JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2011, 59, 747–754 747
DOI:10.1021/jf1038426

Lipid Fatty Acid Profile Analyses in Liver and Serum in Rats with Nonalcoholic Steatohepatitis Using Improved Gas Chromatography−Mass Spectrometry Methodology

Xianli Wu,*[†,‡] Yudong Tong,[†,‡] Kartik Shankar,[†,§] January N. Baumgardner,[†,‖] Jie Kang,[†,‡] Jamie Badeaux,[†] Thomas M. Badger,[†,‡,§] and Martin J. J. Ronis[†,§,‖]

[†]Arkansas Children's Nutrition Center, [‡]Department of Physiology and Biophysics, [§]Department of Pediatrics, and [‖]Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, 15 Children's Way, Little Rock, Arkansas 72202, United States

Fatty acids (FAs) are essential components of lipids and exhibit important biological functions. The analyses of FAs are routinely carried out by gas chromatography−mass spectrometry after multistep sample preparation. In this study, several key experimental factors were carefully examined, validated, and optimized to analyze free fatty acid (FFA) and FA profiles of triglycerides and phospholipids in serum or tissue samples. These factors included (1) methylation/transesterification reagents, (2) validation of internal standards, and (3) final step concentration of FA methyl esters. This new method was utilized to analyze FFAs and the FA profiles of triglycerides and phospholipids in the serum and liver from a recently established rat model of nonalcoholic steatohepatitis (NASH). In this model, rats were fed a 220 kcal kg $^{-3/4}$ day $^{-1}$ diet containing either 5 or 70% corn oil for 21 days using total enteral nutrition. FA compositions of the serum and liver were found to shift from a pattern dominated by saturated and monounsaturated FAs (C16:0/18:1) to one dominated by polyunsaturated C18:2 derived from dietary linoleic acid. Alteration of FA composition in liver after overfeeding of high polyunsaturated fat diets may contribute to the progression of pathological changes from steatosis to inflammation, necrosis, and fibrosis observed in NASH.

KEYWORDS: GC/MS; fatty acid; NASH; triglyceride; phospholipid; liver

INTRODUCTION

Fatty acids (FAs) are essential components of lipids and exhibit important biological functions in various tissues. As principle components in membranes, the chain length, degree of saturation, and other structural features of fatty acids are determinants of the physicochemical properties of biological membranes. In recent years, FAs have been suggested to play important roles in intracellular signaling. Free fatty acids (FFAs), their acyl Co-A esters, and metabolites have been implicated as intracellular signaling molecules involved in turning on nuclear receptors such as the peroxisome proliferator activator receptors (PPARs), which regulate lipid and carbohydrate metabolism, transport, and cellular proliferation (*1−3*). Lipid peroxidation product protein adducts may disrupt normal protein function and contribute to cellular injury, which have been shown to be associated with triglyceride (TG) lipid droplets in liver cells (*4*, *5*). Different FA compositions of TGs may change the size of the droplet and the nature of associated proteins or adducts and thus change signaling pathways (*6*). Phospholipids (PLs) constitute ~60% of the lipid mass of an eukaryotic cell membrane (*7*). FA analysis of PLs, especially in mitochondrial membranes, may also provide clues to mechanisms of cellular injury. In one report, the more unsaturated the FAs in the lipids are, the more likely reactive oxygen species species will perpetuate in membranes and cause mitochondrial permeability leading to apoptosis/necrosis (*8*).

In light of these new findings, the analysis of FA profiles in tissues has become increasingly important as end points in understanding the roles of FAs in physiological or pathological processes. The conventional method for such analysis typically consists of lipid extraction, lipid class segregation, esterification of FAs, chromatographic separation, sequential mass spectrometric identification, and quantitation of the FA esters. For this laborious multistep procedure, each step must be optimized and carefully conducted to achieve optimal results (*9*).

There are only a few papers published that analyzed FFAs and FA profiles in TGs and PLs in animal tissues (*10−12*). None of them carefully validated their methods. We had observed significant variation among samples and replicates using unvalidated methods, in which some very important factors were either neglected or not validated. For instance, the recovery tests were rarely conducted to evaluate the completeness or loss during sample preparation. Moreover, analyzing whole FA profiles, ranging from short to long chain, saturated to polyunsaturated, is very different from analyzing one or several targeted FAs. Short-chain FA methyl esters (FAMEs) are more volatile than

*To whom correspondence should be addressed. Tel: 501-364-2813. Fax: 501-364-3161. E-mail: wuxianli@uams.edu.

© 2010 American Chemical Society
Published on Web 12/16/2010

long-chain FA methyl esters. Without a proper sample handling strategy, the majority of short-chain FAMEs may be lost during esterification and following drying-out steps (*13*).

The first objective of the present study was to optimize several key factors in analyzing FFA and FA profiles of lipids in tissue samples, primarily in liver. Because the theory and general procedures of FA analysis have been well established (*7*, *9*, *14−16*), we focused on several practical aspects including: (1) methylation or transesterification reagents, (2) validation of internal standards (ISs), and (3) last step solvent evaporation of FAMEs.

Using the validated method, FFA and FA profiles of TG and PL in serum and liver from a recently established rat model of nonalcoholic steatohepatitis (NASH) were analyzed (*17*). Although various pathological, biochemical, and molecular parameters of this NASH model have been published (*17*), the FA profiles have awaited improvement of analytical techniques. The current study was designed to fill this gap and provide insights into the role of dietary polyunsaturated FAs (PUFAs) in the progression of the pathological changes in this model.

MATERIALS AND METHODS

Chemicals and Reagents. Thirty-seven FAME standards, glyceryl triheptadecanoate (C17:0 TG), tricosanoic acid (C23:0), heptadecenoic acid (C17:1), nonanoic acid (C9:0), pentadecanoic acid (C15:0), hepadecanoic acid (C17:0), nonadecanoic acid (C19:0), tricosanoic acid (C23:0), *cis*-10-hepadecanoic acid (C17:1), and FAMEs of C9:0, C15:0, C17:0, C19:0, C23:0, and C17:1 were purchased from Sigma-Aldrich (St. Louis, MO). Nonadecatrienoic acid (C19:3), tricosapentaenoic acid (C23:5), and their methyl esters were purchased from Laredan Fine Chemicals (Malmo, Sweden). 1,2-Dinonadecanoyl-*sn*-clycero-3-phospholine (C19:0 PC) was purchased from Avanti Polar Lipids (Alabaster, AL). Sulfuric acid (99.999%), heptane, sodium hydroxide, anhydrous methanol (MeOH), and boron trifluoride methanol solution (14%) were also purchased from Sigma-Aldrich. All other reagents were high-performance liquid chromatography (HPLC) grade. Chloroform, ethanol, hexane, acetic acid, ethanolic phosphomolybdic acid, and ethyl ether were purchased from Fisher Scientific (Pittsburgh, PA). The IS solutions were made in $CHCl_3$ with a concentration of 1 μg/μL and an aliquot in a HPLC vial and stored at −20 °C. Thin-layer chromatography (TLC) plates (Partisil LK6DF Silica Gel 60 Å, 20 cm × 20 cm, 200 μm) were obtained from Whatman (Maidstone, England). Ethanolic phosphomolybdic acid (20%; v/v) was purchased from Sigma-Aldrich. A 2% ethanolic sulfuric acid solution (2% $H_2SO_4 \cdot MeOH$) was made by adding 2 mL of sulfuric acid (99.999%) into 98 mL of anhydrous MeOH.

Gas Chromatography−Electron Ionization Mass Spectrometry (GC/EI-MS). The gas chromatography/mass spectrometry system (GC/MS) consisted of a GC-2010 with a GC/MS-QP 2010 Mass Spectrometer (Shimadzu, Kyoto, Japan). A Supelco SP-2340 fused silica capillary column (30 m × 0.25 mm × 0.2 μm, Bellefonte, PA) was used, with temperature gradients from 50 to 100 °C at 14 °C/min, then to 145 at 9 °C/min, then to 170 at 7 °C/min, and held for 3 min, then to 185 at 4.75 °C/min and held for 1 min, then to 225 at 15 °C/min and held for 3.5 min. The carrier gas (helium) flow was 7.0 mL/min. Injector, ion source, and interface temperatures were 250 °C. GC settings were as follows: injection mode split; flow control mode linear velocity; pressure, 53.5 kPa; total flow, 7.0 mL/min; linear velocity, 18.2 cm/s; purge flow, 1.0 mL/min; and split ratio, 5.0. The mass spectrum was acquired from 40 to 400 *m*/*z* with an interval of 0.4 s, threshold of 100, and scan speed of 1000.

Compound Identification and Quantitation. FA peaks were identified based on comparing retention times with external standards or similarity search. Quantitation was conducted by using the peak area of the most intensive ion of each peak. For recovery tests, quantitation was made using external standards. One set of C9:0/C15:0/C17:0/C19:0/C23:0/C17:1/C19:3/C23:5 mixed FAME standards was used to create a standard curve for the recovery test.

For quantitation of FFAs and FA profiles in tissue samples, an IS method was used. Briefly, one set of FAME standards (37 FAME) was used for peak identification and to generate a relative response factor (RRF) (*14*) for IS quantitation.

Tricosanoic acid (C23:0), glyceryl triheptadecanoate (C17:0 TG), and 1,2-dinonadecanoyl-*sn*-clycero-3-phospholine (C19:0 PC) were used as ISs for quantitation of FFAs and FA profiles in TG and PL, respectively, using the following equation:

$$M_X = M_I(A_X^*/A_I^*) \cdot (SA_I \cdot C_X)/(SA_X \cdot C_I)$$

where M_I is the mass of IS FA, M_X is the mass of target FA, A_I* is the peak area of IS FA (sample), A_X* is the peak area of unknown FA (sample), SA_I is the peak area of IS FA (standard), C_X is the concentration of target FA (standard), SA_X is the peak area of target FA (standard), and C_I is the concentration of IS FA (standard).

In this equation, $(SA_I \cdot C_X)/(SA_X \cdot C_I)$ was also referred to as RRF, which reflects the different detector response to IS and the analyte. RRFs were determined by running known amounts of 37 FAME standards that contained IS FA.

Methylation/Transesterification. Two methods were used for comparison.

Method 1. Two milliliters of 2% $H_2SO_4 \cdot MeOH$ was added to screw cap glass tubes, containing FA standards or scraped silica gel. The tubes were purged with N_2, sealed with vortex, and transferred to a heating block for 70 min at 80 °C. The reaction was terminated followed by the addition of 500 μL of 1 N sodium hydroxide.

Method 2. Two milliliters of borontrifluoride (BF_3) in MeOH (14%) ($BF_3 \cdot MeOH$) was added to screw cap glass tubes, containing FA standards or scraped silica gel. The tube was purged with N_2, sealed, vortexed, and transferred to a heating block for 60 min at 90 °C, and the reaction was terminated by placing the tubes on ice.

Table 1. Recovery Tests of FFA, PL, and TG Standards by Two Methylation/Transesterification Reagents[a]

	method 1 ($BF_3 \cdot MeOH$)			method 2 ($H_2SO_4 \cdot MeOH$)		
FAME	mean (%)	SD (%)	RSD (%)	mean (%)	SD (%)	RSD (%)
saturated FFA	56.6	16.9	29.9	67.4	4.4	6.5
nonanoic acid (C9:0) ME in FFA fraction	58.9	3.4		62.4	3.0	
pentadecanoic acid (C15:0) ME in FFA fraction	32.1	3.8		65.5	3.3	
hepadecanoic acid (C17:0) ME in FFA fraction	55.5	3.2		67.2	4.5	
tricosanic acid (C23:0) ME in FFA fraction	79.9	4.9		74.3	4.8	
unsaturated FFA	60.2	2.9	4.7	65.5	4.1	6.2
cis-10-hepadecanoid acid (C17:1) ME in FFA fraction	56.4	4.5		61.5	4.3	
7,10,13-nonadecenoic acid (C19:3) ME in FFA fraction	60.8	3.2		63.9	3.1	
8,11,14,17,20-tricosanoic acid (C23:5) ME in FFA fraction	63.3	3.5		71.1	4.4	
PL						
nonadecanoic acid (C19:0) ME in PL fraction	66.4	6.6		67.3	5.0	
TG						
hepadecanoic acid (C17:0) ME in TG fraction	65.4	5.5		69.1	6.6	
overall	59.9	12.0	20.0	66.9	3.9	5.8

[a] Data were expressed as means ± SDs (n = 4).

Total FAMEs were extracted by heptane (1 mL × 3). The heptane layers were combined, and the total volume was brought up to 3 mL with heptane for GC/MS.

Animals and Diets. *Animals and Diets.* Male Sprague−Dawley rats (175 g) were purchased from Harlan Sprague−Dawley (Indianapolis, IN). Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved animal facility. Animal maintenance and experimental treatments were conducted in accordance with ethical guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

Rats were randomly assigned to two experimental groups before an intragastric cannula was surgically inserted and infused ig with water for 7 days until presurgical weight returned. Thereafter, the rats were fed by total enteral nutrition (TEN) using previously published liquid diet compositions (*17*). TEN rats were fed isocaloric 220 kcal kg $^{-3/4}$ day $^{-1}$ diets, which is 17% in excess of caloric intake recommended by the National Research Council (*17*) . One group received a low-fat TEN diet containing 5% total calories as corn oil (CO), 79% carbohydrate calories (dextrose/maltodextrin), and 16% calories as protein (hydrolyzed whey), while the second group received a high-fat TEN diet containing 70% total calories as CO, 14% carbohydrate calories, and 16% protein calories. Rats were euthanized after 21 days of diet infusion, and serum and livers were collected and stored at −70 °C until use.

Pathological Evaluation. Liver pathology was assessed by Oil Red O staining of liver sections according to the method described previously (*17*).

Sample Preparation. *Extraction of Total Lipids from Serum.* Before extraction, 50 μL of C17:0 TG and 50 μL of C23:0 FFA (1 μg/μL for both standards) were added as ISs. Total lipids were extracted with ethyl ether (2 mL × 2) and dried under N_2.

Extraction of Total Lipids from Liver. Liver homogenates were extracted with chloroform−MeOH (2:1; v/v). Glyceryl triheptadecanoate (150 μg), C23 tricosanoic acid (100 μg), and 1,2-dinonadecanoyl-*sn*-clycero-3-phospholine (150 μg) were added into homogenates as ISs. The chloroform phase was collected and dried under N_2.

Separation of Lipid Classes by TLC and Transesterification. Samples were reconstituted with chloroform and loaded onto TLC plates. Glyceryl triheptadecanoate, tricosanoic acid, and 1,2-dinonadecanoyl-*sn*-clycero-3-phospholine were also loaded as reference compounds. TLC plates were developed with hexane:ether:acetic acid (60: 13: 0.65). A 20% ethanolic phosphomolybdic acid was sprayed onto the lane where the standard was spotted, and the plate was heated for visualization. FFA, TG, and PL were located based on the R_f of the three reference compounds. Total FFA, TG, and PL spots were scraped and directly esterfied by sulfuric acid in MeOH or with boron−trifluoride−MeOH reagent (BF_3−MeOH).

Last step Solvent Evaporation

Experiment 1. C9:0 and C23:0 were dissolved in anhydrous MeOH and directly esterified by sulfuric acid. After direct methylation, half of the samples were extracted with heptane and directly analyzed by GC/MS. The other half was extracted with hexane followed by solvent evaporation under N_2. The residue was reconstituted with same volume of heptane for GC/MS analysis.

Experiment 2. C9:0, C23:0, C17:0 TG, and C19:0 PL were spiked in liver samples, and FAMEs of C9:0, C23:0, C17:0, and C19:0 were prepared by the above-mentioned method with sulfuric acid. Their recoveries were calculated with and without last step solvent evaporation described in Experiment 1.

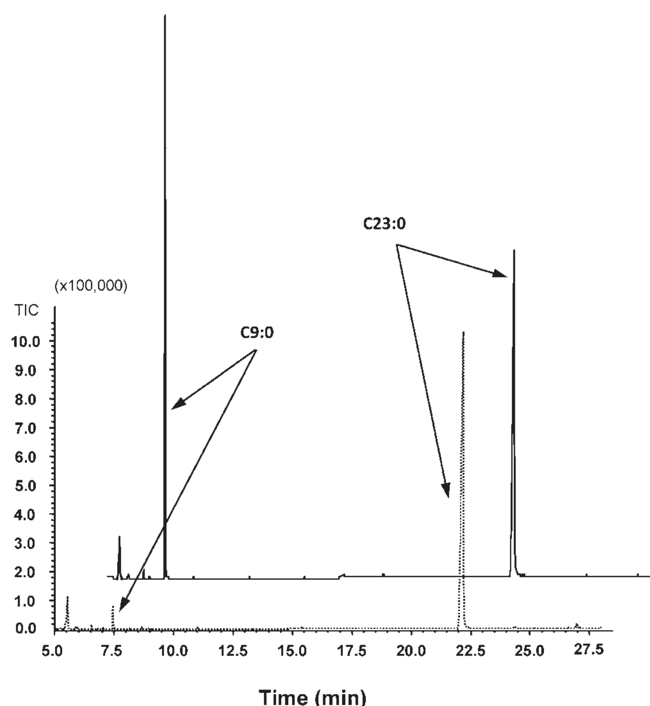

Figure 1. Profiles of C9:0 ME and C23:0 ME, which were made through methylation by 2% H_2SO_4−MeOH with (dotted line) and without (solid line) final step solvent evaporation, respectively (under the same scale in the MS spectrum).

Table 3. .Linearity and Detection Limit of Eight Odd Number Carbon FAMEs

FAME	linearity (*r*)[a]	DL (pg)[b]
nonanoic acid (C9:0) ME	0.9999	50
tricosanoic acid (C23:0) ME	0.9999	80
hepadecanoic acid (C17:0) ME	0.9999	50
nonadecanoic acid (C19:0) ME	0.9999	80
pentadecanoic acid (C15:0) ME	0.9999	50
cis-10-hepadecanoic acid (C17:1) ME	0.9999	100
7,10,13-nonadecenoic acid (C19:3) ME	0.9999	100
8,11,14,17,20-tricosanoic acid (C23:5) ME	0.9999	200

[a] Calibration range for correlation coefficient 1−50 ng. [b] DL, detection limit.

Table 2. Recoveries of Standards Spiked in Liver with or without Final Solvent Evaporation[a]

FAME	H_2SO_4·MeOH (final solvent evaporation)		H_2SO_4·MeOH (no final solvent evaporation)	
	mean (%)	SD (%)	mean (%)	SD (%)
		FFA		
nonanoic acid (C9:0) ME in FFA fraction	8.6	5.4	62.4	3.0
tricosanoic acid (C23:0) ME in FFA fraction	45.2	3.0	74.3	4.8
		PL		
nonadecanoic acid (C19:0) ME in PL fraction	36.8	4.4	67.3	5.0
		TG		
heptadecanoic acid (C17:0) ME in TG fraction	30.1	5.8	69.1	6.6

[a] Data were expressed as means ± SDs (*n* = 4).

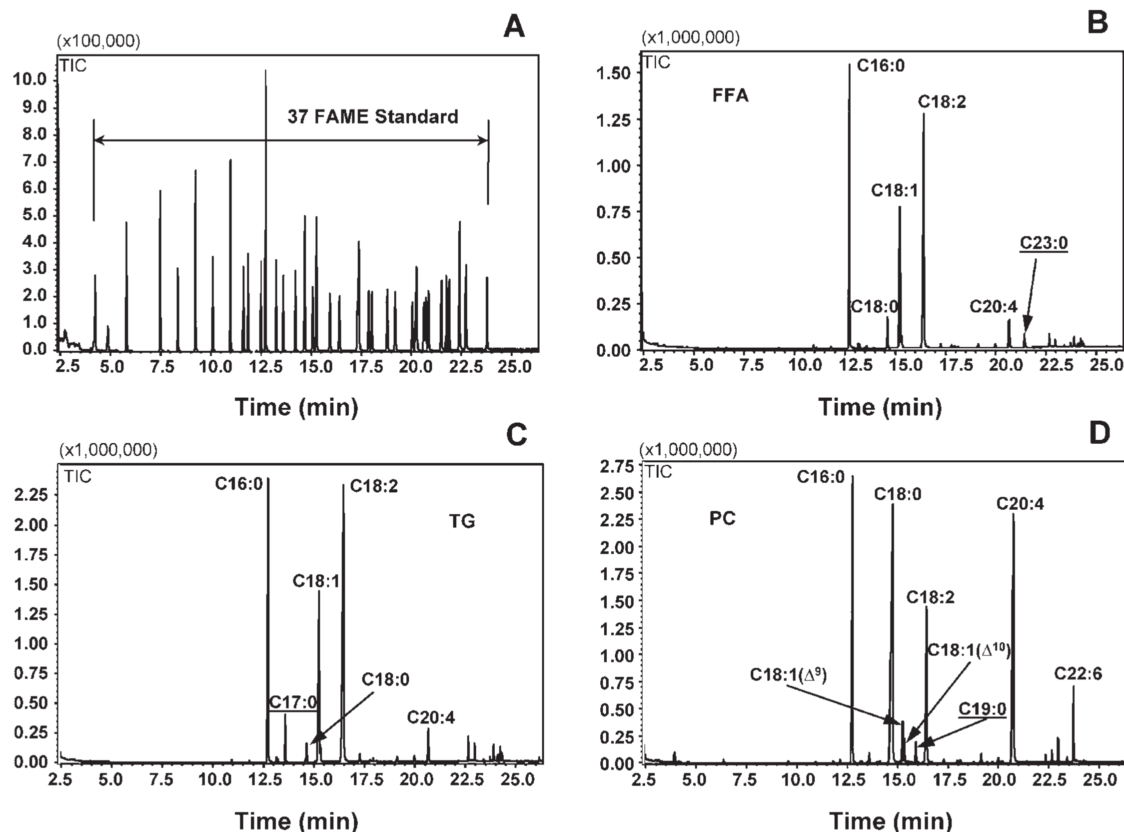

Figure 2. Representative chromatograms of 37 FAME standards (**A**) and the fatty acid profiles of FFAs (**B**), TG (**C**), and PL (**D**). C23:0, C17:0, and C19:0 are ISs in the fractions of FFA, TG, and PL.

Table 4. Recoveries of Three ISs Spiked in Rat Liver Samples over 29 Days

FAME	mean (%)	range (%)	SD (%)	RSD (%)
tricosanoic acid (C23:0) ME in FFA fraction	74.7	56.8−87.5	7.3	9.8
heptadecanoic acid (C17:0) ME in TG fraction	68.8	57.9−79.8	6.9	10.0
nonadecanoic acid (C19:0) ME in PL fraction	71.3	57.6−82.2	7.4	10.4

Statistical Analysis. Data are expressed as means ± standard errors of the mean (SEM). Student's *t* test was used to analyze differences between two groups. A value of $P < 0.05$ was considered significant unless otherwise mentioned. SigmaStat software package version 3.0 (SPSS, Chicago, IL) was used to perform all statistical tests.

RESULTS AND DISCUSSION

Validation of New GC/MS Method for Analysis of Lipid FA Profiles. To be analyzed by GC, FAs must be converted into volatile FA esters, in most cases, FAMEs. For lipids such as TG and PL, traditionally, this process includes two steps. First, the FAs are split off by saponification with sodium hydroxide or potassium hydroxide and then methylated (*12*, *14*). However, direct esterification of lipids ("alcoholysis") proceeds far more rapidly than saponification, with hydrolysis and esterification taking place in one step (transesterification), which uses only one reagent. The methylation/transesterification reagent must be carefully selected to make sure that the majority of FFAs or FA chains of PL/TG are converted into FAME (*14*). Derivatization methods in preparing FAMEs have been well documented (*12*−*14*, *18*). Two reagents, boron−trifluoride−MeOH reagent (BF_3−MeOH) or acid-catalyzed reagents [in particularly, sulfuric acid−MeOH reagent (H_2SO_4−MeOH)], are the most widely used (*19*). Both of them have been successfully used in methylation of FFA, and BF_3−MeOH has been used in transesterification of TG and PL (*11*, *20*). However, to our knowledge, there are no studies employing H_2SO_4−MeOH in transesterification of TG and PL of biological samples, and there are no studies to compare the effectiveness of the two reagents in the methylation of different FFAs and transesterification of TG and PL. In this study, these two most widely used methylation/transesterification reagents were compared for their effectiveness in making FAMEs.

Despite its popularity, BF_3−MeOH has disadvantages. This reagent is highly hazardous, expensive, and has a limited shelf life even when kept refrigerated, and deterioration can lead to artifacts. In our experiments, we found the formation of boron trimethoxide, and it can also add MeOH to double bonds (personal communication with Dr. W. W. Christie).

In our study, we compared BF_3−MeOH and H_2SO_4−MeOH in methylation and transesterification of FFA, TG, and PL. Odd number carbon FAs, which differ in chain length and degrees of saturation as well as TG or PL containing odd number carbon FA chains, were spiked in rat liver, and their recoveries were measured (**Table 1**). The overall average recovery of these FFAs, TG, and PL was 66.9% for 2% H_2SO_4−MeOH method vs 59.9% for BF_3−MeOH method, indicating that 2% H_2SO_4−MeOH provided better recoveries. The 2% H_2SO_4−MeOH not only provided better recovery but also generated less variation. Relative standard deviations (RSDs) calculated for saturated FFA, unsaturated FFA, and overall were 6.5, 6.2, and 5.8% for 2% H_2SO_4−MeOH method, whereas these values with BF_3−MeOH method were 29.9, 4.7, and 20.0%. This is critical for quantification

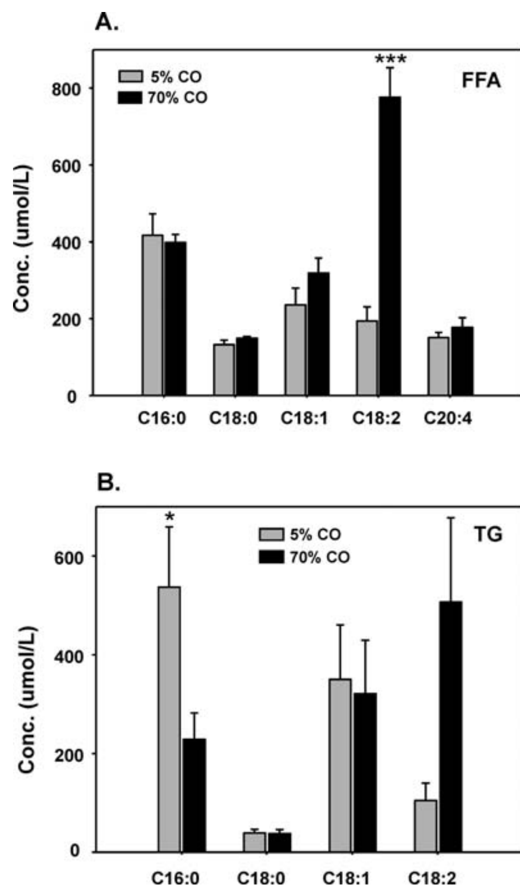

Figure 3. Serum fatty acid profiles of FFA (**A**) and TG (**B**) from rat fed either 5% CO diet or 70% CO diet (data expressed as means ± SEMs, $n = 5$; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

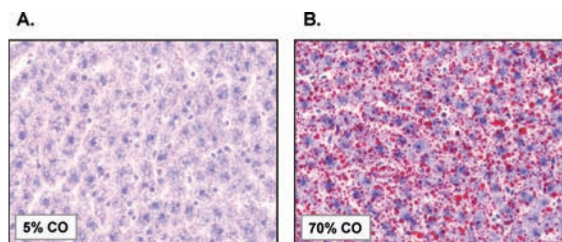

Figure 4. Representative Oil Red O stained liver sections from rats fed a diet containing 5% CO (**A**, 5% CO) or 70% CO (**B**, 70% CO).

with ISs. We also obtained better baseline in MS spectrum by using 2% H_2SO_4−MeOH, which improves quantitation of minor compounds by increasing the signal-to-noise ratio to promote detection at lower concentrations. In conclusion, we found 2% H_2SO_4−MeOH to be superior to BF_3−MeOH in making FAME from FFAs, TG, and PL.

The use of the IS for quantitation helps to balance out any loses of sample inherent to the sample preparation method or to inconsistent sample injection. However, for this to be true, the IS must be closely similar to the unknown analytes. In analyzing FAs with wide ranges of chain length and degrees of saturation, ISs must be validated and carefully selected. In some cases, if necessary, more than one IS may have to be used. Unfortunately, the validation of IS(s) has rarely been done. In this study, we validated tricosanoic acid (C23:0) as IS for FFA, glyceryl triheptadecanoate (containing three C17:0 chains, C17:0 TG) as IS for TG, and 1,2-dinonadecanoyl-*sn*-clycero-3-phospholine (containing two C19:0 chains, C19:0 PC) as IS for PL.

For validation of ISs, recovery tests were conducted after spiking C9:0, C15:0, C17:0, C23:0, C17:1, C19:3, C23:5, C17:0 TG, and C19:0 PC in liver samples as described early. Odd number carbon FFAs do not exist in liver and represent FFAs of short or long chains and with different saturation degrees. Because of availability, only one standard from TG (C17:0 TG) and one from PC (C19:0 PC) were adopted for recovery tests. The 2% H_2SO_4−MeOH was used as a methylation/transesterification reagent. The results (**Table 1**) showed that the recoveries of most standards were within the range 60−70%, except C23:0, which was slightly higher (74.3%). For quantification of FFAs, C23:0 was found to be acceptable but not perfect as IS. However, considering that the majority of FAs in liver are in the C16−C20 range, choosing C23:0 can reduce the interference with analytes. For PC and TG, C17:0 TG and C19:0 PC also displayed the recoveries of C17:0 and C19:0 in the 65−70% range. Thus, we think that C17:0 TG and C19:0 PC are valid ISs for TG and PC, respectively.

In the conventional procedure, after derivatization, FAMEs are extracted with organic solvents (e.g., hexane). Organic solvent is then evaporated and reconstituted in a smaller volume of organic solvent for GC or GC/MS analyses (*21−24*). The main purpose of this step is to concentrate the sample solution. However, this step may account for major loss of FAME, especially for short-chain FAMEs due to higher volatility (*11*,*13*,*14*). Nevertheless, except for one paper in which a simple comparison was mentioned (*11*), no other studies have addressed this issue carefully. In the current study, two experiments were conducted to evaluate the loss of FAME in this single step. In the first experiment, a quick test was done using C9:0 and C23:0. After methylation by 2% H_2SO_4−MeOH, their GC/MS chromatograms with and without final step solvent evaporation were shown in **Figure 1**. Comparing to direct heptane extraction, final step solvent evaporation accounted for major loss of short chain FA C9:0. For C23:0, the loss was minor because of much lower volatility of C23:0 methyl ester. In the second experiment, the recoveries of four standards spiked into liver samples, C9:0, C23:0, C17:0 TG, and C19:0 PC, were conducted by exactly the same procedures with and without final step solvent evaporation (**Table 2**). With last step solvent evaporation, recoveries of these four standards were all lower than those from procedure without last step solvent evaporation. Not only were recoveries lower, but the variations among samples were also greater (8.6−45.2% vs 62.4−74.3%) (**Table 2**). These clearly showed that this solvent evaporation step must be avoided. The main purpose of this step is to increase sample concentration, which is crucial for analyzing minor FAMEs. We increased sample concentration by (1) using more sample and (2) using less organic solvent for the final step extraction. There are other alternatives to increase sensitivity, such as analyzing pentafluorobenzyl (PFB) esters by negative chemical ionization (NCI) (*25*), but for other esters, standards are usually not commercially available, which limits usage in quantitation.

Because the current method was developed to analyze FAs in animal tissue samples, which in many cases are in tiny amounts, the linearity within the range of very low concentration (1−50 ng) and detection limit were assessed using eight odd number carbon FAME standards (**Table 3**). All standards displayed very good correlation coefficients (r) > 0.9999. The detection limits of the eight FAME standards were estimated in the range of 50−200 pg by serial dilution. Therefore, this method is ideal for analyzing trace amounts of FAs in animal tissue samples.

The representative chromatograms of 37 FAME standards and the fatty acid profiles of the three fractions FFA, TG, and PL are presented in **Figure 2**. Reproducibility and robustness of this

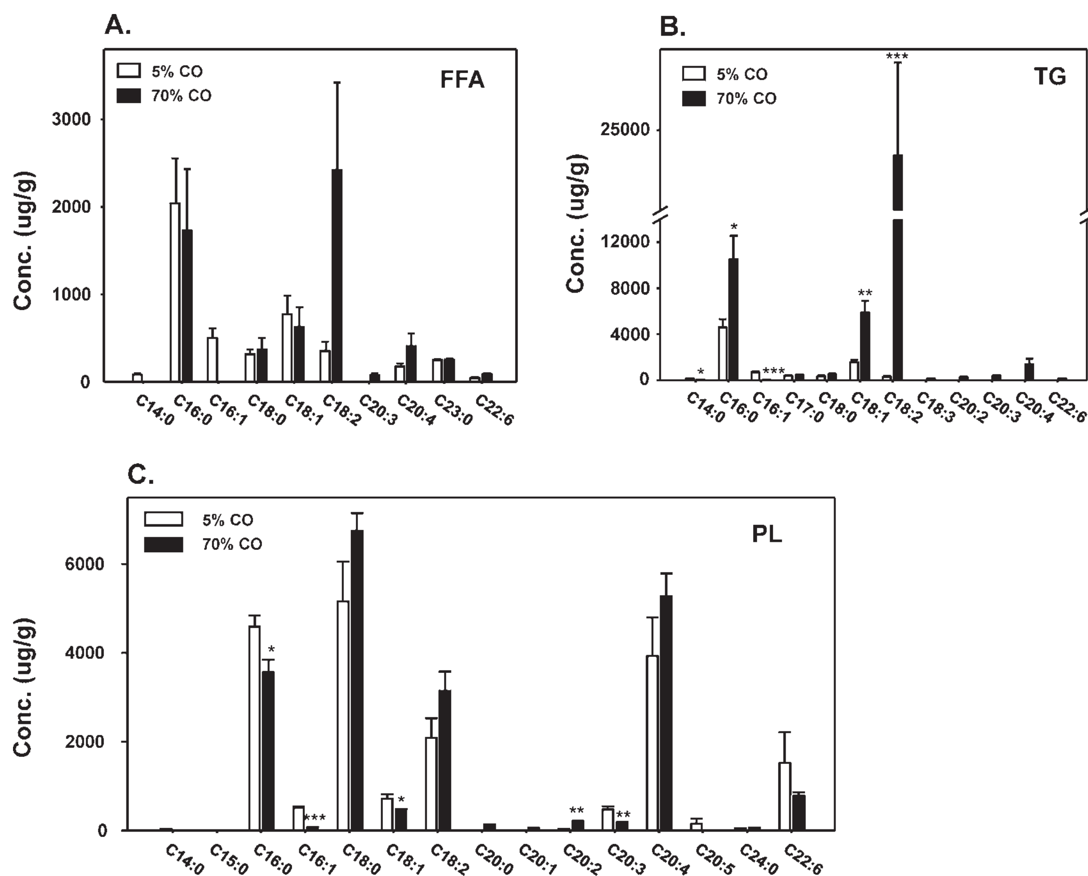

Figure 5. Liver fatty acid profiles of FFA (**A**), TG (**B**), and PL (**C**) from rat fed either 5% CO diet or 70% CO diet (data expressed as means ± SEMs, $n = 5$; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

FA analysis method were evaluated by monitoring the recoveries of three ISs day to day for 29 days (**Table 4**). Most recoveries fell in the 60−80% range, and their RSDs were around 10%.

FFAs and FA Profiles in Rat Serum and Liver. Dietary lipids directly influence FA composition in animal tissues, which in turn can alter their physiological or pathological consequences. In a newly established NASH model, rats fed a high polyunsaturated fat diet were found to develop steatoheptatitis similar to clinical NASH (*17*). By using the above validated GC/MS method, FFAs and FA profile of TGs were analyzed in serum from rats fed a diet containing 70% (total calories) or 5% CO (*5*, *17*). CO contains 55% PUFAs, 30% monounsaturated FAs, and 15% saturated FAs. Of the saturated FAs, 80% are palmitic acid (lipid number of C16:0), 14% stearic acid (C18:0), and 3% arachidic acid (C20:0). Over 99% of the monounsaturated FAs are oleic acid (C18:1). Ninety-eight percent of the PUFAs are the linoleic acid (C18:2). We have previously demonstrated that in this model overfeeding of either diet for 21 days resulted in obesity with identical gain in body weight and adiposity (*17*). However, in the 70% CO diet, total serum TGs were elevated from 5.8 ± 0.8 to 10.3 ± 0.6 mg/mL ($P < 0.05$), and serum NEFA concentrations were elevated from 0.6 ± 0.08 to 1.3 ± 0.3 mM ($P < 0.05$) (*17*). In serum from rats fed 5% CO, palmitic acid (C16:0) was the major FA in NEFA and TG (**Figure 3**). In 5% CO diet, 76% total calories are derived from carbohydrates (*17*). Palmitic acid is the major FA synthesized from carbohydrates. In serum from rats fed 70% CO, the composition and ratio of FFAs in NEFA and the FA profile of TG generally reflected that found in the dietary CO (**Figure 3**), indicating that FAs in CO were proportionally absorbed and incorporated into VLDL secreted from the liver.

In the NASH model, there were dramatic increases in liver TG content relative to livers of rats overfed 70% CO and the appearance of macro- and microsteatosis as indicated by Oil Red O staining (**Figure 4**). After 3 weeks of TEN feeding, the appearance of steatosis in the 70% CO group was accompanied by the development of oxidative stress, inflammation, and evidence of hepatocyte injury (ballooning) with an increase in serum ALT values from 19 ± 1.9 to 70 ± 4.0 U/L ($P < 0.05$), indicative of necrosis (*17*). Rats overfed the 70% CO diet for longer periods of 9 weeks had progressive liver pathology resembling that observed in NASH patients, which is characterized by further lipid accumulation, appearance of necrotic foci, stellate cell activation, and the development of fibrosis (*5*). FFAs and the FA profiles of TG and PL in livers of rats in the present study are presented in **Figure 5A−C**. FFAs displayed a similar pattern among 5 and 70% CO groups in FFAs as those in the serum. Some minor FAs including C14:0, C20:3, C20:4, C23:0, and C22:6 were also detected. A dramatic difference between the two diet groups was observed in FA composition of TG, which were present at levels >5-fold higher in the livers of rats fed 70% CO as compared to those fed 5% CO. The majority of FAs in TG from the 70% CO group were PUFAs; C18:2 is the predominant FA among them. PUFAs are vulnerable to oxidation by free radicals and regarded as a major source for lipid peroxidation (*26*). Oxidative stress has been proposed as a major mechanism leading to NASH pathology, and the antioxidant *N*-acetylcysteine has been found to attenuate progression of NASH (*5*). Lipid peroxidation product protein adducts such as those formed with hydroxynonenal (HNE) and malondialdehyde (MDA) have been shown to be associated with TG

lipid droplets in liver cells (*4*, *5*). It is likely that the FA side chains on the surface of lipid droplets appearing in liver after high fat overfeeding represent a major substrate for lipid peroxidation reactions and for the formation of HNE/MDA adducts with droplet-associated proteins such as perilipin and adipophilin. Such adducts may disrupt normal protein function and contribute to cellular injury (*27*). It is likely that adduct formation is highly dependent on FA composition of the TGs in the lipid droplets; therefore, that fewer adducts would be formed in steatotic livers following the feeding of saturated or monounsaturated fats as compared to polyunsaturated or w-3 fats such as fish oil. This is consistent with studies of alcoholic liver injury in which severity of hepatic steatohepatitis has been found to be greater in animals fed ethanol in the presence of CO or fish oil as compared to those fed the same level of ethanol in the presence of saturated or monounsaturated fats (*28*).

In liver PL, the FA composition in the two diet groups was remarkably similar. Stearic acid (C18:0) and arachidonic acid (C20:4) appeared to be the major FAs. Stearic acid and arachidonic acid were most likely synthesized from dietary linoleic acid by hydrogenation and desaturation−elongation, respectively (*29*). Although levels of 18:0, 18:2, 20:2, and 20:4 were somewhat higher in PLs from the liver of rats fed 70% CO (as compared to 5% CO), this did not reach statistical significance except for 20:2. However, there was a significant decrease in the content of saturated and monounsaturated FAs 16:0, 16:1, and 18:1.

In conclusion, after careful examination of several key practical factors in GC/MS analysis of FA profiles in animal tissue, we propose a new validated procedure for analyzing FA profiles in different fractions of lipids from animal tissue, including FFAs, TG, and PL. Using this procedure, lipid FA profiles were analyzed in serum and liver from newly developed rat model of NASH for the first time. The FA composition of FFAs and TGs in liver and plasma was closely related to dietary lipids. Alteration of FA composition in liver FFAs and TGs to enrich the proportion of PUFAs susceptible to lipid peroxidation may contribute to progression of injury following development of steatosis.

ABBREVIATIONS USED

BF_3, borontrifluoride; CO, corn oil; CV, coefficient of variation; EI, electron ionization; FAME, fatty acid methyl ester(s); FFA, free fatty acid; GC/MS, gas chromatography/mass spectrometry; HCl, hydrochloric acid; H_2SO_4, sulfuric acid; IS, internal standard; MeOH, methanol; PC, phosphatidyl choline; PL, phospholipid; R_F, relative migration; RRF, relative response factor; RSD, relative standard deviation; TG, triglycerides; TLC, thin-layer chromatography; TEN, total enteral nutrition; NASH, nonalcoholic steatohepatitis.

ACKNOWLEDGMENT

We acknowledge Matthew E. Ferguson and Renee Till for their technical assistance.

LITERATURE CITED

(1) Jump, D. B. Fatty acid regulation of gene transcription. *Crit. Rev. Clin. Lab. Sci.* **2004**, *41*, 41−78.
(2) Hihi, A. K.; Michalik, L.; Wahli, W. PPARs: Transcriptional effectors of fatty acids and their derivatives. *Cell. Mol. Life Sci.* **2002**, *59*, 790−798.
(3) Grimaldi, P. A. Fatty acid regulation of gene expression. *Curr. Opin. Clin. Nutr. Metab. Care* **2001**, *4*, 433−437.
(4) Kharbanda, K. K.; Todero, S. L.; Shubert, K. A.; Sorrell, M. F.; Tuma, D. J. Malondialdehyde-acetaldehyde-protein adducts increase secretion of chemokines by rat hepatic stellate cells. *Alcohol* **2001**, *25*, 123−128.
(5) Baumgardner, J. N.; Shankar, K.; Hennings, L.; Albano, E.; Badger, T. M.; Ronis, M. J. N-acetylcysteine attenuates progression of liver pathology in a rat model of nonalcoholic steatohepatitis. *J. Nutr.* **2008**, *138*, 1872−1879.
(6) Poli, G.; Biasi, F.; Leonarduzzi, G. 4-Hydroxynonenal-protein adducts: A reliable biomarker of lipid oxidation in liver diseases. *Mol. Aspects Med.* **2008**, *29*, 67−71.
(7) Peterson, B. L.; Cummings, B. S. A review of chromatographic methods for the assessment of phospholipids in biological samples. *Biomed. Chromatogr.* **2006**, *20*, 227−243.
(8) Rohrbach, S. Effects of dietary polyunsaturated fatty acids on mitochondria. *Curr. Pharm. Des.* **2009**, *15*, 4103−4116.
(9) Myher, J. J.; Kuksis, A. General strategies in chromatographic analysis of lipids. *J. Chromatogr. B: Biomed. Appl.* **1995**, *671*, 3−33.
(10) Gutiérrez, A. M.; Reboredo, G. R.; Catalá, A. Fatty acid profiles and lipid peroxidation of microsomes and mitochondria from liver, heart and brain of Cairina moschata. *Int. J. Biochem. Cell Biol.* **2002**, *34*, 605−612.
(11) Han, J.; Hamilton, J. A.; Kirkland, J. L.; Corkey, B. E.; Guo, W. Medium-chain oil reduces fat mass and down-regulates expression of adipogenic genes in rats. *Obes. Res.* **2003**, *11*, 734−744.
(12) Nikolaidis, M. G.; Petridou, A.; Mougios, V. Comparison of the phospholipid and triacylglycerol fatty acid profile of rat serum, skeletal muscle and heart. *Physiol. Res.* **2006**, *55*, 259−265.
(13) Rosenfeld, J. M. Application of analytical derivatizations to the quantitative and qualitative determination of fatty acids. *Anal. Chim. Acta* **2002**, *465*, 93−100.
(14) Eder, K. Gas chromatographic analysis of fatty acid methyl esters. *J. Chromatogr. B: Biomed. Appl.* **1995**, *671*, 113−131.
(15) Gutnikov, G. Fatty acid profiles of lipid samples. *J. Chromatogr. B: Biomed. Appl.* **1995**, *671*, 71−89.
(16) Seppänen-Laakso, T.; Laakso, I.; Hiltunen, R. Analysis of fatty acids by gas chromatography, and its relevance to research on health and nutrition. *Anal. Chim. Acta* **2002**, *465*, 39−62.
(17) Baumgardner, J. N.; Shankar, K.; Hennings, L.; Badger, T. M.; Ronis, M. J. A new model for nonalcoholic steatohepatitis in the rat utilizing total enteral nutrition to overfeed a high-polyunsaturated fat diet. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2008**, *294*, G27−G38.
(18) Shantha, N. C.; Napolitano, G. E. Gas chromatography of fatty acids. *J. Chromatogr.* **1992**, *624*, 37−51.
(19) Antolin, E. M.; Delange, D. M.; Canavaciolo, V. G. Evaluation of five methods for derivatization and GC determination of a mixture of very long chain fatty acids (C24:0-C36:0). *J. Pharm. Biomed. Anal.* **2008**, *46*, 194−199.
(20) Morrison, W. R.; Smith, L. M. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride−methanol. *J. Lipid Res.* **1964**, *5*, 600−608.
(21) Basconcillo, L. S.; McCarry, B. E. Comparison of three GC/MS methodologies for the analysis of fatty acids in Sinorhizobium meliloti: Development of a micro-scale, one-vial method. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2008**, *871*, 22−31.
(22) Morimoto, M.; Reitz, R. C.; Morin, R. J.; Nguyen, K.; Ingelman-Sundberg, M.; French, S. W. CYP-2E1 inhibitors partially ameliorate the changes in hepatic fatty acid composition induced in rats by chronic administration of ethanol and a high fat diet. *J. Nutr.* **1995**, *125*, 2953−2964.
(23) Ronis, M. J.; Korourian, S.; Zipperman, M.; Hakkak, R.; Badger, T. M. Dietary saturated fat reduces alcoholic hepatotoxicity in rats by altering fatty acid metabolism and membrane composition. *J. Nutr.* **2004**, *134*, 904−912.
(24) Yi, L. Z.; He, J.; Liang, Y. Z.; Yuan, D. L.; Chau, F. T. Plasma fatty acid metabolic profiling and biomarkers of type 2 diabetes mellitus based on GC/MS and PLS-LDA. *FEBS Lett.* **2006**, *580*, 6837 6845.
(25) Pawlosky, R. J.; Sprecher, H. W.; Salem, N., Jr. High sensitivity negative ion GC-MS method for detection of desaturated and chain-elongated products of deuterated linoleic and linolenic acids. *J. Lipid Res.* **1992**, *33*, 1711−1717.

(26) Niki, E.; Yoshida, Y.; Saito, Y.; Noguchi, N. Lipid peroxidation: mechanisms, inhibition, and biological effects. *Biochem. Biophys. Res. Commun.* **2005**, *338*, 668−676.
(27) Hartley, D. P.; Kolaja, K. L.; Reichard, J.; Petersen, D. R. 4-Hydroxynonenal and malondialdehyde hepatic protein adducts in rats treated with carbon tetrachloride: Immunochemical detection and lobular localization. *Toxicol. Appl. Pharmacol.* **1999**, *161*, 23−33.
(28) Nanji, A. A.; Su, G. L.; Laposata, M.; French, S. W. Pathogenesis of alcoholic liver disease--recent advances. *Alcohol.: Clin. Exp. Res.* **2002**, *26*, 731−736.
(29) Zhou, L.; Nilsson, A. Sources of eicosanoid precursor fatty acid pools in tissues. *J. Lipid Res.* **2001**, *42*, 1521−1542.

Received for review October 1, 2010. Revised manuscript received November 22, 2010. Accepted November 30, 2010. This study was supported by the U.S. Department of Agriculture (6251-51000-005-02S). Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.