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Elimination of *n*-butylated hydroxytoluene methylation during fatty acid analysis by gas chromatography

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Abstract

An improved method for fatty acids analysis with optimum recovery of highly polyunsaturated fatty acids methyl esters in biological systems is presented. The method is based on transesterification of phospholipid and triacylglycerols to fatty acid methyl esters using a commercially available reagent, Methyl-Prep II. Without proper precautions, as much as 50% of *n*-butylated hydroxytoluene (BHT) added to prevent oxidation of polyunsaturated fatty acids, could be methylated during the transesterification step. Methylated BHT elutes close to 14:0 (myristic acid) and no longer functions as an antioxidant, but the modified conditions virtually eliminate the methylation of BHT. Sample extraction and methylation was completed in 30 min at room temperature. A chelator (diethylenetriamine-pentaacetic acid; DTPA) is also added to prevent peroxidation of metal catalyzed free radical chain reactions. The standard deviations of the major fatty acids from multiple human plasma samples prepared on different days were less than 5%. The recovery of arachidonic acid, 20:4, from plasma was improved using the new method, and the recovery for docosahexaenoic acid, 22:6, spiked to human plasma was found to be 99%.

Keywords: *n*-Butylated hydroxytoluene; Hydroxytoluene, *n*-butylated; Fatty acids

1. Introduction

Fatty acid composition is frequently determined by transesterification to form fatty acid methyl esters (FAMEs), followed by gas chromatography [1–4]. The fatty acid composition of the human macula and peripheral retina of the human eye shows five major fatty acids, palmitic (16:0), stearic (18:0), oleic (18:1), arachidonic (20:4), and docosahexaenoic (22:6), and has been determined in relative amounts [5,6]. To determine absolute values of fatty acids in the eye tissues by using an internal standard, a human plasma pool was established for quality control as was done previously for vitamin E analysis [7]. However, initially it was found that there were significant variations in fatty acid content between different samples of the same human plasma pool. It was reported by Stone et al. that addition of antioxidants such as *n*-butylated hydroxytoluene (BHT) can improve recovery of polyunsaturated fatty acids (PUFA) [8]. Heckers et al. found that BHT can be methylated during transesterification [9], which causes it to lose its antioxidant properties. The antioxidant function of BHT is dependent on the

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capability of the phenol group to donate a proton to neutralize a radical [10]. Once the hydroxyl group in BHT is derivatized (e.g. methylated), no proton can be easily abstracted. It is the purpose of this study to eliminate BHT methylation and to devise a reproducible lipid extraction and fatty acid transesterification procedure, using a human plasma pool for quality control, which can then be applied to determine fatty acid levels of various regions of human eye tissues.

2. Experimental

2.1. Reagents

Methanolic (*m*-trifluoromethylphenyl) trimethyl ammonium hydroxide (Methyl-Prep II), 0.2 *M*, for transesterification was obtained from Alltech Associates (Deerfield, IL, USA). Methanol was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Dehydrated ethanol, 200 proof, was obtained from Midwest Grain Products (Perkin, IL, USA). Hexane, dichloromethane, anhydrous sodium sulfate and iso-octane were gas chromatography–mass spectrometry (GC–MS) for residue analysis grade from EM Science (Gibbstown, NJ, USA). Butylated hydroxytoluene (BHT), diheptadecanoic acid phosphatidylcholine (di-17:0 PC) and diethylenetriamine-pentaacetic acid (DTPA) were from Sigma (St. Louis, MO, USA). 1-Palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphatidylcholine (16:0)(22:6) PC was a gift from Edward A. Dratz, who originally purchased it from Avanti Polar Lipids (Birmingham, AL, USA) and stored it in CH_2Cl_2 at −120°C for 6 years.

Human plasma was obtained from Bozeman Deaconess Hospital (Bozeman, MT, USA), fractionated in hundreds of 1-ml aliquots and stored at −85°C as described elsewhere [7].

2.2. Preparation of FAME

Method A

The lipid extraction of fatty acids was modified from our previously reported method [6] by adding an internal standard. Human plasma (50 μl) and 20 μl of di-17:0 PC (0.75 μg/μl in ethanol) were mixed with 950 μl phosphate-buffered saline solution (PBS) at pH 7.4, followed by addition of 1 ml BHT (100 μg/ml in methanol) and 2 ml CH_2Cl_2. After vortex mixing for 3 min, the mixture was centrifuged for 3 min at 1800 *g*. The lower layer (CH_2Cl_2 phase) containing the extracted lipids was carefully collected and passed through a pasteur pipet containing ~150 mg anhydrous Na_2SO_4 to remove any traces of moisture. The CH_2Cl_2 was evaporated completely with argon. To the dry residue, 50 μl CH_2Cl_2 were added to dissolve the lipids followed by 25 μl Methyl-Prep II to convert the fatty acids to their methyl esters; 1–3 μl of the CH_2Cl_2–Methyl-Prep II mixture were injected on the capillary GC column after a 10-min incubation at room temperature. Various modifications were made in Method A and are described as Methods B, C and D.

Method B

To ensure complete transesterification, the incubation with Methyl Prep II was carried out in a sand bath for 3 min at 50°C and then for 10 min at room temperature. To reduce the background of the residual Methyl-Prep II base, the methylated sample was evaporated to dryness and dissolved in 50 μl iso-octane [11], after which 1 μl was injected onto the GC or GC–MS system.

Method C

To enhance purification of the samples, the dry methylated extract (Method B) was mixed with 1.0 ml PBS buffer and 1.0 ml methanol and briefly vortex mixed. Subsequently, 2 ml of hexane were added, the sample was vortexed for 3 min, and then centrifuged for 3 min at 1800 *g*. The hexane upper phase was collected and evaporated with argon. The residue was dissolved in 50 μl iso-octane and 1 μl was injected onto the GC system.

Method D

The lipid extract was made with Method A, either with or without 2 m*M* DTPA in the PBS buffer, and was evaporated completely under argon. Methylene chloride (50 μl) and 25 μl of Methyl-Prep II were added and the mixture was incubated for 10 min or 30 min at room temperature, immediately followed by injection of 1 μl into the GC system. Subsequently 1.0 ml of PBS, either with or without 2 m*M*

DTPA, and 1.0 ml methanol were added to the methylated sample, followed by addition of 2 ml hexane and vortex mixed for 3 min. The mixture was then centrifuged for 3 min at 1800 *g*. The hexane phase was collected as described above or the vials containing the sample were immediately put in dry ice powder to freeze the lower phase (water phase), so that the upper phase (hexane phase) could be poured off. The upper phase was then evaporated to a small volume of about 100 μl, and then 50 μl iso-octane were added. The sample was evaporated with argon to about 50 μl for GC and GC–MS assay.

2.3. GC and GC–MS assay

Fatty acid analysis was carried out on a Model HP 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) with a split–splitless injector and a flame ionization detector that was connected with a RC 501 Chromatopac integrator (Shimadzu, Columbia, MD, USA) to record gas chromatograms. A DB-23 capillary column (15 m $\times$ 0.2 mm I.D., 0.15 μm film thickness) was used. Dried helium was used as carrier gas at a pressure of 35 kPa. Fuel gases used were air and hydrogen at flow-rates chosen to give maximum sensitivity which are 350 kPa and 42 kPa, respectively. The injector port and interphase were kept at 260°C. The oven temperature was held at 50°C for 2 min, followed by an increase to 110°C at a rate of 20°C/min, held at 110°C for 1 min followed by a 5°C/min increase to a final temperature of 240°C, and held for 10 min. Peak height ratios were used for quantitation because they gave slightly more reproducible results than peak area ratios on the Shimadzu integrator used in this study (data not shown).

GC–MS analysis was performed on a HP 5890 Series II gas chromatograph in combination with a HP 5971 mass-selective-detector. The identification of FAMEs were obtained by the analysis of *m/z* numbers in combination with searching and matching the standard spectra from a library data base.

3. Results

The fatty acid composition of human plasma is known [12,13]. Seven major fatty acids are present, which include 14:0 (myristate), 16:0 Palmitate), 16:1 (palmitoleate), 18:0 (stearate), 18:1 (oleate), 18:2 (linoleate) and 20:4 (arachidonate).

Using Method A, there was a large variation in fatty acid content of plasma samples from our plasma pool with standard deviations of up to 15% for the polyunsaturated fatty acids (data not shown). Furthermore, the residual Methyl-Prep II base caused a large background on the capillary column. Further analyses using Method B to ensure complete transesterification and to reduce background were performed. Using Method B (Fig. 1a), the recovery of BHT (retention time 11 min) is very poor, and a part of the BHT was methylated (retention time 13.5 min). The two large peaks at 4 and 6.2 min are residues from the Methyl-Prep II reagent [1]. Method C was then used to clean up the samples by removing the Methyl Prep II residue, and the chromatogram (Fig. 1b) indicates that the Methyl-Prep II residue is reduced (one peak at 4 min eliminated, and the peak at 6.2 min is smaller), but there is still a significant methylation of the BHT (13.5 min). The recovery of BHT was estimated to be much better as compared with Method B, but methylated BHT still was ~15% of total BHT.

GC–MS was used to identify the peaks at ~11 and 13.5 min as BHT (Fig. 1c) and methylated BHT (Fig. 1d), respectively. The peak at *m/z* 220 (Fig. 1c) is BHT (M=220) and the peak at *m/z* 205, is BHT minus a methyl group. The peak at *m/z* 234 is methylated BHT (Fig. 1d) and the peak at *m/z* 219 is methylated BHT minus a methyl group.

Since BHT methylation was not observed during our previous study, the original method (Method A) was tried again and it was confirmed that using this approach no BHT was methylated (data not shown). In Method D, samples were extracted as described in Method A, but DTPA was added to the PBS buffer. The samples were injected directly onto GC (without evaporation or re-extraction) and it was confirmed that BHT was not methylated under these conditions (Fig. 2a).

To improve the reproducibility of the method while eliminating BHT methylation, we further changed Method D to re-extract the samples, and the result is shown in Fig. 2b. The profiles from directly injected FAMEs and purified (re-extracted in hexane) FAMEs obtained by Method D are compared in Fig.

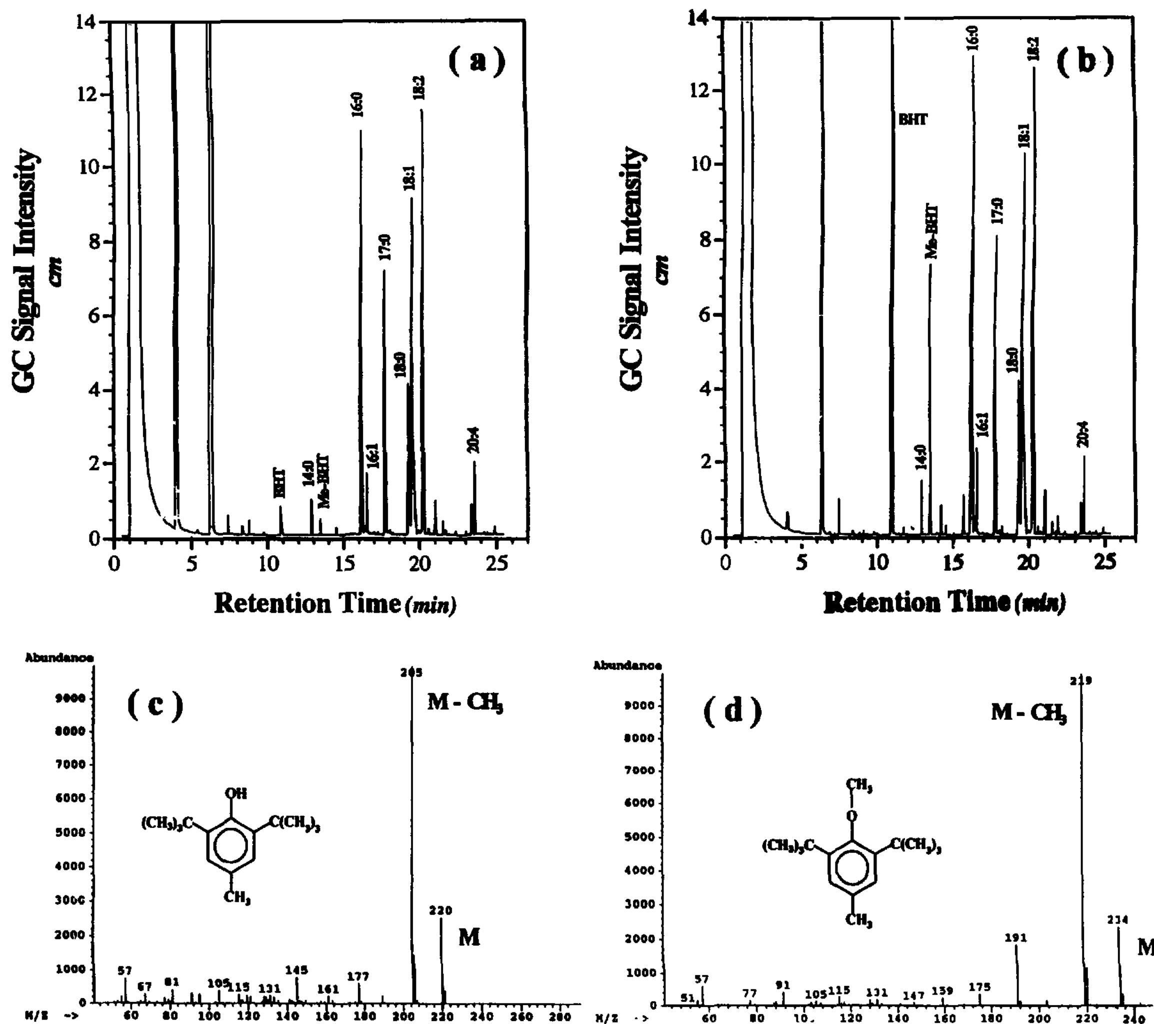

Fig. 1. GC chromatograms and GC–MS spectra from FAMEs prepared by Methods B and C. (a) The extract in CH_2Cl_2 was methylated with Methyl-Prep II for 3 min at 50°C and the mixture was further incubated for 10 min at room temperature, and then immediately blown down completely and 50 μl iso-octane was added to re-dissolve the methylated extract for GC assay; (b) after injection of an aliquot of (a) into GC, the remainder of the sample was immediately re-extracted in hexane (see Method C). The hexane layer was taken off and was evaporated completely, and 25 μl iso-octane were added for GC assay again. GC–MS spectra of BHT (c) and methylated BHT (d) from sample (a). A similar GC–MS spectrum (not shown) was recorded from injection (b).

2a and Fig. 2b. There are numerous small impurities in Fig. 2a that are much less pronounced in Fig. 2b. The large peaks from (*m*-trifluoromethylphenyl) trimethylammonium salts [1] at 4 and 6.2 min in the original GC profile were removed almost completely (4 min) or reduced in size (6.2 min) after re-extraction in hexane. The standard deviation values of FAMEs ($n = 6$) with Method D are smaller than those with Method A (data not shown). In addition, FAMEs were found to be stable during a week's monitoring of the same sample (data not shown). The methylated BHT was minimal, if present at all, and was no longer detectable by GC–MS. The standard deviation values of the ratios of FAMEs and internal standard (17:0) were less than 5% (Table 1). Another important improvement was the recovery of 20:4 with Method D (Fig. 2b), which was one-fold greater than that with Methods B or C (Fig. 1a and Fig. 1b).

BHT is an antioxidant that has already been used

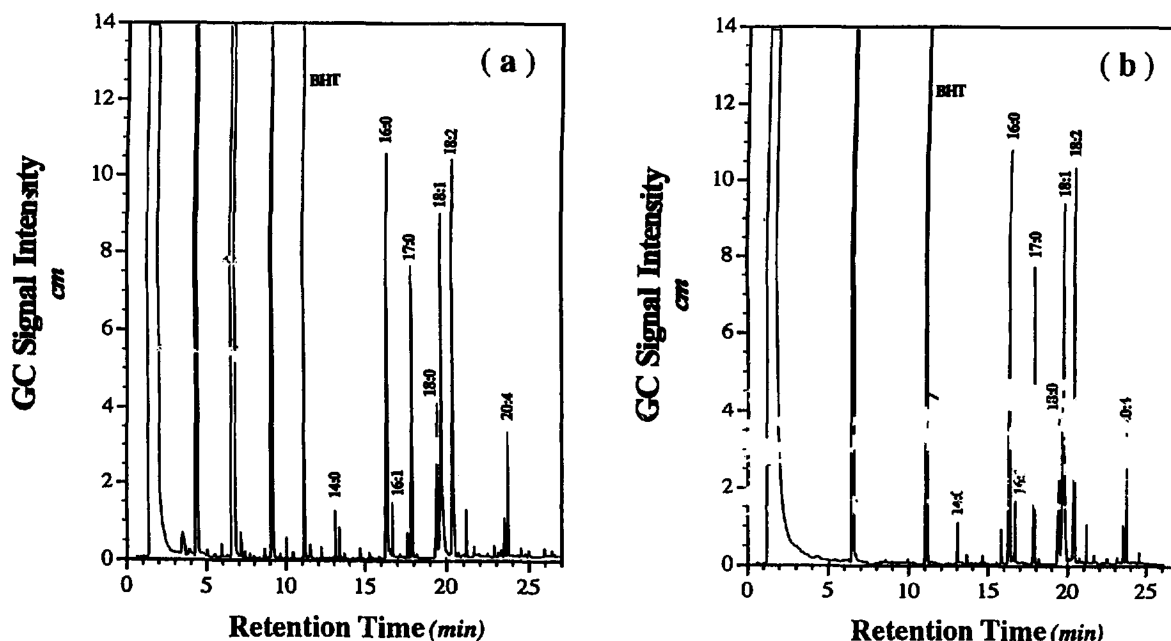

Fig. 2. GC chromatograms obtained from methylated extracts prepared by Method D. (a) FAMEs were immediately injected into GC after methylation without evaporation or re-extraction; (b) after the sample was re-extracted in hexane, 50 μl iso-octane were added and the sample evaporated to ~50 μl with argon for GC assay. See description of Method D for more detail.

in fatty acid assay systems to limit lipid peroxidation during analysis. Since transition metals can cause lipid peroxidation and human plasma contains iron [14,15] chelators are often used in addition in various experimental systems. We determined whether a chelator, which increases the complexity of the assay system and might be unnecessary, should also be included in the system. Therefore, we compared the ratios of fatty acid peaks with internal standard (17:0) in the absence and the presence of DTPA in the test system. The standard deviation values from samples with DTPA are smaller than those from samples without DTPA, indicating that the transition metals cause lipid peroxidation in this

Table 1
Effects of chelator and transesterification time on accuracy of fatty acids assay[a]

Fatty acid	30 min (n = 6)[b] without DTPA		30 min (n=6) with DTPA		10 min (n=4) with DTPA	
	Peak ratio[c]	μg/100 μl[d]	Peak ratio[c]	μg/100 μl[d]	Peak ratio[c]	μg/100 μl[d]
14:0	0.18±0.02	3.8±0.4	0.14±0.01	3.0±0.2	0.14±0.01	3.0±0.2
16:0	1.35±0.05	28.6±1.1	1.28±0.03	27.1±0.6	1.40±0.01	29.7±0.2
16:1	0.23±0.02	4.9±0.4	0.20±0.02	4.2±0.4	0.22±0.01	4.7±0.2
18:0	0.40±0.03	8.5±0.6	0.40±0.01	8.5±0.2	0.51±0.01	10.8±0.2
18:1	0.98±0.04	20.8±0.9	0.98±0.01	20.8±0.2	1.24±0.01	26.3±0.2
18:2	1.10±0.04	23.3±0.9	1.10±0.01	23.3±0.2	1.32±0.02	28.0±0.4
20:4	0.29±0.06	6.2±1.3	0.28±0.04	5.9±0.9	0.41±0.02	8.7±0.4

[a]All data are obtained with Method D, including re-extraction to clean up the samples.
[b]Times shown above are the durations of the methylation. The data of samples methylated for 30 min in the absence and presence of 2 mM DTPA were obtained on one day; samples methylated for 10 min with DTPA present were prepared on a different day.
[c]The number represents peak height ratios of fatty acid versus internal standard each.
[d]Calculated concentrations of each fatty acid based on the peak height ratio.

Table 2
Recoveries of fatty acids 16:0 and 22:6 in plasma

Fatty acid	PC[a]		PL[a]		PC + PL		Recovery (%)
	Peak ratio[b]	μg/100 μl[c]	Peak ratio[b]	μg/100 μl[c]	Peak ratio[b]	μg/100 μl[c]	
16:0	1.19±0.02	25.2±0.4	1.96±0.03	41.6±0.6	3.11±0.03	65.9±0.6	101
22:6	1.12±0.04	23.7±0.9	0.10±0.02	2.12±0.2	1.23±0.03	26.1±0.6	99

[a]PC is the standard (16:0)(22:6) PC and PL is plasma.
[b]The numbers shown are the peak height rations of fatty acids versus the internal standard; each date pair represent four analyses. Method D was used with methylation time of 10 min at room temperature.
[c]Calculated concentration of each fatty acid based on the peak height ratio.

assay system and use of the chelator gives more accurate results. In addition, the recovery of 18:0, 18:1. 18:2, and especially of 20:4 obtained by 10 min methylation time is greater than with 30 min reaction time, causing some scatter in the data as shown in Table 1.

To examine the reliability of our modified method for highly polyunsaturated fatty acids present in the retina, we performed an experiment using the internal addition method [16] and calculated the recoveries of 16:0 and 22:6 in plasma. Three separate samples of (16:0)(22:6) PC (50 μl of 1 μg/μl), 50 μl plasma and 50 μl plasma spiked with 50 μl PC were extracted and derivatized using Method D and analyzed in quadruplicate. The standard deviations from each set of samples are small (Table 2). The recoveries for 16:0 and 22:6 were calculated to be 101% and 99%, respectively. It is noteworthy that the 22:6 did not decrease significantly relative to 16:0 in the (16:0)(22:6) PC sample, which had been stored for 6 years in methylene chloride at −120°C.

4. Discussion

Fatty acids are easily converted to their methyl esters using a variety of acidic or basic reagents [3]. Methyl-Prep II was first introduced by McCreary et al. [1] as a reagent that would yield quantitative methanolysis at room temperature, while requiring a minimum of time-consuming manipulations. Another reason that we choose Methyl-Prep II as a transesterification reagent is that because of its mild nature, it allows detection of hydroxy fatty acids derived from phospholipid peroxides [17] and it also quantitatively releases PUFA from phospholipids [2,6]. However, we found that the FAME composition of plasma using the unmodified method (Method A) results in large standard deviation values. What are the major factors causing such poor reproducibility? It has been reported previously [3,18] that there are four factors which may cause analytical errors in fatty acid analyses, including methylation, extraction of FAMEs into organic solvent, evaporative losses and saponification of FAMEs when an alkaline reagent is used. In Method B, although Methyl-Prep II reportedly worked at room temperature [1], we initially heated the reaction mixture for 3 min at 50°C, based on the consideration that a high temperature was needed for complete methylation reaction. However, this did not improve our reproducibility. Furthermore, the data from methylation analyses (Table 1) show that completeness of fatty acid methylation is not a major factor because it is a fast reaction and the measurements are reproducible with methanolysis for 10 min at room temperature. FAMEs extract quantitatively into hexane and are stable in iso-octane [11]. Thus, the major causes of assay error could be oxidation of fatty acids, BHT methylation, and evaporative losses of FAMEs.

Since BHT is the principal antioxidant during the determination of fatty acid composition and polyunsaturated fatty acids are particularly susceptible to peroxidation [19], it is impossible to obtain an accurate and reproducible result when BHT looses its antioxidant property. Both Methods B and C yielded methylated BHT (Fig. 1). However, in Method B the recovery of BHT and methylated BHT was very low probably because it formed a complex with Methyl-Prep II, which is insoluble in iso-octane. Once the Methyl-Prep II was removed (Method C), the re-

covery of BHT was improved, and the GC signal of methylated BHT is large (Fig. 1b). Heckers et al. [9] reported an artifact of BF_3-methanol methylation in samples containing BHT. The peak was not identified, but was reported by Bondia et al. to be methylated BHT [12] and its relationship relative to BHT is similar, as what we found for methylated BHT. Using transesterification Method D, methylated BHT could not be detected by GC–MS and the standard deviations were small. In addition, the ratio of 20:4 to the 17:0 internal standard is larger (Fig. 2). It is evident that the modified method protects BHT against methylation and thus, fatty acids from oxidation, supporting our conclusion that BHT methylation is one of the factors affecting reproducibility of the assay.

The loss of FAMEs was also found to significantly depend on the evaporation step; in particular, the GC signal of FAMEs is very weak when a sample is evaporated to complete dryness after transesterification. It is known that lipids are not easily evaporated, but their methyl esters possess higher volatility, which has been applied to GC analysis. Since the volatility of individual FAMEs is different, it is difficult to control FAMEs level at a fixed ratio when a sample is completely evaporated by argon. This may be the reason why standard deviation values from the ratios of FAMEs and internal standard are large with Methods B and C. Method D proved to protect FAMEs from losses because the sample was no longer completely evaporated after methylation of fatty acids. As shown in Fig. 2, the GC signal intensity of FAMEs prepared by modified method is the same as that from Methods B and C with only one-third of their injection volume. The re-extraction purifies FAMEs from the mixture of base methylation reagent products, because hexane can extract FAMEs efficiently [4,12,20] and water can wash the side products from methylated extracts [3] and reduce the noise level of the GC signal (Fig. 2b).

Another modification in Method D is the use of the chelator, DTPA. In our previous work, we found that the use of EDTA, in the presence of a transition metal such as iron, increases the EPR signal intensity of DMPO-OH (unpublished data by L.-Y. Zang and H.P. Misra), indicating that EDTA–metal is capable of producing the hydroxyl radical (·OH). This effect could be eliminated by using DTPA instead of EDTA. Polyunsaturated fatty acids are particularly susceptible to peroxidation [19]; once initiated, this process proceeds as a free radical chain reaction. The ·OH is one of the species that is able to initiate peroxidation, and it can be generated in transition metal ion catalyzed reactions such as the Fenton reaction. Isolated plasma and membrane fractions often reduce oxygen to form superoxide radical ($O_2^{\cdot-}$), which dismutates to hydrogen peroxide (H_2O_2), that can then react with Fe^{2+} or Cu^+ to form ·OH. It is difficult to obtain accurate and reproducible data for the determinations of fatty acids without precautions that eliminate these reactive species. In the present study, DTPA was used in fatty acids assay and gave accurate and reproducible results (see Table 1). The amounts of fatty acids (μg/100 μl) are in agreement with previous reports [11,12].

The reliability of Method D for quantitative recovery of highly polyunsaturated fatty acids like 22:6 that are present in high quantities in the retina [5,6] has been examined using the internal addition method. As shown in Table 2, the standard deviation values are low (less than 5%), and the recoveries for 16:0 and 22:6 are high (101% and 99%, respectively), which indicates that under the conditions used, no losses of 22:6 occur.

Our data show that use of Method D transesterification and re-extraction eliminates most by-products of Methyl-Prep II reaction. Method D also gives reproducible results, low background noise and preserves BHT in its active antioxidant form, which provides a safeguard against inaccuracy due to lipid peroxidation. All above data support that our modified method is very applicable to fatty acids analysis in biological systems. It is recommended that Method D be used with 2 m*M* DTPA, in aqueous buffer, methylation with Methyl-Prep II at room temperature for 10 min, re-extraction into hexane and dissolving of the residue in iso-octane, without ever taking the sample to complete dryness.

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