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A Rapid, Micro FAME Preparation Method for Vegetable Oil Fatty Acid Analysis by Gas Chromatography

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Abstract A 30-min, micro-base-catalyzed method for vegetable oil fatty acid methyl ester (FAME) preparation was developed using only 1 mg of oil sample by limiting the solvent volumes used. This method was primarily developed to quickly analyze fatty acid composition of CLA-rich soy oil but can be further applicable to pure vegetable oils. Existing base-catalyzed FAME preparation methods are not appropriate to use because they are either rapid but not micro, or micro but not rapid, or are rapid and micro but use acidification in the final step of FAME preparation, which would isomerize oils containing conjugated fatty acids. Serial dilutions of a mixed commercial FAME reference standard were prepared and analyzed by GC with a flame ionization detector (FID) with maximum instrument sensitivity. The novel method was also used to prepare soy oil FAMEs for GC-FID analysis. There were no statistically significant differences ($P < 0.05$) in fatty acid data from the FAME reference standard dilutions. Similarly, there was no statistical significant difference ($P < 0.05$) between results obtained for all the soy oil dilutions and the control method. This technique is a rapid method for preparing small pure oil samples as FAMEs for GC-FID analysis.

Keywords Fatty acid composition · Rapid micro-method · FAME preparation · Base catalysis · Oil analysis · Gas chromatography · Flame ionization detector

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Introduction

Fatty acid analysis has become increasingly important due to growing awareness of lipids associated with nutritional and health benefits [1–3]. The beneficial relation between n-3 fatty acids and chronic diseases such as atherosclerosis [4] or rheumatoid arthritis [5] has been known for a number of years. Fatty acids are determined as fatty acid methyl esters (FAMEs) mainly by gas-liquid chromatography (GLC) with flame ionization detectors (FID).

Acid-catalyzed and base-catalyzed methods are commonly used for FAME derivatization. Acid-catalyzed methods [6–9], not only produce methyl esters by transesterification of triacylglycerols (TAGs) but also esterify free fatty acids in the presence of methanol. Three commonly used acid catalytic reagents are hydrochloric acid, sulfuric acid and boron tri-fluoride, all in methanol. Heating is required to accelerate the reaction but high concentrations should be avoided, to ensure other reactions do not occur [10]. Presently, there are a number of micro and macro-acid-catalyzed methods available [11–20]. However, they are time consuming taking from 1 to 16 h for FAME preparation. Hence, a rapid micro-scale FAME preparation method would be valuable.

Morrison and Smith developed a rapid, acid-catalyzed semi-micro-method for analyzing 4–16 mg of TAGs taking about 30 min for the FAME preparation [10]. Palmquist and Jenkins [11] reported a one-step, 3-h acid esterification process using methanolic HCl with sample size of 10–50 mg of fatty acids. A rapid, micro-scale FAME preparation method and a rapid GC method were developed by Mondello et al. [12] to analyze 20-μL samples of cod liver oil as FAMEs. The overall time necessary for sample preparation and simultaneous GC analysis was 7.5 min. However, this acid-catalyzed method uses boron

tri-fluoride in methanol and cannot be used to analyze oils with conjugated fatty acids because it can cause geometrical isomerization of conjugated unsaturated fatty acids and is therefore not suitable for CLA analysis. Slover and Lanza [13] developed a FAME method which took 1 h for FAME preparation while using 20–60 mg of TAG samples. A one-step reaction, micro-method of direct esterification was reported by Lepage and Roy [14], which used 100-µL of plasma or bile sample and took 1–16 h of FAME preparation time, and was applicable for simple and complex lipids. The drawback of this method is the longer methylation time and expensive reagents. A continuous derivatization system to prepare FAMEs and their determination by on-line GLC was reported by Ballesteros et al. [15] using 10–120 mg of oil samples requiring 30 min of FAME reaction times. This method is rapid, but cannot use 1 mg of oil samples. Masood et al. [16] reported a time consuming, micro-method using 50-µL of human plasma samples with 3 h FAME reaction time. Heating blocks and a centrifuge are required in this method, which is expensive.

Stoffel et al. [17] reported a micro-method for analyzing 1–10 mg fatty acid samples with reaction times of 3 h. A simple, rapid micro-method was developed by Husek et al. [18] and Woo and Kim [19] using 100 µL of serum samples and fatty acids and takes 30 min for FAME preparation. However, these two methods use acid-catalysis and are not applicable to oils containing conjugated fatty acids due to isomerization during FAME preparation. Sukhija and Palmquist [20] developed an acid-catalyzed semi-micro-method using 10–50 mg of fatty acids and it takes 3 h of reaction time for methylation of fatty acids. Use of acid-catalyzed methods for FAME methylation is not applicable for oils containing isomers of conjugated fatty acids because acid catalysis can cause conjugated fatty acid geometrical isomerization, with an increase in the relative proportion of *trans, trans* isomers [21]. A further disadvantage of using acid-catalysts is that they are toxic and expensive.

In contrast to acid-catalysis, alkaline-catalysis transesterifies neutral lipids in anhydrous methanol more quickly and does not degrade fatty acids nor isomerize double bonds. However, alkaline-catalysis is unable to esterify free fatty acids. Furthermore, the reaction requires more rigid anhydrous conditions than acid-catalysis because the presence of water leads to irreversible hydrolysis of lipids [22]. Sodium methoxide is the most popular alkaline-catalyst with others including potassium hydroxide and sodium hydroxide in methanol. Rapid procedures have been reported with these catalysts taking <1 h for FAME preparation [23, 24]. Some micro and macro-methods using base-catalysts have been reported that analyze small oil samples but the FAME preparation takes 30 min to 12 h [25, 26]. A one-vial, small-scale method for the analysis of bacterial fatty acids, avoiding the needs for extraction and sample handling, was developed by Basconcillo and McCarry [25]. A 1,000-fold reduction in sample size was reported in this method. The disadvantage of this method was the 3–12 h reaction time for FAME preparation. Birch et al. [26] and Carvalho and Malcata [27] reported analysis of fatty acids using a 1–2 mg sample size with a FAME preparation time of 30 min. These two methods are micro and rapid but consist of an acidification step using HCl at the end and is thus not applicable to oils containing conjugated fatty acids. O'Fallon et al. [28] reported use of both acid-catalyzed and base-catalyzed methods using 20 µL of oil with 2 h required to complete the FAME preparation. The disadvantage of this method is longer methylation time and expensive instrumentation for FAME analysis. A few micro-methods using a base-catalyst were also reported [29]. Gehrke and Goerlitz [29] developed a semi-micro-method analyzing 50-mg sample size with a FAME reaction time of 8 h. This is an effective method but very time consuming.

The reason for the following study was to develop a rapid, micro, base-catalyzed method to quickly measure the CLA isomers in small TAG fractions of CLA-rich soy oil obtained after photo-isomerization [30]. Existing base-catalyzed methods are not appropriate because they are either rapid but not micro [31], or micro but not rapid [29], or are rapid and micro but use acidification in the final step [26] of FAME preparation which would isomerize the CLA fatty acids. The goal of this study was to produce a simple, one-step, one-vial, base-catalyzed micro-method to quickly convert vegetable oil fatty acid to their FAMEs for subsequent GC analysis. Mixed FAME standard and soy oil were used to evaluate this method as fatty acid composition of both the FAME standard and soy oil is well established.

The specific objectives of this paper are: (1) to determine the accuracy, precision and sensitivity of mixed standard fatty acids, as FAME dilutions by GC-FID and (2) to develop a rapid, base-catalyzed micro-method for FAME derivatization from soy oil for fatty acid GC-FID analysis.

Experimental Procedures

Materials

Refined, bleached, deodorized soy oil (Wesson, ConAgra, Irvine, CA, USA) was obtained from a local grocery store with linoleic acid (55%) and linolenic acid (6%), as measured by GC [32]. Sodium methoxide and anhydrous sodium sulfate (EM Science, Darmstadt, Germany) were used for methyl ester preparation.

Fatty Acid Analysis of FAME Samples Obtained by Serial Dilution of a Commercial FAME Standard

Sample Preparation

A 50-mg sample of mixed FAME's (AOCS RM-1 mix, Matreya LLC, Pleasant Gap, PA, USA) consisting of methyl stearate (3%), methyl palmitate (6%), *cis*-9 methyl oleate (35%), *cis,cis*-9,12 methyl linoleate (50%), *cis*-9,12,15 methyl linolenate (3%) and methyl arachidate (3%) FAME's were obtained. Serial dilutions of the FAME mixture with hexane were made in triplicates to obtain FAME concentrations of 15,000 (control), 5,000, 1,000, 500 and 100 µg/mL. A 1% heptadecanoic methyl ester (HME C17:0, Sigma-Aldrich, St Louis, MO, USA) in hexane was prepared and HME equivalent to 5% of the total FAME was added to each dilution as an internal standard.

Gas Chromatographic Analysis

Methyl esters were analyzed for each replicate by GC [32] in triplicate using a SP 2560 fused silica capillary column (100 m × 0.25 mm i.d. × 0.2 µm film thickness; Supelco Inc., Bellefonte, PA, USA) with a FID (model 3800, Varian, Walnut Creek, CA, USA). The samples were injected by an autosampler (Varian, Walnut Creek, CA, USA). The sensitivity of the GC instrument was manually maximized in the Galaxie Chromatography Workstation software (version 1.9.3.2) to 12 in order to provide sufficient sensitivity. The FID settings were as follows: heater = 250 °C, sensitivity = 12, He gas = 30 mL/min, H_2 = 31 mL/min and air = 296 mL/min and oven program time of 111 min. Fatty acid concentrations were calculated by the following equation:

$$\%\ \text{FA Conc.} = [\text{Int. Std Conc.}(5\%) \times \text{Sample Peak} \times \text{Relative Response Factor}] / \text{Int. Std Sample Peak} \qquad (1)$$

Soy Oil Fatty Acid Analysis Using a Novel Micro FAME Derivatization Method

Conventional Macro-Method for FAME Preparation

Methyl esters were prepared from the soy oil by a base-catalyzed method [31]. One hundred milligrams of soy oil was accurately weighed using a weighing balance (Mettler Toledo Classic, AB204-S) into a 25-mL centrifuge tube using a class-A pipette, and 500 µL of 1% HME (C17:0, internal standard manually added), 2 mL of toluene and 4 mL of 0.5 M sodium methoxide in methanol were added to the centrifuge tube and then purged with nitrogen gas. The centrifuge tube was heated to 50 °C for 10–12 min and then cooled for 5 min. To inhibit formation of sodium hydroxide, which could hydrolyze methyl esters to free fatty acids, 0.2 mL glacial acetic acid was added to the centrifuge tube. Five milliliters of distilled water was added to the centrifuge tube followed by 5 mL of hexane, and the tube was vortexed for 2 min. The hexane layer was extracted and dried over anhydrous sodium sulfate in a 7-mL glass vial for 15–20 s. Another 5 mL of hexane was added to the centrifuge tube, the tube was vortexed for another 2 min, and the hexane layer was dried over anhydrous sodium sulfate prior to GC analysis [32].

Micro-Method for FAME Preparation

Triplicate soy oil samples of 100, 75, 50, 25, 5, 4, 3, 2 and 1 mg were accurately weighed using a weighing balance (Mettler Toledo Classic AB204-S) in 25-mL centrifuge tubes using a class-A pipette. A 1% HME solution in hexane was prepared. HME equivalent to 5% of the oil weight was manually added to each centrifuge tube as an internal standard using a class-A pipette. One milliliter of toluene and 4 mL of 0.5 M sodium methoxide in methanol were added to each centrifuge tube and purged with nitrogen gas. The centrifuge tubes were heated to 50 °C in a water bath for 10–12 min and then cooled for 5 min. To inhibit formation of sodium hydroxide, which could hydrolyze methyl esters to free fatty acids, 0.2 mL glacial acetic acid was added to the centrifuge tube. Five milliliters of distilled water was added to each centrifuge tube followed by 1 mL of hexane, and the tubes were vortexed for 2 min. The hexane layer was extracted and dried over anhydrous sodium sulfate in a 7-mL glass vial for 15–20 s. The total time taken in this method for FAME preparation was 30 min prior to GC analysis [32].

Effect of Sample Weight Variability on Fatty Acid Composition of 100- and 1-mg Oil Samples

There was a higher variability in weighing 1-mg oil samples as compared to 100-mg samples. In order to minimize the variability caused by weighing small oil samples, sample weight was added to the equation (Eq. 2). Triplicate soy oil samples of approximately 100 mg (A) and 1 mg (B) (nominal weights) were accurately weighed, using a weighing balance (Mettler Toledo Classic AB204-S) in 25-mL centrifuge tubes using a class-A pipette and the weights were recorded (actual weights). The weighed oil samples (A) were converted to FAMEs using the conventional macro-method [31] and the weighed oil samples (B) were converted to FAMEs using the micro-method. The fatty acid composition of the FAMEs (A + B) was determined by GC [32].

Gas Chromatographic Analysis

Methyl esters were analyzed for each replicate by GC in triplicate using the method as described previously. The fatty acid concentrations were calculated by the new equation (Eq. 2) to account for the weight of the sample which is as follows:

$$\% \text{ FA Conc.} = [\text{Int. Std Conc.}(5\%) \times \text{Sample Peak} \times \text{Relative Response Factor} \times \text{Weight Correction Factor})/\text{Int. Std Sample Peak} \quad (2)$$

where, Weight Correction Factor = nominal weight (mg)/ actual weight (mg)

Statistical Data Analysis

Data were analyzed by analysis of variance using the JMP version 7.1 (SAS Inst, Cary, NC, USA). Least significant differences were calculated to compare mean values among three replications within each fatty acid for significant differences using the Tukey–Kramer honestly significant difference test.

Orthogonal regression was used to compare the mean values obtained from commercial AOCS FAME standards containing 15,000 and 100 µg/mL and soy oil samples containing 100 and 1 mg. Orthogonal regression was preferred over linear regression as linear regression is used to minimize the sum of the squared vertical distances from the data points to the fitted line [33]. Orthogonal regression minimizes the orthogonal (perpendicular) distances from the data points to the fitted line reducing the overall variance. Significance was established at $P < 0.05$.

Results and Discussion

Fatty Acid Analysis of FAME Samples Obtained by Serial Dilution of a Commercial FAME Standard

Figure 1 shows fatty acid composition of serial dilutions of a commercial FAME standard as determined by GC-FAME analysis [32]. No statistically significant differences were observed ($P < 0.05$) in the overall fatty acid means between the FAME dilutions analyzed for any specific fatty acid measurement. This indicates that there is no loss of sensitivity in GC analysis of FAME's in the 100–15,000 µg/mL range.

Figure 2 shows a correlation using orthogonal regression between commercial FAME standard concentrations of 15,000 and 100 µg/mL as determined by GC. The correlation coefficient (R^2) obtained between the two

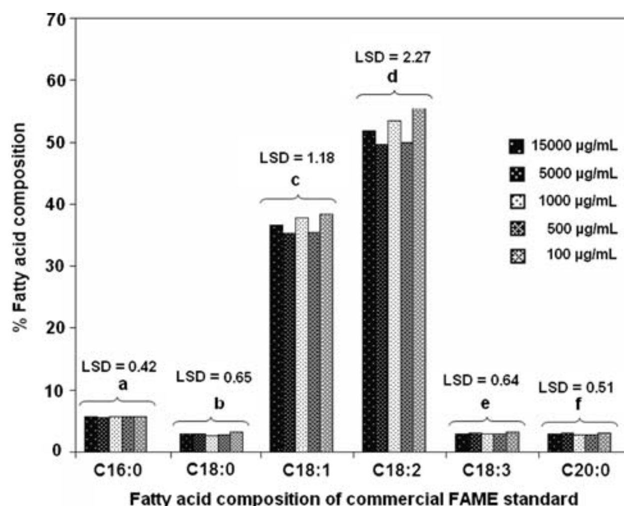

Fig. 1 Fatty acid composition of a commercial FAME standard at various dilutions obtained by GC. Replications (n) = 3. Mean values with different letters differ significantly, $P < 0.05$

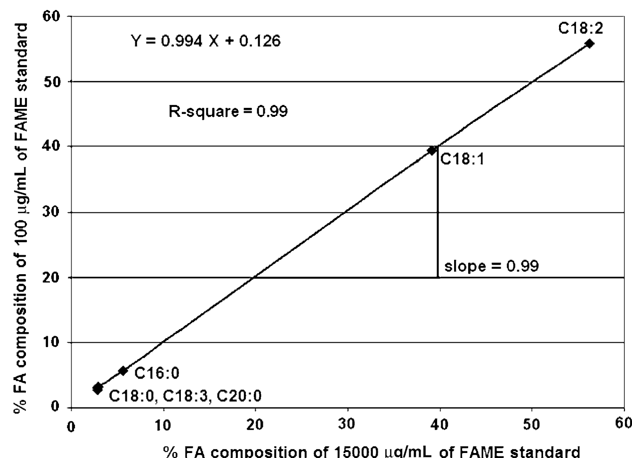

Fig. 2 Correlation using orthogonal regression between fatty acid composition of FAME dilutions of 100 and 15,000 µg/mL determined by GC (adapted from [32])

concentrations was 0.99 indicating no effect of dilution on the fatty acid levels obtained by GC FAME analysis.

Soy Oil Fatty Acid Analysis Using a Novel Micro FAME Derivatization Method

Figure 3 shows fatty acid composition of various dilutions of soy oil as determined by GC-FAME analysis using the novel solvent system with sample weight taken into account to minimize variability. No statistically significant differences ($P < 0.05$) were observed in levels of each fatty acid obtained by the novel solvent system between all soy oil dilutions. Furthermore, there was no statistically significant difference between oil analyzed by the standard

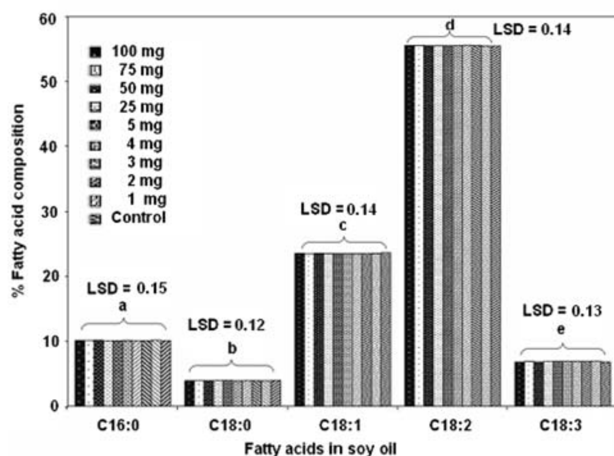

Fig. 3 Fatty acid composition of soy oil at various dilutions by novel micro-method relative to a control analysis conducted by a conventional macro-method (adapted from [31]). Replication (n) = 3. Mean values with different letters differ significantly, $P < 0.05$

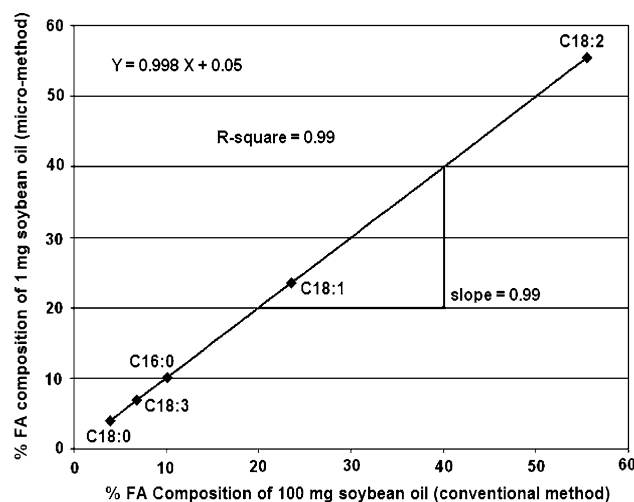

Fig. 4 Correlation using orthogonal regression between fatty acid composition of 1 mg of soy oil determined by the micro-method and 100 mg soy oil determined by the conventional macro-method (adapted from [31])

method (control) [31] and experimental data obtained by the novel solvent system. This indicates that there was no loss of sensitivity in GC analysis of FAMEs in the 1–100 mg oil range.

Figure 4 shows a correlation using orthogonal regression between fatty acid composition of 1 mg oil measured by the micro-method and 100 mg oil as determined by the conventional macro method [31]. The correlation coefficient (R^2) obtained between the two concentrations was 0.99 indicating no effect of dilution on the fatty acid profiles obtained by GC FAME analysis.

Table 1 Effect of sample weight variability on fatty acid composition of 100- and 1-mg oil samples

Sample weights (mg)	% Fatty acid composition in soy oil				
	C16:0	C18:0	C18:1	C18:2	C18:3
99.98	10.80*a*	3.97*a*	23.42*a*	55.67*a*	6.14*a*
100.07	10.77*a*	3.88*a*	23.49*a*	55.68*a*	6.18*a*
99.99	10.88*a*	3.92*a*	23.44*a*	55.74*a*	6.10*a*
1.01	10.80*a*	3.95*a*	23.56*a*	55.64*a*	6.06*a*
1.04	10.75*a*	3.96*a*	23.58*a*	55.64*a*	6.09*a*
0.99	10.76*a*	3.94*a*	23.50*a*	55.70*a*	6.12*a*

Values are % fatty acid mean values of triplicate analyses of three replications within each oil sample weight

Fatty acids in same column with same letters are not significantly different ($P < 0.05$) as measured by ANOVA using Tukey–Kramer HSD test

Effect of Sample Weight Variability on Fatty Acid Composition of 100- and 1-mg Oil Samples

Table 1 shows fatty acid composition of 100- and 1-mg oil samples determined by taking sample weight into account, to minimize variability. No statistically significant differences ($P < 0.05$) were observed in levels of each fatty acid in soy oil. Furthermore, there was no statistically significant difference between 100 mg oil samples converted to FAMEs by the conventional macro-method [31] and 1 mg oil samples converted to FAMEs by the novel solvent system.

In summary, we developed a 30-min, micro-base-catalyzed method for vegetable oil fatty acid determination using a novel FAME derivatization method with modifications in the GC-FID sensitivity for relatively small pure oil samples without loss of sensitivity or accuracy.

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