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Determination of Lipid Hydroperoxides in Serum Iodometry and High Performance Liquid Chromatography Compared

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Summary: It is postulated that lipid peroxidation plays a role in the pathogenesis of a variety of diseases. Efforts have therefore been made to develop reliable and practicable procedures for quantifying lipid peroxidation products such as lipid hydroperoxides in biological specimens. An iodometric cholesterol colour reagent (Merck, Darmstadt, Germany) can be used to measure lipid hydroperoxides in isolated low density lipoproteins without lipid extraction. This method has been validated with respect to its analytical performance and suitability for serum samples by comparing it with a high performance liquid chromatography technique. The method was found to have acceptable performance characteristics with aqueous fatty acid hydroperoxide solutions (linoleic acid) and isolated low density lipoproteins, but it cannot be applied to native serum samples without extraction of lipids.

Introduction

Lipid peroxidation has attracted considerable attention during recent years (1–3). It has been linked to carcinogenesis (4), aging (5) and to a variety of other diseases including atherosclerosis (6). The oxidative modification of low density lipoproteins in particular is considered an important factor in the development of atherosclerotic vascular lesions (7, 8).

The peroxidation of unsaturated fatty acids is a radical chain reaction which is initiated by the abstraction of a hydrogen atom from a methylene group of the fatty acid chain. The carbon radical formed by this reaction tends to stabilize by molecular rearrangement leading to conjugated double bonds. By the reaction with oxygen a reactive peroxy radical is generated which can abstract a hydrogen atom from another lipid molecule. This propagation stage of lipid peroxidation leads to the formation of cytotoxic hydroperoxides (9). On heating or with metal catalysts these lipid hydroperoxides are broken down to lipid alkoxy and peroxy radicals, initiating further radical chain

reactions. Lipid alkoxy radicals are particularly prone to the so-called beta cleavage reaction, leading to the formation of cytotoxic and genotoxic aldehydes (10).

The commonly used thiobarbituric acid method measures these aldehydic products of lipid peroxidation. It is relatively non-specific and is also unreliable in biological samples such as serum (11). More specific assays have recently been developed to quantify lipid peroxidation products, among which luminometric techniques coupled to high performance liquid chromatography have proved to be reliable for the measurement of lipid hydroperoxides (12–14). However, these techniques are time consuming and require sample preparation and special equipment, thereby hampering their application to routine measurements.

A simple and specific spectrophotometric method for following the formation of lipid hydroperoxides during the in vitro oxidation of low density lipoproteins by copper ions was recently reported by *El Saadani* et al. (15). The method is based on the oxidation of iodide to iodine by lipid hydroperoxides, uses commercially available reagents (cholesterol colour reagent, Merck, Darmstadt, Germany), and does not

* This work is part of the doctoral thesis of V. Schettler.

require extraction of lipoprotein lipids. However, the authors neither validated their method with respect to standard lipid solutions nor to serum samples. The objective of the present study was to investigate whether the method is reliable and can be applied to biological samples such as human serum without extraction. For this purpose we compared it with a high performance liquid chromatography technique using chemiluminescence detection for the quantification of lipid hydroperoxides (14).

Materials and Methods

Reagents

Ascorbic acid, linoleic acid, glycine, phenylalanine, tyrosine, methionine, lysine, arginine, cysteine, histidine, tryptophan, asparagine, glutamine, aspartic acid, glutamic acid, proline, poly-proline, poly-cysteine, poly-histidine, poly-methionine, poly-tryptophan, poly-phenylalanine, apolipoprotein B100, transferrin, butylated hydroxy toluene, and lipoxidase (linoleate: oxygen oxidoreductase from soyabean, EC 1.13.11.12)¹⁾ were purchased from Sigma Chemical Co. St. Louis Mo. USA. H_2O_2 (Perhydrol®), cholesterol colour reagent (CHOD-iodide method), chloroform, triton-X-100, sodium azide, alkylbenzyltrimethylammonium chloride, iron(II) sulphate, copper(II) sulphate 5-hydrate, ammonium heptamolybdate, potassium hydrogenphosphate, potassium dihydrogenphosphate, butanol, methanol, hydrochloric acid (5 mol/l), sodium hydroxide (5 mol/l), and boric acid were from Merck, Darmstadt, Germany. Ethanol was from J. T. Baker B.V., Deventer, The Netherlands. Human albumin was from Behringwerke AG, Marburg, Germany. All reagents were analytical grade.

Blood samples

Serum samples were obtained from freshly drawn venous blood specimens. Collection tubes did not contain any anticoagulant but were prepared with the antioxidant, butylated hydroxy toluene, in order to avoid non-specific peroxidation. For this purpose a 10 mmol/l stock solution of butylated hydroxy toluene was prepared in ethanol and 10 µl of this solution were added to the collection tubes prior to blood sampling. Tubes were filled with 10 ml blood to yield a final butylated hydroxy toluene concentration of 100 µmol/l. For recovery experiments multiple samples were combined to obtain a serum pool.

Isolation of low density lipoproteins

Low density lipoproteins were isolated by ultracentrifugation as described by Havel (15). Low density lipoproteins were kept under nitrogen during the whole isolation procedure in order to protect the lipoprotein lipids against non-specific peroxidation. For this purpose all solutions were saturated with nitrogen before and after ultracentrifugation and the isolated low density lipoprotein preparations were dialysed against a nitrogen saturated TRIS-HCl buffer (5 mmol/l, pH 7.4) containing 1 mmol/l EDTA.

Oxidation of lipids

Linoleic acid

Linoleic acid was oxidized using lipoxidase as previously described (14). Two hundred microliters of stock solution corresponding to 20 mg linoleic acid (stock solution: 100 g linoleic acid per litre $CHCl_3$) were evaporated under N_2 and resuspended in 2 ml borate buffer (50 mmol/l, pH 9.4). Lipoxidase (815 U) was added in 6 aliquots (10 µl) every 10 min during 1 h of incubation at 37 °C in a shaking water bath. At the end of incubation the oxidized fatty acid was extracted twice with 2 ml diethyl ether at acidic pH (20 µl 5 mol/l HCl). The ether phases were combined and evaporated under N_2 and the oxidized fatty acid was redissolved in ethanol at the desired concentrations and stored at -20 °C.

Serum samples

Serum samples (2 ml) were incubated in a shaking water bath at 37 °C for 120 min. Lipoxidase (746.9 kU) dissolved in 5.5 ml borate buffer (50 mmol/l, pH 9.3) was added in 7 aliquots (50 µl) every 5 min during the first 30 min of incubation and then in 6 additional aliquots (50 µl) every 10 min over the next 60 min. Incubations were stopped by cooling (0 °C). Control incubations with borate buffer instead of lipoxidase were run in parallel.

Low density lipoproteins

Low density lipoproteins (1 g protein per litre) were oxidized by $CuSO_4$. For this purpose low density lipoproteins were dialysed (5 h, 20 °C) against EDTA-free TRIS-HCl buffer (5 mmol/l, pH 7.4) containing 20 µmol/l $CuSO_4$ as described by Jürgens et al. (17).

Extraction of lipids

Lipid extraction was performed by the Bligh & Dyer procedure (18). The chloroform layer was evaporated under N_2 and the dried lipid residue was redissolved in ethanol.

Iodometric assay

Lipid hydroperoxide determination with the iodometric assay (cholesterol colour reagent, cholesterol oxidase-iodide method, Merck, Darmstadt, Germany) was performed as described by El Saadani et al. (15). Lipids were added in ethanol to the reagent mixture. Blank readings were made after the addition of pure ethanol. H_2O_2 was diluted with H_2O and analysed at the indicated concentrations. Samples were incubated at room temperature for either 30 min or 60 min in the dark.

Amino acids (1 g/l), polypeptides (1 g/l), albumin (1 g/l), apolipoprotein B100 (1 g/l), Fe_2SO_4 (250 µmol/l), and $CuSO_4$ (250 µmol/l) were dissolved in water and 100 µl of these samples were also mixed with 1 ml of the reagent mixture. Ascorbic acid was dissolved in water and was added at the indicated concentrations together with 40 µmol/l H_2O_2 or oxidized low density lipoproteins.

A "blank" reagent mixture without iodine, prepared according to the manufacturer's instructions, consisted of 0.15 mmol/l sodium azide, 2 g/l triton-X-100, 0.1 g/l alkylbenzyltrimethylammonium chloride, and 0.01 mmol/l ammonium heptamolybdate in a potassium phosphate buffer (0.2 mol/l, pH 7.0).

Absorbances were determined at 365 nm with a Beckman DU-8 spectrophotometer (Beckman Instruments, Irvine, CA, USA). Wavelength scanning between 250 nm and 450 nm was performed with the same instrument. Lipid hydroperoxide and

¹⁾ Enzyme
Lipoxidase: Linoleate oxygen oxidoreductase (EC 1.13.11.12)

H₂O₂ concentrations were calculated using a molar extinction coefficient of $2.46 \cdot 10^3 \text{ m}^2/\text{mol}$ and $2.45 \cdot 10^3 \text{ m}^2/\text{mol}$ respectively as published by *El Saadani et al.* (15).

High performance liquid chromatography determination of lipid hydroperoxides

For measurement of lipid hydroperoxides by high performance liquid chromatography we modified a method originally described by *Frei et al.* (12). For the luminometric detection of hydroperoxides, a modification of an assay cocktail originally published by *Myiazawa et al.* (13) was used. The modified cocktail consisted of 6.4 $\mu\text{mol/l}$ cytochrome c and 0.1 $\mu\text{mol/l}$ luminol. Two columns (Si 60 and ODS RP) were combined in order to increase the separation performance (14). Reduced ubiquinone has been shown to cause hydroperoxide non-specific signals in the luminescence detection described by *Frei et al.* (12), but it does not interfere in our assay (14). For the quantification of the lipid hydroperoxides by high performance liquid chromatography, integrated peak areas were compared with peak areas obtained with an external linoleic acid hydroperoxide standard. The amount of hydroperoxide contained in this standard fatty acid was determined iodometrically (15).

Other assays

Protein was determined by the *Lowry* procedure (19). Cholesterol measurements in serum and low density lipoprotein samples were performed enzymatically using the peroxidase/4-aminophenazone technique (Boehringer Mannheim, Mannheim, Germany). Bilirubin was determined according to *Jendrassik & Grof* (20) using a commercially available reagent kit (Boehringer Mannheim, Mannheim, Germany) and an automatic analyser (Hitachi 717, Boehringer Mannheim, Mannheim, Germany).

Reducing substances contained in serum samples were determined with a commercially available test kit (Boehringer Mannheim, Mannheim, Germany) which is based on the reduction of the tetrazolium salt 3-(4,5 dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide to formazan.

Recovery experiments

Hydroperoxides were determined in serum samples which had been spiked with oxidized low density lipoproteins. They were also determined in serum samples without added oxidized low density lipoproteins, as well as in oxidized low density lipoproteins alone. For this purpose 300 μl of a serum pool were mixed with 200 μl of an oxidized low density lipoprotein preparation, resulting in a total sample volume of 500 μl . The volume of the serum samples which were not spiked with oxidized low density lipoproteins and the volume of the oxidized low density lipoprotein preparation itself was also corrected to 500 μl with sodium chloride (0.9 g/l). Samples were assayed by the iodometric method, either directly or after extraction. For the high performance liquid chromatography detection of hydroperoxides, samples (500 μl) were extracted with 500 μl butanol.

Results

Iodide oxidation as measured by the iodometric method is linearly related to the hydroperoxide concentration up to at least 40 $\mu\text{mol/l}$ of either H₂O₂ or linoleic acid hydroperoxides (fig. 1). Linoleic acid concentrations above 250 $\mu\text{mol/l}$ caused turbidity thus precluding hydroperoxide measurement.

Fig. 1. Relationship between absorbance at 365 nm (iodometric method), hydrogen peroxide (open symbols) and linoleic acid hydroperoxide (closed symbols) concentrations. Samples were treated as described in Materials and Methods. Values are means of duplicate determinations. The linoleic acid contained 0.16 μmol hydroperoxide per mol fatty acid.

Within day and day to day imprecision of the iodometric method is summarized in table 1.

With oxidized lipid samples the incubation time of 30 min was not sufficient to complete the reaction. It took at least 60 min at room temperature before the conversion of iodide to iodine was complete with 50 $\mu\text{mol/l}$ linoleic acid hydroperoxide or 40 $\mu\text{mol/l}$ low density lipoprotein lipid hydroperoxide (fig. 2).

When serum samples were analysed iodometrically, interindividual absorbance differences (365 nm) were observed ($\bar{x} \pm s$: 0.23 ± 0.01 ; range: 0.11–0.34; $n = 30$).

In order to investigate whether these absorbances were hydroperoxide-specific, other serum components such as proteins (albumin, apolipoprotein B100), amino acids, polypeptides or metal ions (copper, iron), which have been suggested to interfere with iodometry

Tab. 1. Imprecision of the iodometric method.

Type of imprecision	Sample	n	$\bar{x} \pm s$ ($\mu\text{mol/l}$)	CV (%)
Within day	LDL lipid hydroperoxides	20	13.25 ± 0.47	3.5
	Linoleic acid hydroperoxides	20	5.13 ± 0.53	10.3
Day to day	LDL lipid hydroperoxides	5	12.48 ± 1.23	9.8
	Linoleic acid hydroperoxides	5	7.40 ± 1.48	20.0

Fig. 2. Kinetics of iodine oxidation using either low density lipoprotein lipid hydroperoxide (closed symbols) or linoleic acid hydroperoxide (open symbols). Samples were incubated as described in Material and Methods. Values are means of duplicate determinations. The linoleic acid contained 0.2 μmol hydroperoxide per mol fatty acid.

(21), were tested in the assay at the concentrations given in Materials and Methods. Of the compounds tested only an aqueous solution of ferrous sulphate (250 $\mu\text{mol/l}$) caused a change in absorbance (0.1) during 60 min of incubation. Copper ions did not react at a concentration of 250 $\mu\text{mol/l}$. Transferrin was also ineffective in catalysing the oxidation of iodide.

No increase of absorbance (365 nm) was found during a 60 min incubation of serum samples with the iodometric reagent ($A_{60\text{min}} - A_{0\text{min}} = \bar{x} \pm s$: 0.01 ± 0.01 ; range: 0.00–0.04; $n = 30$). This was not due to the antioxidant, butylated hydroxytoluene, contained in serum samples, since up to 100 $\mu\text{mol/l}$ butylated hydroxytoluene were without effect on the oxidation of iodide by 11 $\mu\text{mol/l}$ linoleic acid hydroperoxide.

In order to confirm that the absorbance differences seen between serum samples were independent of the oxidation of iodide by hydroperoxides, a reaction mixture without iodide was prepared according to the manufacturer's instructions (Merck, Darmstadt, Germany). Addition of serum samples to this "blank" reagent mixture also caused absorptions which were not different from those obtained with the same samples using the complete colour reagent ($A_{60\text{min}} - A_{0\text{min}} = \bar{x} \pm s$: 0.01 ± 0.01 ; range: 0.00–0.04; $n = 30$). Control incubations with 40 $\mu\text{mol/l}$ H_2O_2 or 33 $\mu\text{mol/l}$ linoleic acid hydroperoxides caused no absorbance at 365 nm with this "blank" reagent mixture.

Further evidence that the absorbances obtained with the iodometric reagent and serum samples were not due to the oxidation of iodide by hydroperoxides is provided by wavelength scanning between 250 nm and 450 nm. Whereas 20 $\mu\text{mol/l}$ H_2O_2 caused a peak at 365 nm with the iodometric reagent, no such peak

Fig. 3. Wavelength scanning (250 nm–450 nm) of serum (dashed line), 20 $\mu\text{mol/l}$ hydrogen peroxide (solid line), and 5 $\mu\text{mol/l}$ β -carotene (dashed and dotted line) incubated for 60 min with the iodometric reagent. Experimental details were as described in Materials and Methods.

was observed with a serum sample showing an absorbance of 0.20, which theoretically corresponds to 8.1 $\mu\text{mol/l}$ lipid hydroperoxide (fig. 3). β -Carotene (5 $\mu\text{mol/l}$), which is found in low density lipoproteins (22), also absorbed (0.21) at 365 nm (fig. 3). Non-specific absorbances of serum samples were not related to serum bilirubin concentrations ($r = 0.08$; $p > 0.05$; $n = 20$), or to triacylglycerol concentrations ($r = 0.4$; $p > 0.05$; $n = 20$), but showed a weak positive and significant correlation with serum total cholesterol ($r = 0.5$; $p < 0.05$; $n = 20$), and low density lipoprotein cholesterol ($r = 0.5$; $p < 0.05$; $n = 20$).

Serum samples did not contain lipid hydroperoxides, as shown by the high performance liquid chromatography method. Figure 4 shows the high performance liquid chromatographic analysis of serum samples with and without addition of an oxidized low density lipoprotein preparation. Lipid hydroperoxides were seen predominantly in the cholesterol ester fraction of the oxidized low density lipoprotein preparation (fig. 4). The exogenously added low density lipoprotein lipid hydroperoxides were not detectable iodometrically unless the lipids were extracted (tab. 2). Peroxidation of serum lipids was also induced enzymatically in 9 serum samples using lipoxidase. In contrast to the high performance liquid chromatography method, these enzymatically generated endogenous lipid hydroperoxides were not reliably measured with the iodometric method (fig. 5). The yield of lipid hydroperoxides iodometrically recovered from serum was inversely related to the reducing capacity of serum samples which was determined by the reduction of the tetrazolium salt 3-(4,5 dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) ($r = -0.67$, $p < 0.1$, $y = -7.99x + 1.67$).

Fig. 4. High performance liquid chromatography separation of a butanol extract from native serum (chromatogram A), an oxidized low density lipoprotein preparation (chromatogram B) and the oxidized low density lipoprotein preparation added to serum (chromatogram C). α -tocopherol elutes after 10 min (negative signal), cholesterol ester hydroperoxides after 32 min (positive signal). Experimental details were as described in Materials and Methods.

Physiological concentrations of ascorbic acid (60 $\mu\text{mol/l}$) reduced the iodometric signal obtained with H_2O_2 (40 $\mu\text{mol/l}$) and oxidized low density lipoproteins (5.4 $\mu\text{mol/l}$). High concentrations of ascorbic

Fig. 5. Comparison between the iodometric method and the high performance liquid chromatography method. Lipid hydroperoxides were generated in serum ($n = 9$) by lipoxidase treatment as described in Materials and Methods. Values are means of duplicate determinations.

acid (280 $\mu\text{mol/l}$) totally precluded iodometric measurements (tab. 3). Ascorbic acid had the same effect, irrespective of whether it was added before or after incubation of hydroperoxides with the iodometric reagent (tab. 3).

Discussion

The importance of lipid peroxidation in biological systems has been recognized during recent years (1–11). Efforts are being made to develop reliable and practicable methods to measure lipid peroxidation products such as lipid hydroperoxides in biological specimens (11–15). The oxidation of iodide to iodine by hydroperoxides has been frequently used to determine lipid peroxidation (21, 23–25, 26). *El Saadani* et al. have used a commercially available iodometric reagent mixture to monitor the formation of low density lipoprotein lipid hydroperoxides in vitro (15). This assay is convenient and easy to use. However, it has not been shown whether this method is reliable, and whether it can also be applied to biological samples other than lipoprotein preparations such as serum. This iodometric method was therefore compared with a high performance liquid chromatography method (14).

Tab. 2. Recovery of LDL lipid hydroperoxides from serum samples added as oxidized low density lipoprotein lipids – comparison between iodometry and HPLC.

Method	Lipid hydroperoxides ($\mu\text{mol/l}$)			Recovery
	Serum	LDL	Serum + LDL	
Iodometry without extraction	<1	36.0	<1	0%
Iodometry with extraction	<1	35.0	26.0	74,3%
HPLC	<1	37.0	35.0	94.6%

Values are means of duplicate determinations from one representative experiment.

Tab. 3. Effect of ascorbic acid on the iodometric method.
Ascorbic acid was added before and after a 60 min incubation of either 40 µmol/l hydrogen peroxide (H₂O₂) or 5.4 µmol/l low density lipoprotein lipid hydroperoxide with the iodometric colour reagent.

Sample			Hydroperoxides measured (µmol/l)
H ₂ O ₂			40.0 ± 0.5
H ₂ O ₂ plus 60 µmol/l ascorbic acid	before incubation		34.5 ± 0.7
H ₂ O ₂ plus 280 µmol/l ascorbic acid	before incubation		< 1
H ₂ O ₂ plus 60 µmol/l ascorbic acid	after incubation		35.0 ± 0.7
H ₂ O ₂ plus 280 µmol/l ascorbic acid	after incubation		< 1
LDL			5.4 ± 0.6
LDL plus 60 µmol/l ascorbic acid	before incubation		2.4 ± 0.7
LDL plus 280 µmol/l ascorbic acid	before incubation		< 1
LDL plus 60 µmol/l ascorbic acid	after incubation		2.7 ± 0.6
LDL plus 280 µmol/l ascorbic acid	after incubation		< 1

Values are given as means ± s, n = 5.

The iodometric assay is not specific for hydroperoxides, since iron in an aqueous solution can also lead to the oxidation of iodide. However, iron is transported in serum tightly bound to transferrin and iron-saturated transferrin did not cause any signal with the reagent. It is therefore unlikely that serum metals interfere with the assay.

Absorbances observed at 365 nm with serum samples were non-specific and independent of the oxidation of iodine. Serum samples caused no time-related increase in absorbance at 365 nm and the incubation of serum samples with a “blank” reagent without iodide caused almost the same absorbances as those obtained with the complete iodometric reagent.

Lipid hydroperoxides added to serum in the form of an oxidized low density lipoprotein preparation were not detected iodometrically unless the lipids were extracted (tab. 2). Lipid hydroperoxides, enzymatically generated in serum samples by lipoxidase treatment, were also not reliably detectable with the iodometric which can react with the iodine formed. For example, ascorbic acid, which has a redox potential lower than method (fig. 5). This is possibly due to the presence of reducing activity, which was detected in serum, and iodine (E_o = 0.085 V vs. E_o = 0.535 V), decreased the iodometric signal at physiological concentrations, while at high concentrations it totally precluded iodometric measurement of H₂O₂. This interference was caused by the reduction of the iodine already formed by the oxidation of iodide in the presence of hydroperoxides (tab. 3). Other reducing substances such as glutathione or uric acid, which we did not investigate, may have similar effects.

The high performance liquid chromatography method shows hydroperoxides predominantly in the cholesterol ester fraction of oxidized serum and low density

lipoproteins (fig. 4). This is in agreement with data of *Frei et al.*, who used a similar high performance liquid chromatography method and found that cholesterol ester and phospholipid hydroperoxides in particular are stable in human plasma (27).

The non-specific absorbances seen with serum at 365 nm were not related to bilirubin and triacylglycerol concentrations, but showed a positive and significant correlation with total cholesterol and low density lipoprotein cholesterol levels. Similar non-specific absorbance readings were observed with β-carotene (fig. 3) which is transported in lipoproteins (22). The limited lipid dissolving capacity of the iodometric reagent, which we have observed with linoleic acid or isolated low density lipoproteins, causes minor turbidities which may also contribute to non-specific background absorbances. Neither albumin, nor apolipoprotein B100, nor a variety of polypeptides and amino acids were found to interfere with the iodometric assay at the concentrations tested.

As previously suggested by *Thomas et al.* (21) and *Cramer et al.* (26) the present results confirm that it is necessary to extract lipid hydroperoxides from serum before running an iodometric assay. *Cramer et al.* (26) report 4.0 ± 1.7 µmol/l hydroperoxides in extracted human plasma samples using their iodometric method, whereas we were not able to detect lipid hydroperoxides in serum with the high performance liquid chromatography method (fig. 4). This is in agreement with *Frei et al.*, who also found no lipid hydroperoxides in human plasma using a similar chemiluminescence high performance liquid chromatography assay (12). The discrepancies might be due to the lack of protection against oxidation employed during blood sampling and storage. *Cramer et al.* analysed pooled plasma samples which had been frozen and thawed without added antioxidants (26). Fur-

thermore, the possibility cannot be excluded that individuals whose antioxidant defenses are depleted might develop circulating lipid hydroperoxides.

The results of this investigation show that the method published by *El Saadani* et al. (15) is a reliable tool to monitor lipid hydroperoxides in isolated lipoprotein preparations. However, the incubation time of 30 min is not sufficient and should be increased to at least 60 min. Most importantly the method cannot be used for native serum samples without extraction. In addition, the iodometric assay has a relatively unfavourable detection limit (1 µmol/l) compared with the high performance liquid chromatography methods (about 0.05 µmol/l) 12–14). This will also hamper its application in the investigation of tissue samples and membranes where only low concentrations of lipid hydroperoxides can be expected (28, 29).

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