range 0.5 to 36% with increased accuracy. A recent collaborative study organized by Health and Welfare Canada tested a slightly modified procedure of Madison *et al.* (14). Methyl oleate was used instead of methyl linoleate for the development of the calibration curve. A good agreement among the participating laboratories (reproducibility relative standard deviation, RSD_R, ranged between 8.8 to 11.7%) was obtained for samples containing moderate to high content of *trans* unsaturation (15 to 34% *trans*) (see Table 1). However, for sample A (Table 1), that had the lowest *trans* content (5.2%), the agreement among the laboratories was less satisfactory (RSD_R 34.5%). This suggests that accurate measure of low levels of *trans* unsaturation (<5%) by IR is difficult.

Determination of *trans* by FTIR

The newer technique of FTIR offers several advantages over the conventional dispersive IR, including the high signal to noise ratio (S/N) obtained by averaging multitude spectral scans, rapid and comprehensive data collection allowing simple integration of peaks and digital background substraction (15). Use of computerized FTIR eliminates the time consuming tasks encountered with the conventional procedures of manually drawing the baseline and measuring of the peak heights.

Table 1. Statistical Evaluation of GLC-IR Collaborative Study of PHVO Samples

PHVO Sample	IR *trans*			18:1*t*			18:1*c*		
	Mean *	S_R	RSD_R	Mean *	S_R	RSD_R	Mean *	S_R	RSD_R
A	5.17	1.79	34.56	4.88	1.77	36.39	24.93	0.95	3.79
B	15.54	1.76	11.31	14.92	1.41	9.48	24.70	1.75	7.08
C1**	18.92	2.21	11.69	17.37	2.18	12.53	28.11	1.94	6.89
C2**	19.09	1.97	10.32	17.53	1.81	10.34	28.17	2.01	7.14
D	30.06	2.69	8.94	26.64	2.55	9.58	34.38	2.11	6.14
E	34.48	3.90	11.31	32.60	2.53	7.78	34.28	3.61	10.52
R	21.63	1.90	8.79	19.37	1.87	9.65	32.16	2.10	6.53

* Mean for 12 laboratories

S_R = Reproducibility Standard Deviation

RSD_R = Reproducibility Relative Standard Deviation

PHVO = Partially hydrogenated vegetable oil (blend of partially hydrogenated soybean oil and cottonseed oil)

** C1 and C2 are blind duplicates

Lanser and Emken (16) developed a computer assisted procedure for the estimation of isolated *trans* unsaturation in fats, using the peak area of the *trans* absorbance band at 966 cm^{-1} from FTIR spectra of fatty acid methyl esters in carbon disulphide. The area under a peak depends on the baseline chosen. They observed that absorbance minima, more specifically the minimum at the higher wave number, varied with the proportion of *trans* unsaturation. This required adjustment of the baseline according to the *trans* content. Samples with more than 10% *trans* produce an absorption band with minima at 944 and 988 cm^{-1}, whereas at less than 10% *trans*, the peak minima are at 944 and 985 cm^{-1} and below 5% *trans*, the peak minima are at 944 and 973 cm^{-1}. The calculation of *trans* content in hydrogenated oils containing less than 5% was improved by the use of appropriately selected integration limits.

Use of thin cells and neat methyl esters is the basis of the FTIR method proposed by Sleeter and Matlock (15) for determination of *trans* unsaturation in fats. FTIR uses a Michelson Interferometer, which allows all wavelengths of light to pass through the sample simultaneously, whereas with conventional dispersive spectrophotometers, which uses diffraction gratings, only limited amounts of light pass through the sample. Due to this increased amount of light at all wavelengths, FTIR allows analysis of neat products using thin cells with path lengths of ≈0.1 mm, eliminating possible errors due to weighing of sample and dilution with carbon disulphide. Use of carbon disulphide in dispersive instruments frequently leads to stratification, vapor and air bubble formation within the cell. Sleeter and Matlock (15) use neat mixtures of methyl elaidate and methyl linoleate for calibration as proposed by Madison *et al* (14). The area of the *trans* peak was integrated from 945 to 990 cm^{-1}. Quantitation was obtained by fitting measured *trans* areas of the calibration mixtures with a second order polynomial. This provides a correlation coefficient of 0.9998 and standard error of 0.11% over a range of 0 to 50%. *Trans* content can also be determined by measuring peak heights, which give a slightly increased error.

Determination of fatty acid composition

Gas chromatography (GC) of fatty acid methyl esters is undoubtedly the most convenient and widely used analytical method for determining the fatty acid composition (17). Slightly polar stationary phases, such as polyglycol Carbowax-20M, are normally employed for the analysis of fatty acids of natural origin, in which the double bonds of unsaturated fatty acids are almost exclusively of *cis* configuration. However, with these stationary phases, the separation of *cis/trans* isomers is not feasible. With highly polar cyanosilicone stationary phases such as SP-2560, SP-2340, OV-275 or CP-SIL-88 *cis* and *trans* isomers could be separated to a far greater extent than with polar stationary phases.

Based on an interlaboratory study, a 6.1 m x 2 mm (i.d.) column packed with OV-275 has been recommended by both AOCS (18) and AOAC (19) for determination of *trans* unsaturation in partially hydrogenated oils. However, a complete resolution is not feasible with the OV-275 column, since some of the *trans*-monoenes are hidden under the larger *cis* isomer peak (20).

In many lipid laboratories, capillary columns coated with cyanosilicone stationary phases appeared to gain acceptance for *cis/trans* isomer separation (21,22). AOCS (23) and AOAC (24) recently recommended the use of a 60 m x 0.25 mm (i.d.) flexible fused capillary column coated with SP-2340 to determine the general fatty acid composition, including the levels of *cis* and *trans*-octadecenoates of partially hydrogenated oils. This same method is recommended for determination of total *trans* unsaturation. The direct capillary GLC procedure was based on the assumption that 18:1*c* and 18:1*t* isomers are completely separable on the SP-2340 column. However, a complete resolution of 18:1*t* as a group from that of the *cis* isomers is not feasible on SP-2340 (25) or any other cyanosilicone capillary column (2,26). In these columns, the early eluting 18:1*t* isomers with low Δ values are well separated from the 18:1*c* isomers, but the 18:1*t* isomers with high Δ values (i.e. Δ12 and Δ15) overlap with 18:1Δ9*c* (the major 18:1*c* isomer in PHVO). Because of this overlap the direct GLC method greatly underestimates the total 18:1*t* in favor of the *cis* isomers (25). In some margarines, the underestimation in determining the total 18:1*t* can be as high as 32% (26). The levels of 18:1*t* isomers of high Δ values may depend on the hydrogenation conditions and the source oil, and this will result in variation in the extent of overlaps of the isomers from one PHVO to another. The concentration of the methyl esters applied to the GLC could also influence the *cis* and *trans* resolution.

Sampugna *et al* (21) proposed the use of appropriate correction factors to compensate for the *cis* and *trans* overlaps. They found a linear relationship between the correction factors, determined by comparison with results obtained by silver nitrate TLC/GC, for 18:1*t* and 18:1*c* and the proportion of total 18:1 isomers in the sample. GLC combined with other chromatographic techniques (particularly argentation chromatography) has been suggested (20,25,27-29), but these procedures are lengthy and are not suitable for routine analysis of dietary fats.

Combined GLC-IR

Ratnayake et al (26) proposed use of a combined capillary GLC and IR method for the determination of 18:1t and 18:1c isomers and the general fatty acid composition of PHVO. The total trans unsaturation determined by IR was correlated to the capillary GC weight percentages of the component trans fatty acid methyl esters by the mathematical formula: IR *trans* = %18:*t* + 0.84 x %18:2*t* + 1.74 x %18:2*tt* + 0.84 x %18:3*t* where 0.84, 1.74 and 0.84 are the correction factors relating GLC weight percentages to the IR *trans*-equivalents for mono-*trans* octadecadienoic (18:2*t*), *trans,trans*-octadecadienoic (18:2*tt*) and mono-*trans*-octadecatrienoic (18:3*t*) acids, respectively. This formula forms the basis for the determination of 18:1*t* and 18:1*c* in PHVO. GC provides the pro-

portions of 18:2*t*, 18:2*tt* and 18:3*t*, whereas IR yields the total *trans* unsaturation, and 18:1*t* is calculated from the mathematical formula. 18:1*c* is obtained as the difference between total 18:1 and 18:1*t*.

An interlaboratory collaborative study, just concluded, indicated that the GLC-IR method gives reproducible results for PHVO samples containing more than 5% *trans* unsaturation (Table 1). That the GLC-IR method is capable of good precision is demonstrated by the excellent agreement for a pair of duplicate samples (Table 2). For samples with less than 5% *trans* content (sample A in Table 1), the agreement for 18:1*t* between the laboratories was less than satisfactory. This is because, as mentioned previously, the accuracy of measuring *trans* unsaturation by IR is poor with samples containing low levels of *trans* unsaturation. Direct GLC analysis is recommended for such samples, since when *trans* content is <5%, the overlap of 18:1c and 18:1*t* in cyanosilcone capillary columns is almost negligible.

Summary

With any of the current official methods a fairly good quantitative estimate of *trans* unsaturation can be obtained by IR spectrophotometry. Whether low levels of *trans* unsaturation would be determined by IR is doubtful. For these, use of direct GC is recommended. Alternatively, accuracy at lower *trans* levels could be improved with the use of FTIR by analyzing neat methyl esters in 0.1 mm IR cells.

The combined GC-IR method should be useful for routine analysis of *cis* and *trans*-octadecenoates and the general fatty acid composition in dietary fats made from PHVO and animal fats, provided the *trans* content is more than 5%. For samples containing less than 5% *trans*, detailed fatty acid composition and the total *trans* unsaturation are conveniently obtained through

Table 2. Statistical Evaluation of GLC-IR Collaborative Study of PHVO
Blind Duplicates – Samples C1 and C2

	Mean	**S_r**	**S_R**	**RSD_r**	**RSD_R**
IR *trans*	19.04	1.22	2.04	6.42	10.72
18:1*t*	17.45	1.26	1.92	7.24	11.00
18:1*c*	28.16	1.12	1.87	3.97	6.05

* n = 12 laboratories
S_r = Repeatability Standard Deviation
S_R = Reproducibility Standard Deviation
RDS_r= Repeatability Relative Standard Deviation
RSD_R = Reproducibility Relative Standard Deviation

GC analysis alone, without resorting to the use of IR. The GC-IR procedure, however, is not applicable to partially hydrogenated fish oils, because these fats contain a complex mixture of *cis/trans* isomers of polyunsaturated fatty acids with a wider range of chain lengths.

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