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Food Chemistry 98 (2006) 593–598

Food Chemistry

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A simple method for the analysis of *trans* fatty acid with GC–MS and AT™-Silar-90 capillary column

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Received 27 April 2005; accepted 16 May 2005

Abstract

The study was aimed to evaluate a simplified gas chromatography method based on the AOAC method 996.06 to analyze the *trans* fat content in food samples. The gas chromatograph was equipped with mass spectrometer and Alltech AT™-Silar-90 capillary column. Ten kinds of the *trans* fatty acid standard were separated completely from the *cis* standard and the chemical composition of the peaks was verified by using the mass spectrum. Under the optimized conditions, the recovery rate for triheptadecanoin was 99.0%, the correlation coefficients of *trans* fatty acid calibration curve was 0.9998 or higher. It demonstrated that the methylation and hexane extraction procedures used in this method was effective and the result was consistent. The major fatty acids found in the shortening sample were 16:0, 18:0, *trans*-18:1, *cis*-18:1, *cis*-18:2, and *cis*-18:3. The total *trans* fat content in the sample was 283.6 ± 18.2 mg/g. The current method was more convenient. It is adequate for the routine analysis of *trans* fat content in food products with low free fatty acid content.
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Keywords: *Trans* fatty acid; GC–MS

1. Introduction

Trans fatty acids, according to the FDA definition, are unsaturated fatty acids that contain one or more isolated (i.e., nonconjugated) double bonds in a *trans* configuration (Food & Drug Administration, 2003). *Trans* fat in food products is mainly derived from partially hydrogenated oil. Minor amount of *trans* fat is also found in dairy products and ruminant meats due to the biohydrogenation in the rumen. The health risks of *trans* fat have been debated for many years. Epidemiological studies have revealed their association with higher level of total plasma cholesterol and low-density-lipoprotein (LDL) cholesterol, lower level of high-density-lipoprotein (HDL) cholesterol. Consumption of *trans* fat may increase the risk of coronary heart disease (Ascherio, Katan, Zock, Stampfer, & Willett, 1999; Hu et al., 1997; Vijver et al., 2000). Therefore, the FDA issued the rule requiring manufacturers to label *trans* fat on the nutrition facts beginning in January 2006 (Food & Drug Administration, 2003). This rule prompts the urgent need to optimize the method for the analysis of *trans* fatty acid in food products.

The analysis of *trans* fatty acid is extremely challenging and complex because of the wide range of positional monoene, diene, and triene isomers. The analytical methods mainly include infrared spectroscopy, ^{13}C nuclear magnetic resonance spectroscopy, gas chromatography, thin-layer chromatography, high performance liquid chromatography, and mass spectrometry or combined of them (Adlof, 1994; Miyake & Yokomizo, 1998). The infrared method measures the absorption at the 966 cm^{-1} band, which offers the advantage of simplicity and speed, but it is difficult to eliminate the interference of absorption from other functional groups

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0308-8146/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2005.05.013

other than the *trans* band. The interference will decrease the accuracy of the test (Mcdonald & Mossoba, 2002). It was reported that the applicability of attenuated total reflection infrared spectroscopy method was limited to >5% *trans* fat (Adam, Mossoba, Dawson, Chew, & Wasserman, 1999; Mossoba, Adam, & Lee, 2001). The conventional HPLC method does not exhibit enough separation efficiency offered by capillary GC column. Silver ion HPLC is able to separate the *trans* isomer from the *cis*, but for quantitative analysis of *trans* fat, the isolated *trans* fraction still need to be analyzed with subsequent capillary GC column. ^{13}C Nuclear magnetic resonance spectroscopy also need pre-separation and is too expensive. The thin-layer chromatography method is very laborious and inaccurate (McDonald et al., 2002). For food labeling purposes, gas chromatography might be the only choice with appropriate accuracy, convenience, and cost. The GC analysis of *trans* fatty acid is normally performed on high polarity phase column. The most frequently used are SP–2560 and CP™-Sil 88 (Ali, Angyal, Weaver, & Rader, 1997; Bruggen, Duchateau, Mooren, & Oosten, 1998; Ratnayake, Plouffe, Pasquier, & Gagnon, 2002). When appropriate column and operating conditions are selected, gas chromatography will give a good separation of *cis* and *trans* isomers, but no column can completely separate these isomers. Due to the limited availability of the fatty acid standard and the complexity of the fatty acid composition, it is always difficult to identify some peaks with the conventional FID detector. At present, the FDA considers the modified AOAC gas chromatography method 996.06 an appropriate method. In this method, the triacylglycerol (fat) and fatty acids are extracted from food, then methylated to fatty acid methyl ester using BF_3 in methanol, and FAMEs are quantitatively measured by capillary gas chromatography (AOAC, 2000). Nevertheless, the FDA regulations do not specify the methodology that can be used to obtain values for nutrition labeling purposes. Therefore, other simple and reliable methods will be in great need to meet the diversity of manufacturer's conditions. The current study was aimed to find a simplified gas chromatography method based on the AOAC 996.06 to analyze the *trans* fat content in food samples. We employed different methylation procedures and capillary column. The food sample containing *trans* fat was analyzed with the optimized condition. GC was coupled with MS to achieve more accurate peak identification than the FID detector.

2. Materials and methods

2.1. Samples and reagents

Trans and *cis* fatty acid methyl ester (FAME) standards (GLC 481 and A20) and Triheptadecanoin were purchased from Nu-Chek-Prep, Inc (Elysian, MN). Methyl heptadecanoate, sodium methoxide, sodium chloride were from Sigma (St. Louis, MO). HPLC Grade hexane and other organic solvent were purchased from Fisher Chemicals (Fair Lawn, NJ). One shortening sample was obtained from a local grocery store. All chemicals and solvents were of the analytical grade and used without further purification.

2.2. Sample preparation

The fat was converted to FAMEs using sodium methoxide method (Qian, 2003). Briefly, A portion of 100 mg shortening sample was dissolved in 25 ml hexane. An aliquot of 5 ml was transferred into a tube with tight-sealing cap and mixed with 250 μl sodium methoxide (0.5 M) in methanol. The tube was capped tightly and vortexed for 1 min. Five milliliters saturated sodium chloride solution was added and shaken vigorously for 15 s. After 10 min, 3 ml hexane layer was transferred to a vial containing small amount of Na_2SO_4 anhydrous. With tight cap, the hexane phase containing FAMEs was exposed to Na_2SO_4 anhydrous for about 30 min. The hexane phase was transferred to a 1.5 ml vial for subsequent GC–MS analysis. The sample was prepared in triplicate. Three levels of *trans* and *cis* FAME standard solution were prepared in similar procedures, respectively. Constant concentration of internal standard (methyl heptadecanoate, 10 μg/ml) was incorporated into each sample and standard vial.

2.3. GC–MS conditions

Analysis of FAMEs was carried out on Shimazu gas chromatograph (GC-2010, Kyoto, Japan) equipped with Shimazu GCMS-QP2010 mass spectrometer and Alltech AT™-Silar-90 capillary column (30 m × 0.25 mm, 0.2 μm film thickness) (Alltect, Deerfield, IL). For electron ionization, 70 eV was used. The injection mode was split with a split ratio of 50.The injection volume was 1 μl. The carrier gas was helium at a column flow rate of 0.6 ml/min. To optimize the condition, the following three temperature programmings were used. (1) Isothermal at 180 °C; (2) initial temperature 150 °C, hold 10 min, ramp at 5 °C/min, final temperature 200 °C, hold 2 min; (3) initial temperature 150 °C, hold 10 min, ramp at 2.7 °C/min, final temperature 210 °C, hold 3 min. Optimum condition was established based on the resolution of the *trans* fatty acid in the standard and used to analyze the shortening sample.

2.4. Recovery studies of derivatization procedure

Triacylglycerol standard (triheptadecanoin) solution was prepared in hexane at concentration of 2 mg/ml. Five milliliters was transferred into a tube with tight-

sealing cap. Triheptadecanoin was converted to methyl ester and analyzed following the same procedure as the sample. The concentration of methyl heptadecanoate was calculated according to the calibration curve prepared by methyl heptadecanoate. The recovered concentration of triheptadeconoin was converted from the concentration of methyl heptadecanoate. Recovery rate was the ratio of recovered concentration to prepared concentration. Tests were performed in triplicate.

2.5. Calculations and statistic analysis

Peak identification was performed by comparison of the retention time and mass spectrum with respective standard. Quantification of individual *trans* fatty acid was performed using the calibration curve on the basis of the total ion current (TIC) peak area data relative to internal standard. The content of fatty acid was converted to the corresponding triacylglycerol by the conversion factor (AOAC, 2000).

Data analysis was performed on the SPSS (SPSS for Windows, Version 10.0.5., 1999, SPSS Inc., Chicago, IL). Significant difference test among means was inspected using LSD one-way ANOVA multiple groups test.

3. Results and discussion

3.1. Optimization of the GC conditions

The AT™-Silar-90 column has the highly polar cyanopropyl polysiloxane phase. It is similar to the SP-2560 and CP™-Sil 88 column. Primary run of *trans* and *cis* FAME standard have showed that the elution pattern of FAME on AT™-Silar-90 column was similar to that on the SP 2560. The elution order of FAMEs in the standard mixture was listed (Table 1). Previous reports indicated that the most difficult region of separation was the 18:1, 18:2 and 18:3 (Aro et al., 1998; Duchateau, Oosten, & Vasconcellos, 1996). Therefore, the resolution of these regions could be used as an index for optimizing the analytical condition. The values of resolution at isothermal and programmed temperature condition were showed (Table 2). The best separation was achieved at initial temperature 150 °C, holding 10 min, ramp at 2.7 °C /min, final temperature 210 °C, holding 3 min. This condition was used to analyze shortening sample. The TIC chromatogram from the standard mixture under the optimized condition was shown (Fig. 1).

Under isothermal condition, as the column supplier recommended, resolution of the *trans* fatty acids was very poor. The FDA recommended AOAC Official method 996.06 for analysis of fat including *trans* fat also adopted programmed temperature (AOAC, 2000). The better resolution of programmed temperature was attributed to not only the interaction of analyte with stationary phase but also the volatility differences of the analyte, FAMEs.

3.2. Recovery and reproducibility of the method

The procedures for sample preparation were similar to the AOAC Official Method 996.06 with some modifications. In this study, the sodium methoxide methylation procedure was applied. To evaluate the methylation and extraction methods, the recovery and relative standard deviation (RSD) of the recovery were calculated. The recovery rate of triheptadeconoin was 99.0%. RSD was 1.5%. The calibration curve were linear

Table 1
Mixture of FAMEs standard

Peaks[a]	Chain length	FAMEs	Concentration (μg/ml)
1	14:0	Methyl myristate	8
2	14:1 *trans*	Methyl myristelaidate	65
3	15:1 *trans*	Methyl 10 transpentadecenoate	65
4	16:0	Methyl palmitate	120
5	16:1 *trans*	Methyl palmtelaidate	65
6	16:1 *cis*	Methyl palmitoleate	12
7	17:0	Methyl heptadecanoate	10
8	17:1 *trans*	Methyl 10-transheptadecenoate	65
9	18:0	Methyl stearate	112
10	18:1 *trans*	Methyl 6,9,11-transoctadecenoate	195
11	18:1 *cis* 9	Methyl oleate	164
12	18:2 *trans*	Methyl linoelaidate	65
13	18:2 *cis*	Methyl linoleate	28
14	19:1 *trans*	Methyl 7,10-transnonadecenoate	130
15	18:3 *cis*	Methyl linolenate	12
16	20:1 *trans*	Methyl 11-transeicosenoate	65
17	20:2 *trans*	Methyl 11,14-transeicosadienoate	65
18	C22:1 *trans*	Methyl brassidate	65

[a] The peak number corresponds to the number in Fig. 1.

Table 2
Resolution of peaks[a]

Temperature programming	Group one[b]	Group two[c]
1	1.269 a	0 a
2	1.229 b	1.779 b
3	1.306 c	2.411 c

[a] Within the column, means with the same letter were not significantly different ($P < 0.01$) ($n = 3$).
[b] Group one was 18:1 *trans* 11 vs. 18:1 *cis* 9.
[c] Group two was 18:2 *cis* vs. 19:1 *trans* 7.

over the tested concentration ranges with correlation coefficients $R^2 \geqslant 0.9998$. The calibration curve for *trans* 18:1 was shown (Fig. 2). It demonstrated that the methylation procedure and hexane extraction was very effective. The method was also reasonably consistent. Compared with the BF3/methanol method, this method is simpler, less laborious, and easier to handle. Nevertheless, it should be noted that this method is not suitable for the methylation of free fatty acids.

3.3. Trans fat content of shortening samples

To check the applicability of this method, shortening sample was analyzed under the optimized conditions. The chromatogram of FAMEs prepared from the sample was shown (Fig. 3). The major fatty acids found in the sample were 16:0, 18:0, *trans*-18:1, *cis*-18:1, *cis*-18:2, and *cis*-18:3. The complexity of the region around 18:1 was apparent. The *trans*-18:1 consists of a series of peaks, which could not be separated completely with the *cis*-18:1. The double bond configuration of natural 18:1 is *cis*Δ9 shown as peak 5 in the chromatogram. The partial hydrogenation cannot only change the geometrical configuration, but also shift the position of double bond. The double bond may exist anywhere between Δ4 and Δ16. This makes it difficult in complete separation of the 18:1 isomers. However, the majority of *trans*-18:1 fatty acid is *trans*Δ4 to Δ11, which would be eluted before *cis*Δ9 (Aro et al., 1998). According to the chromatogram obtained from FAMEs standard, the *trans*-18:1Δ4–11 was able to be separated from the *cis*-18:1Δ9–15. However, the *trans*-18:1Δ12–16 would overlap with the *cis*-18:1Δ11–15. This overlapping would underestimate the level of *trans*-18:1. Considering that the *trans*Δ12–16 are the minor components in most hydrogenated vegetable oil, the error of *trans* fat determination would be insignificant. This result was in agreement with other studies (Ratnayake et al., 2002).

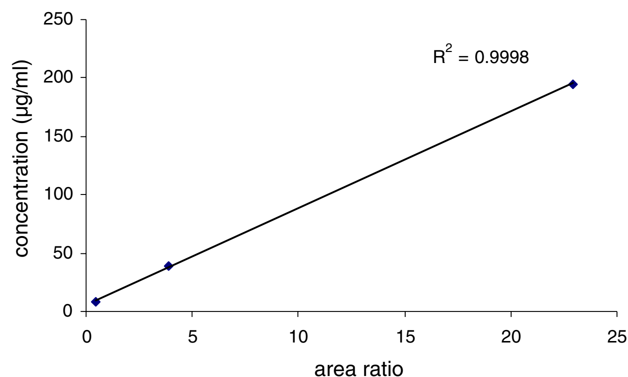

Fig. 2. The calibration curve of *trans*-18:1. The line was not forced through the origin. The area ratio was the area of *trans*-18:1 standard against internal standard.

Peak 6 cannot be identified by comparing the retention time with standard. However, according to the elution order and mass spectrum similarity search, they could be identified as *cis*-18:1 with 98% confidence. These two peaks were quantified using the same response factor of the fatty acid close to them assuming that similar fatty acid having similar response factor. DeVries also recommend modifications

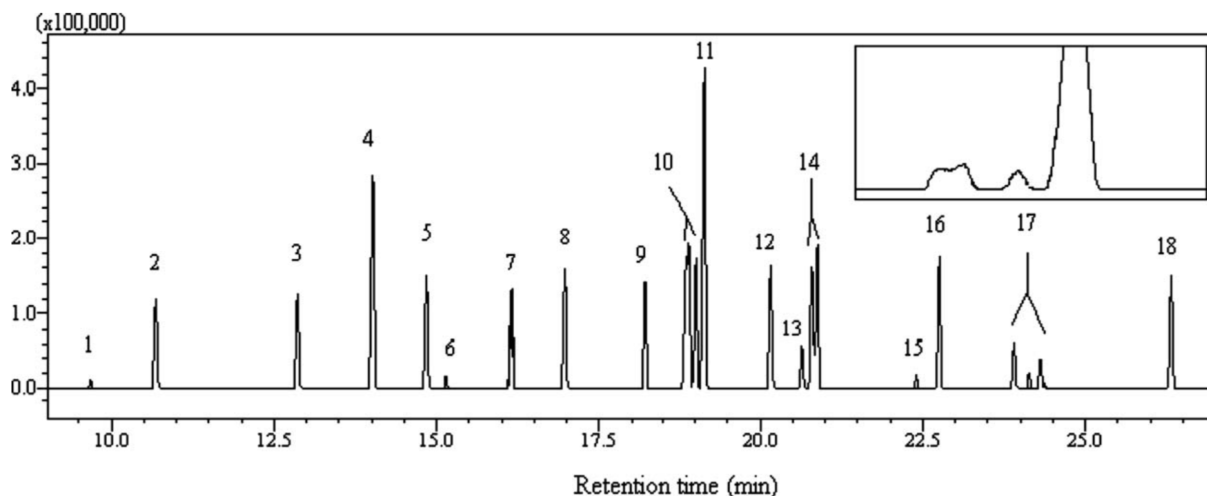

Fig. 1. The TIC chromatogram of FAMEs standard. The peak number corresponds to the number in Table 1. The inserted graph was the enlargement of peaks 10, 11.

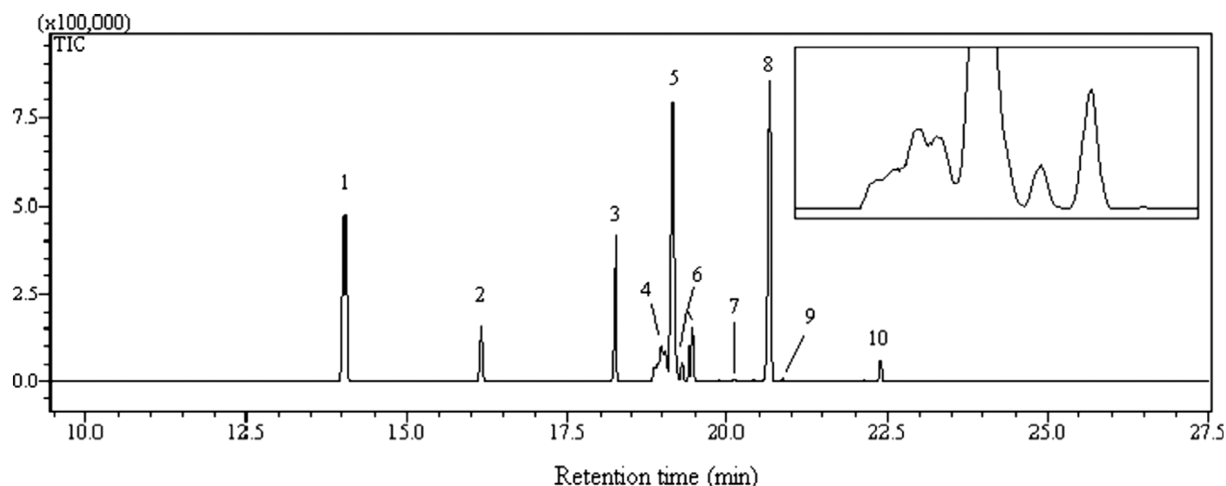

Fig. 3. The TIC chromatogram of shortening sample. Peak identification: 1, 16:0; 2, 17:0, internal standard; 3, 18:0; 4, *trans*-18:1; 5, 6 *cis*-18:1; 7, *trans*-18:2; 8, *cis*-18:2; 9, *trans*-19:1; 10, *cis*-18:3. The inserted graph was the enlargement of peaks 4, 5, 6.

Table 3
Trans fat content of shortening sample

Trans fat type	Mean (mg/g)[a]	Standard deviation (mg/g)
Trans 18:1	280.4 a	18.1
Trans 18:2	1.2 b	0.0
Trans 19:1	2.0 b	0.1
Total *trans* fat	283.6	18.2

[a] Within the column, means with the same letter were not significantly different ($P < 0.01$) ($n = 3$).

in AOAC Official Method 996.06 to quantity unknown or uncalibrated peaks of fatty acid by combination with mass spectral data (DeVries et al., 1999). Coupling with mass spectrometry, the identification of the unknown peaks would be more accurate. It is a great advantage over the conventional FID detector. Other identified *trans* fatty acids were peak 7 and 9, which could be *trans*-18:2 and *trans*-19:1, respectively. These peaks could be separated from adjacent peaks completely. The results of *trans* fat content and total *trans* fat content were shown (Table 3). The *trans*-18:1 was the major component of *trans* fat. The *trans*-18:2 and *trans*-19:1 content were significantly lower than the *trans*-18:1 content.

In this study, the mass spectrometry was used to verify the chemical structure of peaks. Nevertheless, once the chromatography condition was optimized, the quantification of *trans* fat can also be achieved with FID detector.

4. Conclusion

Trans fatty acid in triacylglycerol could be methylated with sodium methoxide and extracted with hexane effectively. The methylated *trans* and *cis* fatty acid could be quantified by the gas chromatography with a polar Alltech AT™-Silar-90 capillary column. This method was more convenient than the AOAC 996.06 method and could be used for the routine analysis of food products.

Acknowledgement

This study was partially funded by the USDA CSREES 1890 land grant university capacity building grant program with contract number 2002-38820-12557 and National Aeronautics and Space Administration (NASA) Tuskegee University center for food and environmental system for human exploration of space. Dr. Robert A Moreau from USDA eastern regional research center has provided consulting service for this study.

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