Measurement of Malondialdehyde Levels in Food by High-Performance Liquid Chromatography with Fluorometric Detection

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A sensitive and reproducible HPLC assay with fluorometric detection was used to measure the malondialdehyde (MDA) concentration in food (butter, margarine, oil, fish, and meat tissue). Samples were homogenized in water supplemented with butylated hydroxytoluene. Proteins were precipitated with ice-cold 5% trichloroacetic acid and removed by centrifugation. The supernatant was incubated in a 0.28% thiobarbituric acid (TBA) mixture from which the oxygen was depleted. Optimal incubation time and temperature, for the TBA treatment, were found to be 30 min and 90 °C, respectively. The MDA−TBA adduct was fractionated by reverse phase HPLC and detected by fluorescence (λ_{EX} = 515 nm; λ_{EM} = 543 nm). Elution was performed at 1 mL/min flow rate with a mixture of acetonitrile and sodium phosphate at pH 7 (15:85 v/v). The described sample preparation procedure minimizes the lipid oxidation and provides high sensitivity (0.01 pmol of MDA), reproducibility, and specificity.

Keywords: *Malondialdehyde; food; lipid peroxidation; HPLC*

INTRODUCTION

Lipid oxidation is a complex process whereby unsaturated lipid material undergoes reaction with molecular oxygen to yield lipid hydroperoxides (Halliwell et al., 1995). Furthermore, lipid peroxidation is a ubiquitous phenomenon first associated with the development of rancidity in food and implicated in the aging process as well as in several degenerative diseases (Rice-Evans and Burdon, 1993; Yu, 1994).

In this context, many analytical methods have been developed to measure lipid oxidation products (Gutteridge and Halliwell, 1990; Kinter, 1995), and procedures of quantitative analysis are being constantly improved.

Lipid oxidation in food is associated with the development of rancidity and oxidative deterioration. These phenomena represent a major cause of loss of nutritional quality as well as a cause of concern for food safety (St. Angelo, 1996). Polyunsaturated fatty acids (PUFA) are susceptible to oxidation during food manipulation, processing, and cooking (Finlay, 1993) and, as a consequence of oxidative damage, aldehydes, ketones, alcohols, acids, or hydrocarbons are produced (St. Angelo, 1996). Among aldehydes, malondialdehyde (MDA), has often been used as marker of oxidative damage in biological samples (Kinter, 1995) and foods (St. Angelo, 1996).

The most widely used assay for the titration of MDA is a test based on the measurement of the pink fluorescent complex (1:2 MDA−TBA) produced upon incubation with 2-thiobarbituric acid (TBA) at low pH and high temperature. The TBA test has been criticized for its low specificity, when performed by spectrophotometric detection, because of the presence of interfering substances that generally lead to an overestimate of the complex (Janero, 1990).

The TBA test in food is generally performed by using various procedures such as direct heating of the sample with TBA (Turner et al., 1954; Yu and Sinnhuber, 1957; Sinnhuber and Yu, 1958) or distillation of the sample (Tarlagdis et al., 1960; Rhee, 1978; Yamauchi et al., 1982; Ke et al., 1984), lipid extraction with organic solvents (Pikul et al., 1983, 1989), or acid extraction of MDA (Witte et al., 1970; Sinnhuber and Yu, 1977; Siu and Draper, 1978; Ohkawa et al., 1979; Newburg and Concon, 1980; Salih et al., 1987; Squires, 1990) followed by acid reaction with TBA.

Recently, spectrophotometric methods to titrate the MDA−TBA complexes have been proposed (Raharjo et al., 1992; Botsoglou et al., 1994). However, low concentrations of MDA cannot routinely be measured unless expensive (gas chromatography/mass spectrometry) and time-consuming methods are used (Yeo et al., 1994).

Despite the improvement in materials and equipments used, HPLC is not a widely used technique in the analysis of MDA in food. In contrast, chromatographic fractionation and fluorometric detection of the complex represent a currently used procedure to measure the MDA concentration in human blood plasma (Tatum et al., 1990; Fukunaga et al., 1993, 1995; Li and Chow, 1994; Suttnar et al., 1997) because of its reproducibility and sensitivity (at the micromolar level).

In this study, a reproducible, sensitive, and rapid HPLC assay, by modification of previously published procedures, was set up. Some experimental conditions for sample processing such as the temperature of incubation with trichloroacetic acid (TCA), supplemen-

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tation with butylated hydroxytoluene (BHT) or metal chelators, and oxygen depletion were preliminarily tested to prevent or suppress sample oxidation. Finally, this method was used to measure the MDA concentration in food.

MATERIALS AND METHODS

Materials. HPLC grade organic solvents were used. Acetonitrile was purchased from Romil (Cambridge, U.K.) and ethanol from Sigma (St. Louis, MO). Aqueous solutions were prepared with Milli-Q purified water. All chemicals were of analytical grade.

Margarine, butter, oil (olive, peanut, and corn), frozen fish (sole, salmon, and cod), and meat (beef, chicken, and pork) were purchased from a local supermarket. In particular, olive oil marked with the label "extra virgin" (corresponding to composition according to the Italian law) was used.

Preparation of Standard MDA. The MDA standard solution was prepared essentially according to a published procedure (Csallany et al., 1984). In detail, 5 mM 1,1,3,3-tetraethoxypropane (TEP) was hydrolyzed by 10 mM HCl at room temperature (rt) for 6 h (Yeo et al., 1994). The concentration of MDA in the standard solution was calculated by measuring the absorbance at 245 nm using $\epsilon = 13\,700$.

HPLC Analysis. The HPLC analysis was performed using a Gilson pumping system (Middleton, WI) equipped with the spectrofluorometric detector RF-551 and the chromatopac integrator C-R5A (Shimadzu, Kyoto, Japan). The MDA−TBA complex was fractionated on a Nova-Pak C_{18} steel column (3.9 × 150 mm) protected by a Sentry guard column Nova-Pak C_{18} (3.9 × 20 mm) (Waters, Milford, MA). The mobile phase was daily prepared and consisted of 5 mM sodium phosphate buffer (pH 7.0) and acetonitrile (85:15 v/v). Sample aliquots (20 μL) were injected, and the chromatographic fractionation was performed at 1 mL/min flow rate (rt). The fluorescence detector was set at $\lambda_{EX} = 515$ nm and $\lambda_{EM} = 543$ nm.

Temperature Effect on the Yield of the MDA−TBA Complex Formation. Aliquots of 180 μL from 0.6 μM MDA or 10 mM HCl (blank) were mixed with 20 μL of ethanolic BHT to give a final concentration of 1000 ppm. After addition of 1 volume of 10% TCA, these solutions were incubated for 10 min at 30, 60, and 90 °C. At the end of incubation, the samples were quickly cooled and centrifuged (5 min, 10000*g*, rt) to recover water condensed on the tube walls. A solution of 0.4% TBA in 2 M acetate buffer at pH 3 was degassed by a vacuum pump (5 min) and flushed with nitrogen for 10 min (TBA mix). The TCA-treated samples were added to 4 volumes of TBA mix, and the resulting solutions were degassed (flushed with nitrogen for 1 min) and then incubated for 30 min at 30, 60, and 90 °C. Finally, the samples were cooled and centrifuged and 20 μL aliquots were fractionated by HPLC.

Effect of Temperature on PUFA Oxidation. Oil aliquots (100 mg) were pipetted into microcentrifuge tubes containing 0.4 mL of Milli-Q water supplemented with different amounts of ethanolic BHT corresponding to final concentrations ranging from 0 to 5000 ppm. One volume of ice-cold 10% TCA was added to the mixture, and then the samples were sonicated (6 min in an ice bath) and incubated at 30 and 60 °C for 10 min. At the end of incubation the samples were cooled, vigorously mixed, and then centrifuged (5 min at 10000g, rt). Aliquots of 300 μL were taken from the aqueous phase and added to 700 μL of TBA mix. The solutions were degassed and then incubated for 30 min at 90 °C. After the incubation, the samples were cooled and particulate material was removed by centrifugation (5 min, 10000g, rt). Finally, 20 μL aliquots of supernatant were analyzed by HPLC.

Sample Preparation. Aliquots (2 g) of fish or meat were transferred into centrifuge tubes and readily homogenized in a solution consisting of 4.75 mL of Milli-Q water and 0.25 mL of ethanolic BHT (1000 ppm final concentration). Aliquots (500 μL) of the homogenate were added to 500 μL of ice-cold 10% TCA. The samples were vigorously mixed (3 min) and proteins removed by centrifugation (5 min, 10000*g*, rt). Butter or margarine aliquots (2.25 g) were weighed into centrifuge tubes and melted at 40 °C in the presence of ethanolic BHT (1000 ppm final concentration). After addition of 2.5 mL of ice-cold 10% TCA, the samples were mixed and then centrifuged, as above. Oil aliquots (100 mg) were pipetted into microcentrifuge tubes containing 0.4 mL of Milli-Q water supplemented with ethanolic BHT (1000 ppm final concentration). One volume of ice-cold 10% TCA was added to the oil samples. The samples were then sonicated and centrifuged as above described.

Aliquots of 300 μL were taken from the sample supernatant, and 700 μL of TBA mix was added. The mixtures were degassed and then incubated for 30 min at 90 °C. At the end of incubation, the samples were cooled, centrifuged (5 min, 10000g, rt) to remove particulate material and, finally, sample aliquots (20 μL) were injected on the HPLC system.

During each experiment, BHT−TCA solutions without the sample or with MDA standards were processed and used as blank or as control, respectively. The blank value was subtracted from the MDA value measured for each sample. The MDA concentration was calculated on the basis of the calibration curve and the values obtained were expressed as nanomoles per gram.

Finally, excitation ($\lambda_{EX} = 515$ nm) and emission ($\lambda_{EM} = 543$ nm) spectra of the fluorescent adduct, formed by the reaction of both standard MDA and food sample with TBA, were recorded.

Analytical Performance. The linear relationship between MDA incubated with TBA and the MDA−TBA complex produced was assessed as follows. Aliquots (180 μL) containing increasing amounts of MDA were mixed with 20 μL of ethanolic BHT (1000 ppm final concentration). Two hundred microliters of TCA was then added to the sample, and the mixture was mixed and centrifuged (5 min, 10000g, rt). Supernatant aliquots (100 μL) were added to 4 volumes of TBA mix and incubated for 30 min at 90 °C. After incubation, aliquots of 20 μL (containing 0.01, 0.05, 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 pmol of reaction products) were injected on the HPLC system. The coefficient of variation (CV%) and linear regression (r^2) were calculated.

Reproducibility was also tested. Each food sample was analyzed in triplicate in the same experiment, and three different experiments were carried out. Both within- and between-assay precision, expressed as CV%, were calculated.

Accuracy was determined by assessing the recovery in spiked samples. A known amount of MDA (160 pmol) was added to food samples (beef, sole, and corn oil) and then processed as described above.

Effect of Supplementation with Metal Chelators. To determine the effect of inorganic ions on the MDA concentration, the effect of metal chelators on the inhibition of artifactual PUFA oxidation was tested. Beef and codfish samples were homogenized in water supplemented with ethanolic BHT (1000 ppm final concentration), in the presence or absence of deferoxamine (50 and 100 μM) or EDTA (50 and 500 μM). The samples were processed as described above, and the MDA concentration was measured.

RESULTS

Determination of the Optimum Temperature Conditions for the Yield of the MDA−TBA Complex. The effect of temperature on the formation of the MDA−TBA complex was determined by using a known amount of MDA standard. Aliquots of the standard, containing 0.6 μM MDA, were incubated (10 min at 30, 60, and 90 °C) with TCA and then treated (30 min at 30, 60, and 90 °C) with TBA as described under Materials and Methods.

Either amount of the complex, formed in the different conditions used, was analyzed by HPLC: 1 pmol of the reaction products was injected.

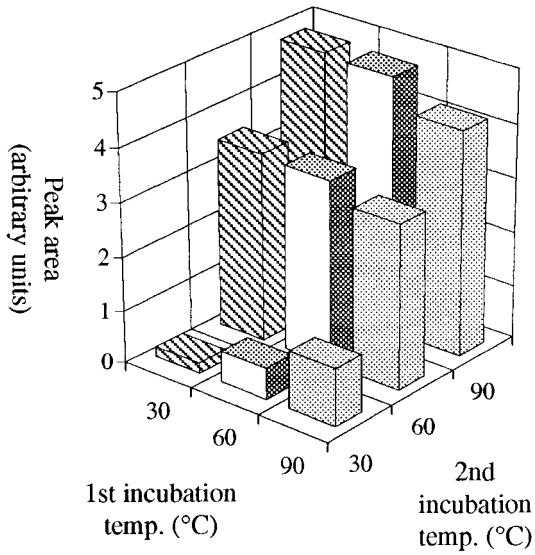

Figure 1. Temperature-dependent formation of the MDA−TBA complex. Aliquots of a standard MDA (0.6 μM) were incubated with TCA (10 min) at 30, 60, or 90 °C (first incubation, *x*-axis). After the TCA treatment, the samples were incubated with TBA for 30 min at 30, 60, or 90 °C (second incubation, *z*-axis). Samples incubated with 10 mM HCl were used as blank for each combination of two temperatures. The amount of MDA analyzed by HPLC corresponds to 1 pmol. The means ± *SD* ($n = 4$) of the peaks are reported and the CV% ranged between 0.1 and 3.9.

Comparable yields of the MDA−TBA complex were found when the sample was treated, at 30 or 60 °C, with TCA and then incubated with TBA at 90 °C (Figure 1). The yield values did not increase when a longer incubation time (up to 60 min) in TBA was used (data not shown).

Analysis of the Temperature-Dependent PUFA Oxidation. The temperature effect on PUFA oxidation was studied. Olive oil samples (100 mg), supplemented with increasing amounts of BHT (0−5000 ppm), were sonicated and incubated (10 min, 30−60 °C) with TCA. After separation of the two phases, aqueous aliquots were incubated (30 min, 90 °C) with TBA to form the MDA adduct. We cannot exclude that other reaction products, possibly formed during the heat treatment, might have interfered with MDA−TBA chromatography and detection. However, the concentration of the molecules that reacted with TBA was higher in the samples treated at 60 °C (5-fold) than that found after 30 °C treatment (Figure 2). A poor antioxidant effect of BHT might be due to a nonhomogeneous dispersion in the samples, possibly because solubility is lower at high temperatures.

Assessment of Linearity, Analytical Range. MDA standard amounts from 0.01 to 4 pmol were injected to test the linear relationship between the amount of the standard and the complex formed with TBA. The variation between assays was always <6% (CV) as measured in duplicate samples from three different experiments. The MDA−TBA peak areas varied linearly over the analytical range employed. The regression equation was $y = 1204.4 + 5614x$ ($r^2 = 0.999$), and the lower limit of detection was set at the lowest standard concentration on the calibration curve.

MDA Titration in Food. In preliminary experiments (data not shown) food samples were incubated in TBA mix, with or without oxygen in the reaction mixture. The concentration of the reaction product in untreated samples was up to 15% higher than those measured in degassed ones.

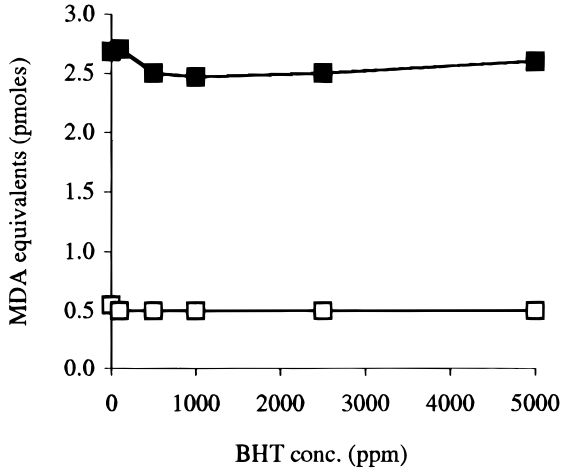

Figure 2. Temperature effect on PUFA oxidation in olive oil. Olive oil samples (100 mg) were supplemented with increasing amounts of ethanolic BHT (0−5000 ppm) and then incubated with TCA for 10 min at 30 (□) and 60 °C (■). After this incubation, 100 μL aliquots from the aqueous phase were mixed with TBA and incubated again (30 min at 90 °C). Average values (±SD) of triplicate samples from three different experiments are reported. Data reported are consistent with the least accurate measurement. The size of the square includes mean and deviations. The CV% was always <10%.

Table 1. MDA Level in Food[a]

sample	MDA level (nmol/g)	within-assay CV%	between-assay CV%
butter	0.16 ± 0.011	1.66	1.44
margarine	1.05 ± 0.063	6.01	5.92
meat			
beef	2.29 ± 0.060	3.99	4.66
chicken	0.44 ± 0.015	3.37	4.23
pork	0.55 ± 0.028	5.07	5.65
fish			
cod	0.74 ± 0.030	3.13	5.63
sole	0.49 ± 0.057	1.16	2.12
salmon	0.23 ± 0.004	1.73	2.23
oil			
olive	2.04 ± 0.017	0.83	3.25
corn	0.33 ± 0.003	2.23	5.21
peanut	0.07 ± 0.002	3.40	7.52

[a] The concentration of MDA was measured in different food samples. MDA was reacted with TBA and the produced MDA−TBA complex was fractionated by HPLC and titrated spectrofluorometrically. The MDA concentration is expressed as nmol/g. Triplicate analyses from three different preparations were reported as mean ± SD. Within- and between-assay precision was calculated and expressed as coefficient of variation (CV%).

MDA was measured by employing the sample preparation and HPLC as detailed under Materials and Methods. The results are reported in Table 1. Good repeatability was achieved: in particular, both within-assay and between-assay precision, expressed as CV%, were <8%.

To evaluate the accuracy of the method, corn oil, beef, and sole tissue sample ($n = 4$) were spiked with a known amount of MDA and then processed according to the procedure described above. The amount of MDA was determined by using the standard curve. Recovery, calculated by using the average concentration in samples, ranged from 90 to 100% (Table 2).

Identification of MDA−TBA in food samples originating from the mixture of the food sample with TBA was confirmed by the retention time and the fluorescence spectra of authentic standard.

Table 2. Recovery of MDA[a]

sample	MDA added (pmol)	MDA detected (pmol)	recovery (%)
beef		3.06	
	0.7	3.78	100.2
sole		0.56	
	0.7	1.26	92
corn oil		0.38	
	0.7	1.08	100

[a] The accuracy of MDA titration in food samples was assayed. Beef, sole, and corn oil ($n = 4$) were spiked, or otherwise, with a known amount of MDA (0.7 pmol). Recovery was calculated by using the average concentration measured in unspiked samples.

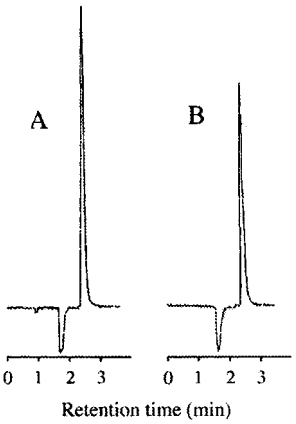

Figure 3. HPLC elution profile of MDA−TBA complex. A known amount (0.8 pmol) of MDA (A) and codfish sample (B) was processed as described under Materials and Methods. The TBA reaction product, fractionated by the HPLC system, was monitored by fluorescence detection at $\lambda_{EX} = 515$ nm and $\lambda_{EM} = 543$ nm. The MDA−TBA complex was eluted in a short time as a sharp single peak.

Both the standard MDA and food sample complex were eluted as a sharp single peak (Figure 3) with the same retention time (~2.3 min), and identical spectra were recorded showing the same excitation maximum at 515 nm and an emission maximum at 543 nm.

Over 2000 analyses were carried out on the same column with no change in the retention time or peak shape.

Effect of Metal Chelators. Metal chelators were added to beef and codfish samples to ascertain whether they could prevent lipid oxidation. In particular, beef or fish muscle tissue was homogenized in a solution containing BHT (1000 ppm) with or without EDTA (50 or 500 μM) or deferoxamine (50 or 100 μM). The samples were processed for MDA titration as described under Materials and Methods. The result obtained (Table 3) indicates that the MDA concentrations did not differ from those measured in untreated samples.

DISCUSSION

Lipids greatly influence food quality: they are of high nutritional value as a source of energy and fat-soluble vitamins and provide organoleptic characters such as color, flavor, aroma, texture, and taste (St. Angelo, 1996). Lipid peroxidation may occur during food manufacturing (Finlay, 1993) and produces rancid off-flavors (Finlay, 1993; Kubow, 1993). Lipid oxidative degradation results in the formation of byproducts that are potentially toxic and adversely affect food quality. Several molecules originate from lipid degradation. Among these products, conjugated dienes, lipoperoxides, aldehydes, or alkanes can be detected and used to evaluate lipid degradation. In particular, the concentration of MDA is the most widely used index of lipid oxidation (Janero, 1990). The titration of MDA is generally based on the sample boiling in the presence of TBA to form the MDA−TBA complex, the amount of which can be determined by UV spectrophotometry.

Yagi proposed to remove proteins and associated lipids before incubation of the sample with TBA at high temperature to prevent PUFA autoxidation during the treatment and, therefore, overestimation of the original MDA levels (Yagi, 1976). Thus, it seems conceivable to prepare the sample in two separate steps, by first removing proteins by precipitation with TCA and then incubating the MDA containing soluble phase with TBA.

Acid and high temperature treatment of the sample was reported to result in a release of the "protein-bound" MDA (Kwon et al., 1965). However, such conditions may cause the oxidation of sample PUFA, leading to the overestimation of the original MDA concentration. Furthermore, our data clearly indicate that BHT, at high temperature, did non prevent lipid degradation during acid treatment.

This method allowed us to titrate MDA in food with good accuracy and reproducibility. The MDA concentration measured in this study was much lower than those obtained by spectrophotometry (Botsoglou et al., 1994; Nourooz-Zadeh et al., 1995; Rhee et al., 1996).

The MDA concentration we found in beef tissue was 2-fold higher when compared with that obtained without acid treatment and derivatization with TBA (Csallany et al., 1984). Presumably, acid extraction results in the release of protein-bound MDA (Bird et al., 1993).

Identification of the MDA−TBA complex from food samples was assessed by comparing the retention time and fluorescence spectrum with those of a reference standard. These MDA−TBA physical−chemical properties are shared by alkanals, alkenals, and alkadienals, which react with TBA to produce red pigments (Kosugi et al., 1987, 1989). However, it was previously reported that these aldehydes interfere little with MDA−TBA detection because the yield of complex formation is lower than that from MDA (Kosugi et al., 1987), particularly when molecular oxygen is absent (Kosugi and Kikugawa, 1985; Kosugi et al., 1988). In agreement with these data, we found lower amounts of complex in samples deprived of oxygen.

The step of sample cleanup before HPLC analysis is generally carried out by means of organic solvents

Table 3. Influence of EDTA and Deferoxamine Addition on Meat MDA Concentrations[a]

		deferoxamine		EDTA	
sample	BHT 1000 ppm	50 μM	100 μM	50 μM	500 μM
beef	2.29 ± 0.06	2.29 ± 0.02	2.28 ± 0.02	2.17 ± 0.03	2.30 ± 0.02
cod	0.74 ± 0.03	0.74 ± 0.08	0.67 ± 0.02	0.735 ± 0.02	0.65 ± 0.07

[a] The effect of metal chelator addition on the MDA concentration was tested. Codfish or beef was homogenized in the presence, or not, of deferoxamine (50 and 100 μM) or EDTA (50 and 500 μM) and then processed as described under Materials and Methods. Data represent the mean of triplicate analyses ± SD. The MDA concentrations are expressed as nmol/g ± SD.

(Lepage et al., 1991; Li and Chow, 1994; Fukunaga et al., 1993; Tatum et al., 1990) or solid phase extraction (Draper et al., 1993). Furthermore, this procedure was suggested as necessary to eliminate the contribution of interfering substances (Draper et al., 1993) or to extend the HPLC column life (Lepage et al., 1991). The cleanup does not improve the MDA−TBA measurement (Bird et al., 1983; Fukunaga et al., 1995) and, furthermore, is organic solvent- and time-consuming (Chirico, 1994). Moreover, we found that omission of the cleanup did not compromise the performance of the HPLC column or the reproducibility of the assay.

During the sample preparation procedure, PUFA oxidation may occur, and thus the MDA concentration is overestimated. The present study was mostly aimed to optimize sample preparation. Our results suggest that the omission of heating during TCA treatment, the addition of antioxidant, and the oxygen depletion prevent the artifactual formation of products interfering with MDA−TBA titration.

Finally, the herein described method offers several advantages over previously published techniques in terms of faster chromatographic fractionation, higher sensitivity, and reproducibility (Hirayama et al., 1983; Csallany et al., 1984; Madére and Behrens, 1992).

ABBREVIATIONS USED

BHT, butylated hydroxytoluene; EDTA, ethylendiaminetetraacetic acid disodium; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; PUFA, polyunsaturated fatty acids; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TEP, 1,1,3,3-tetraethoxypropane.

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