A New Thiobarbituric Acid (TBA) Method for Determining Free Malondialdehyde (MDA) and Hydroperoxides Selectively as a Measure of Lipid Peroxidation[1]

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A new thiobarbituric acid (TBA) method for selectively determining free malondialdehyde (MDA) and lipid hydroperoxides is described. Partitioning of MDA and hydroperoxides in a Bligh and Dyer extraction was studied with pure substances. It was shown that MDA was present in the methanol-water phase exclusively, and the hydroperoxides (polar as well as nonpolar) were found in the chloroform phase. Therefore, the TBA test on these phases determines MDA directly present in the sample and the MDA formed during a ferric ion-catalyzed cleavage of hydroperoxides, respectively. The MDA present in the methanol-water phase was not bound to amino groups. Hydroperoxide values obtained with the present method corresponded well with a colorimetric determination of peroxides and showed better linearity for higher amounts of hydroperoxides. The possibility of using an iron chelator for preventing hydroperoxide cleavage during the TBA reaction, making the determination selective for MDA without Bligh and Dyer extraction, was investigated. The iron chelator did not completely inhibit peroxide cleavage; therefore, it is necessary to perform the extraction before the TBA determination. The method is suitable for samples with low fat content such as cod mince.

The most common method for measuring oxidative changes in biological samples and food products is the thiobarbituric acid (TBA) test based on a spectrophotometric quantitation of a red-violet complex formed with malondialdehyde (MDA) (1). This method determines both the MDA already formed naturally from hydroperoxide cleavage, and the secondary release due to the heating step in the TBA reaction. Therefore, the TBA values obtained are dependent on methodology and not easily reproduced between laboratories. The TBA test is often used for determination of lipid peroxides (2-4), but the present methods measure both free MDA and lipid peroxides. Several investigators have reported selective methods for determining free MDA by HPLC (5-9). Furthermore, HPLC has often been used for determining the MDA-TBA adduct selectively (10-12). However, the adduct measured can arise from both peroxides and free MDA.

In samples with low lipid content the TBA determination on lipids isolated by a Bligh and Dyer extraction (13) has been suggested to increase the sensitivity (14), but the authors did not elucidate whether the reaction was due to MDA, hydroperoxides or both. Neither was the possible loss of MDA by evaporation accounted for.

Albro et al. (15) described a method for measuring free MDA and "labile lipid peroxidation products" selectively. This method, however, is based on the fact that lipids co-precipitate when proteins are precipitated with trichloroacetic acid (TCA) in a microsomal system. The method, therefore, is not applicable for most food products and oils.

A selective TBA determination of free MDA and hydroperoxides, measured as MDA formed during the TBA reaction, has not been reported previously. The purpose of the present work was to develop a selective and sensitive TBA method for lean fish and possibly other food products with low fat content, which determines:

(i) malondialdehyde already present in the sample before the extraction.
(ii) hydroperoxide, measured as malondialdehyde formed during the TBA-test.

The advantage of such a method is a better knowledge of the actual oxidation status. In order to achieve this, the fate of MDA, hydroperoxides and protein-bound MDA during a Bligh and Dyer extraction and the TBA reaction were studied.

MATERIALS

Chloroform, methanol, hexane and diethyl ether were redistilled. All other chemicals used were analytical grade.

ANALYTICAL METHODS

Preparation of MDA standards. Ten µl (9.2 mg) of 1,1-3,3-tetraethoxypropane (TEP, Merck) is accurately diluted to 10 ml with 0.1 N HCl in a screw-capped test tube and immersed into a boiling water bath for five min, then quickly cooled in tap water. A 10^{-4} M stock solution of MDA is prepared by pipetting 2.4 ml of the hydrolyzed acetal into a 100-ml volumetric flask and diluting it to volume with water.

The standards used for the TBA test in the chloroform phases are prepared by adding aliquots of this stock solution (0-20 nmol) to 2.5 ml chloroform. When the TBA reagent containing acetic acid is added, the mixture will turn monophasic.

The standards used for the methanol:water phase (0-12.5 nmol) are prepared by adding the stock solution to 2.5 ml methanol:water (10:9). TBA reagent is added as described later.

Bligh and Dyer extraction (13). Ten g of cod mince is weighed into a glass centrifuge tube (70 ml) and homogenized with a Polytron Kinematica equipped with shaft PTA 20 S for 2 min at speed 7 in a solvent mixture consisting of 10 ml of chloroform and 20 ml of methanol. (The tube is placed in ice during the homogenizations.) Then 10 ml of chloroform are added and homogenization continued for a further 30 sec. Finally, 10 ml of redistilled water are added and the mixture is homogenized for 30 sec. The tube is now centrifuged for 20 min at 4000 g and the aqueous layer transferred to a 50-ml volumetric flask

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which is made to volume with methanol:water (10:9). The chloroform layer is transferred to a 25-ml volumetric flask which is filled to the mark with chloroform.

In the partitioning experiments with hydroperoxides and MDA, eight ml of water were added to the sample before the Bligh and Dyer extraction, corresponding to the water content of 10 g of cod mince.

TBA test in the chloroform phase from a Bligh and Dyer extraction. The present test is based on the method of Ke and Woyewoda (14) in which lipids are extracted from the sample according to Bligh and Dyer (13) before performing the test. In their method the chloroform layer obtained is evaporated, and a known amount of lipid is weighed into a test tube before the TBA reagent is added. This may, however, result in unexpected changes and losses during the evaporation. In the present method this is avoided by analyzing the $CHCl_3$ extract directly. The TBA reagent is therefore in contrast to the original method prepared without $CHCl_3$.

Furthermore, to enhance the cleavage of peroxides to MDA, ferric chloride is added to the reagent as suggested by Asakawa and Matsushita (4). The reagent is prepared from two solutions (A and B) which are mixed not more than 30 min prior to use. Solution A is prepared by dissolving 1.75 g TBA in 30 ml distilled water and adding 270 ml glacial acetic acid. Solution B contains 950 mg sodium sulfite and 22 mg $FeCl_3$, 6 H_2O in 25 ml water. For analysis, 2.5 ml of the $CHCl_3$ phase from a Bligh and Dyer extraction is pipetted into a 30-ml autoclavable glass culture tube (Kimax, USA) with a Teflon-lined screw cap; 4.0 ml of the TBA reagent is added, and the tube is capped tighly. The tubes are heated for 30 min in a boiling water bath and cooled in tap water. During heating and cooling the tubes are covered by a shield for safety reasons. After cooling, 3.5 ml trichloroacetic acid (TCA) solution (5%) is added to each tube, and after mixing thoroughly the tubes are centrifuged for 10 min (1500 g) to separate the two layers. The absorbance of the upper aqueous phase is measured at 532 nm.

TBA test in the aqueous phase. The aqueous phase from the Bligh and Dyer extraction contains methanol and water in the ratio of 10:9. The TBA reagent for the methanol-water phase is prepared by dissolving TBA (1%) in TCA solution (5%).

The sample (2.5 ml) is mixed in a screw capped tube with 2.5 ml TBA reagent. The tubes are heated in a boiling water bath for 30 min, cooled in tap water and centrifuged at 1500 g to assure a clear solution. The absorbance is measured at 532 nm.

Determination of protein bound MDA. Protein bound MDA was hydrolyzed according to the method of Lee and Csallany (16). In this method the sample is hydrolyzed at pH 13 by incubation in 0.5 M NaOH solution at 60°C for 30 min. The hydrolyzed sample is neutralized to pH 8.0 with concentrated HCl, and the amount of MDA generated is determined using the TBA test described for the aqueous phase.

Determination of peroxide value (PV). Peroxide value was determined using the principle of the method of Stine et al. (17). The determination is based on the oxidation of Fe(II) to Fe(III) by peroxides; Fe(III) forms a violet complex with thiocyanate, and this complex is quantitated spectrophotometrically.

Reagents. Ferrous chloride solution: A solution of ferrous chloride is prepared by mixing equal volumes of barium chloride solution (8 mg $BaCl_2$, 2 H_2O/ml) and ferrous sulfate solution (10 mg $FeSO_4$, 7 H_2O/ml). To the mixture is then added concentrated HCl, 20 µl/ml, and the precipitated $BaSO_4$ is allowed to settle for one hr at 4°C before the suspension is centrifuged at 1500 g and a clear supernatant obtained.

Ammonium thiocyanate solution: 300 mg NH_4SCN/ml. Standards are prepared from a stock solution of $FeCl_3(6H_2O)$ 4.8 mg/ml corresponding to one mg Fe(III)/ml. Two hundred and forty mg $FeCl_3(6H_2O)$ are weighed into a flask; three ml concentrated HCl and 200 µl 30% H_2O_2 are added to oxidize Fe(II) eventually present. After 10 min the remaining H_2O_2 is removed by heating the flask in boiling water for 10 min. After cooling, the solution is transferred to a 50-ml volumetric flask and made to volume with water. Six different standard solutions containing 0.05 mg–0.35 mg Fe(III)/ml are prepared by diluting this stock solution with water and HCl-solution making the final concentration of HCl 0.25 M in the standards.

For preparation of the standard curve, 20 µl of the standard solutions (1–6) are mixed with 4.955 ml $CHCl_3$:CH_3OH 3:5 and 25 µl NH_4SCN solution. After 10 min the absorbance is determined at 505 nm.

For determination of the peroxide value in the chloroform phase from Bligh and Dyer extractions, one ml of this phase is mixed with 3.975 ml $CHCl_3$:CH_3OH 3:5 and 25 µl NH_4SCN, and after 10 min the absorbance is determined at 505 nm.

Preparation of methyl linolenate hydroperoxides. Hydroperoxides were prepared from methyl linolenate, 50 mg (Nu Chek Prep, Elysian, Minnesota), which was oxidized in daylight, at room temperature and in a normal atmosphere for one week. The esters were then dissolved in hexane and subjected to separation on a Sep-Pak silica cartridge (Waters). The nonoxidized esters were removed with 25 ml hexane and five ml of a mixture of hexane:diethyl ether (20:1, v/v). The hydroperoxide methyl esters were eluted with five ml diethyl ether. Identifications of fractions were made on Merck Kieselgel 60 TLC plates, 0.25 mm using a solvent mixture containing hexane, diethyl ether, acetic acid (70:30:1, v/v/v); recognition of hydroperoxides was achieved by spraying the plates with a N,N-dimethyl-p-phenylene-diamine reagent (18). If necessary, further purification may be obtained using corresponding TLC plates and a solvent system with hexane, diethyl ether, acetic acid (30:70:1, v/v/v).

Preparation of phosphatidylcholine hydroperoxides. Phosphatidylcholine (PC) isolated from rat liver was oxidized and purified according to the method of Miyazawa et al. (19). The presence of PC hydroperoxide (PC-OOH) was confirmed on Merck Kieselgel 60 TLC plates, 0.25 mm in a developing solvent of hexane, chloroform, methanol, acetic acid (30:40:20:10, v/v/v/v) containing 1.8 g boric acid/100 ml and using the above mentioned spray reagent for hydroperoxides.

Quantitation of phospholipid. Phospholipid was quantitated as inorganic phosphate using the method of Rouser et al. (20).

RESULTS AND DISCUSSION

Partitioning of hydroperoxides in a Bligh and Dyer extraction. Samples of methyl linolenate hydroperoxides

(HPO) were subjected to a Bligh and Dyer extraction after the addition of water and the TBA tests performed on the two phases as described. The methanol-water phase contained only traces of MDA. The results for the chloroform phase are shown in Figure 1. The hydroperoxides are dissolved in the chloroform phase during the extraction, and therefore are determined selectively by the TBA test for this phase. The relation between $OD_{532\,nm}$ and μg hydroperoxides is linear from 0 to 150 μg HPO.

The peroxide values (PV) of the HPO samples were also determined directly without extraction. The results are shown in Figure 2. This method shows linearity to approximately 100 μg HPO; it seems, therefore, that the TBA test has a better linearity.

The partitioning of the more polar phospholipid hydroperoxides was also investigated. Samples of PC-OOH were subjected to extraction, and the distribution was verified by determination of peroxide value as well as the phosphorous content of the chloroform phase. The results from both analyses showed that all phospholipid hydroperoxides were present in the organic phase in a Bligh and Dyer extraction.

Partitioning of MDA. Samples of cod liver oil (10 mg, triplicates, stored for several months) were added known amounts of MDA, extracted according to Bligh and Dyer, and subsequently TBA tests were performed on the chloroform and the methanol-water phases, respectively. The results are shown in Table 1.

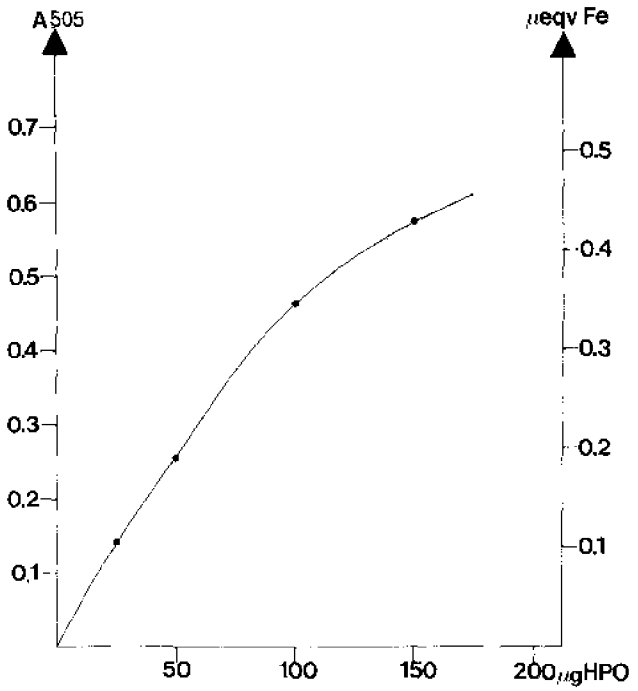

FIG. 2. Measurement of peroxide value of samples of methyl linolenate hydroperoxides (HPO).

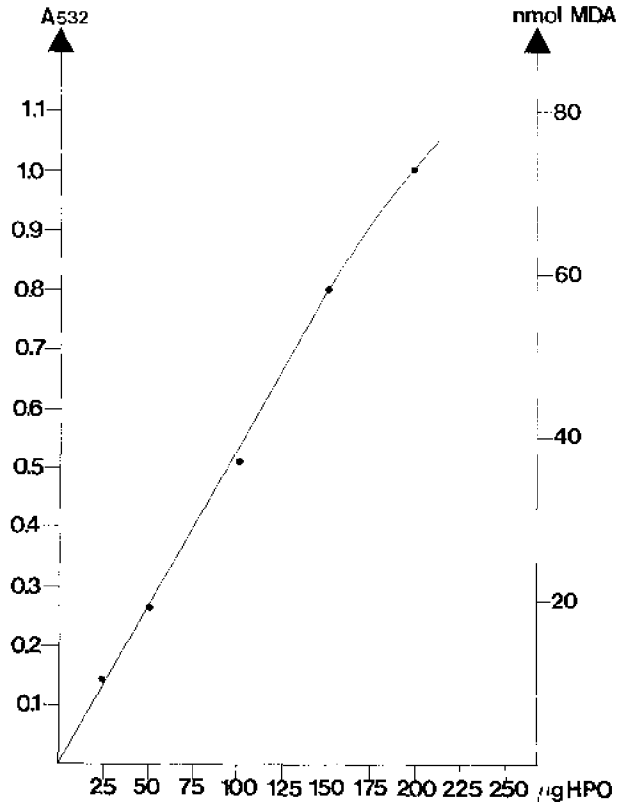

FIG. 1. Measurement of TBA-reactive substances in the chloroform phase from Bligh and Dyer extraction. HPO, methyl linolenate hydroperoxides.

TABLE 1

Distribution of TBA-Reactive Material After Bligh and Dyer Partitioning

	CH_3OH/H_2O	$CHCl_3$
Cod liver oil, Ph.Nord.	3.0 ± 1.0[a] nmol	19.0 ± 0.9
Oil + 15 nmol MDA	16.5 ± 0.9	21.0 ± 0.6
Oil + 30 nmol MDA	27.8 ± 0.4	22.6 ± 0.8

[a]± SEM.

The MDA added was found in the methanol-water phase. The chloroform phase contained lipid-soluble material including hydroperoxides, which to some extent are cleaved during the TBA reaction. A TBA reaction on isolated lipids will measure only hydroperoxides, and MDA already present will avoid determination. Accordingly, the TBA value for the chloroform phase is almost constant despite the added MDA. The level of free MDA in the fish oil is low even though there is a high amount of hydroperoxides.

Bound MDA. When food products containing protein and lipids are subjected to Bligh and Dyer extraction, the proteins will be found mainly in the interfacial material. However, the methanol-water phase will contain amino acids and peptides to some extent.

To investigate whether the TBA value in the methanol-water phase was due only to free MDA, or to a combination of bound and free MDA, experiments with extractions from cod mince were carried out. Cod mince was stored at −18°C for one month to allow the lipids to

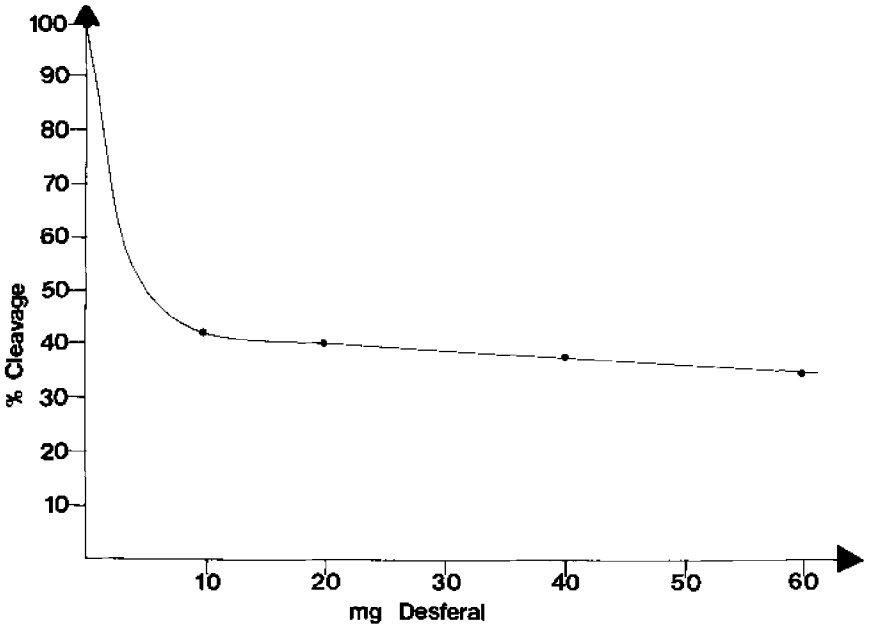

FIG. 3. Inhibition of hydroperoxide cleavage by desferal (shown as percent of original cleavage).

peroxidize, and the MDA produced to bind to the proteins. Ten g of the mince was then extracted according to Bligh and Dyer and the TBA test performed on the two phases as described. Furthermore, the total amount of amino acids in the methanol-water phase was quantitated before and after acid hydrolysis.

The amount of MDA originating from peroxides in the chloroform phase was 12 μmol/kg and in the methanol-water phase 4 μmol/kg, indicating that the mince was oxidized. The methanol-water phase contained 0.53 mg/ml of amino acids totally, corresponding to about 20 mmol/kg. It was found that 85% of this amount were free amino acids. Furthermore, an aliquot of the aqueous phase was evaporated under vacuum in a Büchi Rotavapor® to remove free MDA, and the residue subjected to hydrolysis in a tightly capped test tube by heating it for 30 min in a NaOH solution as described above. MDA released was determined with the TBA test. The hydrolyzed samples contained no MDA; thus, the methanol-water phase contained no bound MDA.

Recently Lee et al. (16) reported the presence of bound MDA in microsomes, using the present method. However, the MDA generated during the described alkaline hydrolysis of animal tissue could originate from hydroperoxides as well as protein bound MDA. A Bligh and Dyer extraction would separate hydroperoxides (chloroform-phase), free MDA (methanol-water phase) and MDA bound in the interfacial material, making selective determinations possible. However, the determination of protein bound MDA in fish mince is difficult, due to the fibrous nature of the interfacial material. Evidence for the presence of protein bound MDA has been found, but further experiments are needed for quantitative evaluations. This aspect may be important, as one of the deleterious problems during storage of cod mince is the change in texture, which is supposed to be related to interactions between the aldehydes and protein.

Experiment with desferal. The cleavage of hydroperoxides during the heating step in the TBA reaction is dependent on metals. It might be expected that addition of an iron chelator in the TBA test could prevent this cleavage and thus determine the MDA originally present. Experiments to elucidate the possibility of using this concept in the selective determination of MDA and hydroperoxide without a partitioning through a Bligh and Dyer extraction were made with desferal (desferrioxamine, Ciba Geigy), a potent iron chelator (binding constant, Fe(III): $K = 10^{31}$).

The TBA test was carried out on 50 μg of methyl linolenate hydroperoxide with varying amounts of desferal. Results in Figure 3 are given as percentages of original cleavage (i.e., without desferal).

Even with the highest amount of the chelator, the cleavage will proceed. Either the chelated iron still acts as a catalyst, or a certain amount of degradation may occur even without Fe(III) present.

It is therefore not possible to avoid the Bligh and Dyer extraction by the use of an iron chelator.

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