Rapid, Sensitive, and Specific Thiobarbituric Acid Method for Measuring Lipid Peroxidation in Animal Tissue, Food, and Feedstuff Samples

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A rapid aqueous acid extraction thiobarbituric acid method for measuring malondialdehyde as a marker of lipid peroxidation in animal tissue, food, and feedstuff samples has been developed. Sample is homogenized with aqueous trichloroacetic acid in the presence of hexane and butylated hydroxytoluene, and the homogenate is centrifuged. Following reaction with thiobarbituric acid reagent, malondialdehyde is directly quantified on the basis of the third-derivative absorption spectrum of the pink complex formed. Further purification is not required because the derivative transformation of the conventional analytical band at around 532 nm virtually eliminates spectral interferences arising from other compounds. The effect of antioxidants and the optimum conditions for the reaction have been established, and the analytical performance of the new method has been evaluated. The applicability of the method on various animal tissue, food, and feedstuff samples has been also tested. Owing to its simplicity and increased sensitivity and specificity, the method may be preferred over other methods for estimating the extent of lipid peroxidation.

Keywords: *Thiobarbituric acid method; derivative spectrophotometry; lipid peroxidation; malondialdehyde*

INTRODUCTION

Monitoring and control of lipid peroxidation in food are of great importance due to increasing demand for good quality products. Lipid peroxidation, the oxidative deterioration of the polyunsaturated lipids of food, leads through formation of hydroperoxides to short-chain aldehydes, ketones, and other oxygenated compounds which are considered to be responsible for the development of rancidity in stored foods (Gray, 1978; Melton, 1983; Wong, 1989) and related to experimental heart disease, cancer, and aging in animals (Chio and Tappel, 1969; Kaunitz and Johnson, 1973; Cutler and Hayward, 1974).

Malondialdehyde (MDA), a major degradation product of lipid hydroperoxides, has attracted much attention as a marker for assessing the extent of lipid peroxidation (Raharjo and Sofos, 1993). The compound is of particular concern since it has been shown to be mutagenic (Basu and Marnet, 1984) and carcinogenic (Shamberger et al., 1974) and implicated in other pathological processes such as the formation of fluorescent pigments typical of cellular aging (Bidlack and Tappel, 1973; Trombly and Tappel, 1975).

The most common method for measuring MDA in food products and biological samples seems to be the thiobarbituric acid (TBA) test, which is based on spectrophotometric quantitation of the pink complex formed after reaction of MDA with two molecules of TBA. The TBA test can be performed (a) by directly heating the sample with TBA followed by separation of the pink complex produced (Turner et al., 1953; Yu and Sinnhuber, 1957; Sinnhuber and Yu, 1958), (b) by distillation of the sample followed by reaction of the distillate with TBA (Tarladgis et al., 1960; Rhee, 1978; Yamauchi et al., 1982; Ke et al., 1984), (c) by extraction of the lipid portion of the sample with organic solvents and reaction of the extract with TBA (Pikul et al., 1983, 1989), and (d) by extraction of MDA using aqueous trichloroacetic (Witte et al., 1970; Sinnhuber and Yu, 1977; Siu and Draper, 1978; Newburg and Concon, 1980) or perchloric (Salih et al., 1987; Pikul et al., 1989) acid and reaction with TBA.

Although the distillation method is the most frequently used procedure and may be regarded as the standard method for MDA analysis, it is more cumbersome and requires more time than the aqueous acid extraction method. Furthermore, heating during distillation enhances the degradation of existing lipid hydroperoxides and, thus, additional MDA and other TBA-reactive substances (TBARS) may be formed even in the presence of metal chelators or phenolic antioxidants (Gutteridge and Quinlam, 1983; Raharjo and Sofos, 1993). The aqueous acid extraction method is also preferred by many workers because it is simple and gives results that are highly correlated with those of distillation (Pikul et al., 1989) and sensory evaluation (Salih et al., 1987) methods. In general, however, all versions of the TBA test have been criticized as being insensitive for the detection of low levels of MDA and nonspecific (Hackett et al., 1988). Other TBARS can interfere with the analysis, overestimating the results (Marcuse and Johansson, 1973; Patton, 1973; Kosugi et al., 1987).

To eliminate interferences, Bird et al. (1983) used high-performance liquid chromatography (HPLC) for

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analyzing MDA in food and feed samples. Other workers applied an additional cleanup step prior to HPLC (Draper and Hadley, 1990; Squires, 1990) or spectrophotometric (Raharjo et al., 1992, 1993) determination using solid-phase extraction (C_{18} cartridge) to remove interfering substances from the reaction mixture. These procedures can provide increased specificity and sensitivity over the conventional TBA methods, but they are rather time-consuming and not practical for routine analysis. Therefore, a simple, rapid, sensitive, and specific method for measuring the extent of lipid peroxidation could be of value.

An interesting alternative to conventional spectrophotometric methods, in cases where spectral interferences obscure the analytical band, is derivative spectrophotometry (O'Haver and Green, 1976). This technique offers, in some instances, improved specificity and sensitivity without any need for lengthy sample clarification (Botsoglou et al., 1993; Fletouris et al., 1993). In this study, the performance of derivative spectrophotometry in developing a new, more reliable method for the determination of MDA in animal tissue, food, and feedstuff samples has been investigated.

MATERIALS AND METHODS

Instrumentation. A Shimadzu Model UV-160A double-beam spectrophotometer with 1-cm absorption cells was used for all measurements. Third-derivative spectra were produced by electronic differentiation ($d^3A/d\lambda^3$, where A is the absorbance and λ is the wavelength) of the normal spectra obtained at a scanning speed of 480 nm/min, using a derivative difference ($\Delta\lambda$) setting of 21 nm. An Ultra-Turrax blender (Janke & Kunkel, GmbH, Germany), a Centra-MP4 centrifuge (IEC), a vortex blender (Heidolph, Germany), and a thermostated water bath, type 3044 (Kottermann, Germany), were also used for sample treatment.

Reagents. Most chemicals and solvents used in this study were of ACS reagent grade. Butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA), ascorbic acid (AA), bovine serum albumin (BSA), and the malondialdehyde precursor, 1,1,3,3-tetraethoxypropane (TEP), were all obtained from Sigma Chemical Co. (St. Louis, MO), whereas trichloroacetic acid (TCA) was from Merck (Germany).

Preparation of MDA Standards. A quantity (73.2 mg) of TEP was accurately weighed in a screw-capped test tube, diluted with 10 mL of 0.1 N HCl, immersed into a boiling water bath for 5 min, and quickly cooled under tap water. Stock solution of MDA (239 μg/mL) was prepared by transferring the hydrolyzed TEP solution into a 100-mL volumetric flask and diluting to volume with water. Working MDA solution (2.39 μg/mL) was prepared by pipetting a 1-mL aliquot of the stock solution into another 100-mL volumetric flask and diluting to volume with water.

Animal Tissue, Food, and Feedstuff Samples. Cow, pork, lamb, and chicken muscle, liver and kidney tissues, sardine, mackerel, and cod fish tissues, meat meal, fish (herring) meal, fish meal, soybean meal, infant milk formula, unsweetened condensed milk, blue cheese, feta cheese, kasseri cheese, processed cheese, coffee cream powder, and butter were all purchased from a local market. Rat muscle and kidney tissues were from the experimental animals of our laboratories.

Analytical Procedure. A 2-g sample was transferred into a 25-mL centrifuge tube, and volumes of 5% aqueous TCA (8 mL) and 0.8% BHT in hexane (5 mL) were successively added. The content of the tube was Ultra-Turraxed for 30 s at high speed and centrifuged for 3 min at 3000g, and the top hexane layer was discarded. The bottom aqueous layer was made to 10-mL volume with 5% TCA, and a 2.5-mL aliquot was pipetted into a screw-capped tube to which a volume (1.5 mL) of 0.8% aqueous TBA was also added. Following incubation for 30 min at 70 °C, the tube was cooled under tap water, and the reaction mixture was submitted to third-derivative spectrophotometry against blank reaction mixture.

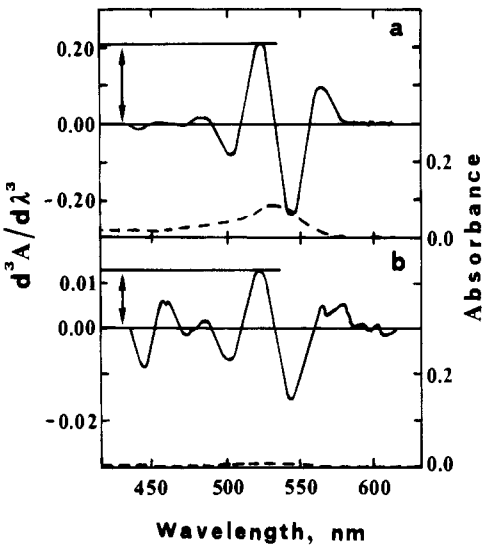

Figure 1. Normal (- - -, right Y-axis) and third-derivative (—, left Y-axis) spectra of MDA–TBA reaction mixtures containing 31.9 (a) and 2.4 ng of MDA/mL (b).

Determination. Aliquots of standard solutions were pipetted into screw-capped tubes and diluted to 2.5-mL volume with 5% TCA. A 1.5-mL volume of 0.8% TBA was added in each tube, and the reaction was carried out as prescribed. Calibration curves were constructed by plotting values of peak height at 521.5 nm, as they are printed on the instrumental chart in arbitrary units, versus known concentration of MDA in the final reaction mixtures. The concentration of MDA in sample extracts was calculated on the basis of the slope and intercept data of the computed least-squares fit of calibration curve. In case the absorbance value exceeded 1.0, sample extract was appropriately diluted with water before final measurement. MDA was determined in samples using the formula

$$\text{MDA content, ppb} = 16CV/W$$

where C is the MDA concentration (ng/mL) in the sample extract according to the calibration curve, V is the dilution factor of sample extract, if any, and W is the weight (g) of the sample.

RESULTS AND DISCUSSION

The performance of derivative versus normal spectrophotometry in the identification of the MDA–TBA complex is shown in Figure 1. When an MDA–TBA reaction mixture containing 31.9 ng/mL standard MDA is submitted to third-derivative spectrophotometry, a series of peaks and troughs appears that may be used to locate and resolve the analytical band; the peak at 521.5 nm and the trough at 544 nm correspond to the inflection points of the conventionally recorded analytical band, while the zero-crossing observed at 532 nm represents the absorption maximum (Figure 1a). The appearance of such extremes improves the resolution so that, while the normal spectrum of a further diluted (2.4 ng/mL) solution gives absolutely no information, its derivative spectrum really does (Figure 1b).

Research based on normal spectrophotometry has, already, pointed out that temperature, time of heating, and pH of the reaction mixture are all critical parameters to the completeness of the MDA–TBA reaction (Raharjo and Sofos, 1993). However, the recommended optimum conditions vary greatly among authors. Heating the reaction mixture at 70 or 80 °C for 30 (McCoy

Table 1. Effect of pH upon the Formation of the MDA–TBA Complex

pH	height[a] of the third-derivative peak[b] at 521.5 nm (arbitrary units)	pH	height[a] of the third-derivative peak[b] at 521.5 nm (arbitrary units)
3.1	0.220	1.6	0.885
2.9	0.298	1.2	1.042
2.7	0.356	1.1	1.047
2.4	0.509	0.5	1.044
2.1	0.689		

[a] Average of triplicate analyses. [b] The reaction mixture contains 147.6 ng of MDA/mL.

et al., 1988) or 90 min (Siu and Draper, 1978), respectively, or boiling for 10 (Buttkus and Bose, 1972), 15 (Shamberger et al., 1977), 20 (Kosugi et al., 1989), 25 (Braddock and Petrus, 1971), 30 (Salih et al., 1987), 35 (Sidwell et al., 1955), 40 (Yu and Sinnhuber, 1957), 45 (Ke et al., 1984), 55 (Tarladgis et al., 1960), and 60 min (Pikul et al., 1989) are all suggested to be optimum heating conditions. On the other hand, some of the proposed optimum pH conditions include acidification of the reaction mixture at pH values of 3.5 (Ohkawa et al., 1979), 3.0 (Draper et al., 1984), and 1.5 (Tarladgis et al., 1960). Due to this inconsistency, reexamination of the optimum reaction conditions through the use of derivative spectrophotometry was carried out.

The data presented in Tables 1 and 2 do not fully support the above view. Lowering the pH of the reaction mixture causes the derivative response to arrive at a maximum (Table 1), whereas when the temperature is decreased from 100 to 70 °C, the yield of the reaction becomes higher but the reaction time is also markedly increased (Table 2). Heating times longer than 30 min at 100 °C or 50 min at 80 °C were found to result in turbidity development and, consequently, in high erroneous values when measurements were made by normal spectrophotometry. This might account for the results presented by Tarladgis et al. (1962) and Yu and Sinnhuber (1964), who also observed spectral interferences to the TBA reaction during heat treatment in the presence of acids and attributed them to decomposition of the TBA reagent. Derivative processing, however, eliminated this turbid background, and quantitation of the MDA–TBA complex could be made possible even in such turbid solutions.

Summarizing the data of Tables 1 and 2, it can be assumed that a pH value lower than 1.2 and a heating time of 30 min at 70 °C might be the optimum conditions for MDA–TBA reaction. Regression analysis of the data obtained by running a series of working MDA solutions showed the response to be linear in the range examined (Table 3). Under the mentioned conditions, as low as 2.6 ng of MDA/mL of reaction mixture could be precisely measured on the basis of the height of the third-derivative peak at 521.5 nm (Table 3). Considering sample size and total extract dilution during the analytical process, this corresponds to a limit of determination (*ca.* 20 μg/kg of sample), which is much lower than that that has been estimated for the conventional spectrophotometric methods (Raharjo et al., 1993) and comparable to that for solid-phase extraction (Raharjo et al., 1993) and HPLC (Bird et al., 1983; Squires, 1990) methods.

The extraction of MDA from samples was carried out with 5% TCA in the presence of BHT and hexane, a modification of the aqueous acid extraction method of Witte et al. (1970). Using hexane, some purification of the extract could be effected, and the appearance of turbidity, the major shortcoming observed in all extraction methods for samples high in fat, was eliminated. The method of Witte et al. (1970) did not include antioxidants during sample blending, and neither do many aqueous extraction methods in current use (Shamberger et al., 1977; Newburg and Concon, 1980; Bird et al., 1983; Csallany et al., 1984; Lee and Csallany, 1987; Schmedes and Holmer, 1989). Some investigators, however, have stated that lipid peroxidation may be a serious concern unless antioxidants such as BHT, AA, butylated hydroxyanisole, propyl gallate, sodium sulfite, and tocopherol or chelating agents such as EDTA are added before sample processing (Siu and Draper, 1978; Rhee, 1978; Pikul et al., 1983; Ke et al., 1984; Salih et al., 1987, 1989; Squires, 1990). To determine whether autoxidation occurred during sample treatment, various concentrations of selected antioxidants and EDTA were tested using 2-days-refrigerated and 2-months-frozen stored mackerel and cod samples, respectively. The hydrophobic BHT and the hydrophilic AA were used as test antioxidants because the former is considered to be more effective than other commonly used antioxidants (Khayat and Schwall, 1983) while the latter, although antioxidant, exhibits also prooxidant activity (Benedict et al., 1975).

Table 4 shows that significant lipid peroxidation occurred when samples were blended with TCA. Hexane added before blending was effective for reducing further lipid peroxidation, but it had no effect when added after blending, a finding suggesting that blending was the major cause of sample autoxidation. On the other hand, addition of EDTA before blending in the presence of hexane had a more pronounced effect on the suppression of lipid peroxidation. This is consistent with previous findings suggesting that the prooxidant action of some metal ions in foods can be greatly retarded by the addition of EDTA due to formation of complexes with low reduction potentials favoring an antioxidant effect (Richardson and Korycka-Dahl, 1983). Table 4 also shows that AA exhibited a definite prooxidant action. The univalent reduction of metal ions by AA followed by the reduction of hydroperoxides to yield hydroxyl radical may provide one basis for the prooxidant function of AA (Kanner et al, 1977; Kanner, 1994). However, combinations of AA and metal ions can be prooxidant or antioxidant depending upon their relative concentrations (Kanner et al., 1977). A number of possible mechanisms have been suggested for this apparent paradox, including reductive activation of metal ions, increased levels of the supposed prooxidant ascorbyl radical, and formation of unspecified ascorbate–metal complexes that may differentially affect propagation and termination reactions of lipid oxidation (Richardson and Korycka-Dahl, 1983; Kanner, 1994). Numerous studies in this area have not succeeded in defining the complex behavior of AA in the oxidative stability of lipids.

On the other hand, BHT showed an outstanding antioxidant activity. At the lower BHT level tested, some decomposition of lipid peroxides still occurred, whereas at higher levels decomposition was fully suppressed even when no EDTA was concurrently added. These findings lend support to previous results suggesting that full protection against autoxidation can be assured when BHT is added to the sample before the homogenization process (Pikul et al., 1983, 1989).

Table 2. Effect of Temperature and Reaction Time upon the Formation of the MDA–TBA Complex

temp (°C)	height[a] of the third-derivative peak[b] at 521.5 nm (arbitrary units) at reaction time of											
	5 min	10 min	15 min	20 min	25 min	30 min	40 min	50 min	60 min	80 min	100 min	120 min
100	0.349	0.414	0.417	0.420	0.416	0.415	0.407	0.398	0.395			
80	0.305	0.401	0.422	0.433	0.436	0.434	0.432	0.431	0.423	0.423	0.423	0.409
70	0.196	0.339	0.390	0.423	0.448	0.449	0.450	0.448	0.449	0.452	0.449	0.448

[a] Average of triplicate analyses. [b] The reaction mixture contains 63.8 ng of MDA/mL.

Table 3. Raw Data and Regression Equation of Calibration Curve for MDA Quantitation by Third-Derivative Spectrophotometry

concn of standard (ng of MDA/mL of reaction mixture)	peak height,[a] mean ± SD ($n = 5$)	RSD (%)
2.6	0.017 ± 0.001	5.9
5.3	0.038 ± 0.002	5.3
10.6	0.069 ± 0.001	0.7
31.9	0.219 ± 0.004	1.8
42.6	0.301 ± 0.002	0.7
53.2	0.378 ± 0.003	0.8
79.8	0.565 ± 0.002	0.4
106.4	0.758 ± 0.001	0.1
133.0	0.936 ± 0.002	0.2

[a] Peak height is expressed in arbitrary units. Regression equation: $Y = -(2.7 \times 10^{-3}) + (7.1 \times 10^{-3})X$, where Y represents peak height and X the concentration of MDA in reaction mixture (ng/mL). Correlation coefficient: 0.9999.

Table 4. Effect of Adding AA, BHT, or EDTA before Blending on Malondialdehyde Values of Mackerel and Cod Samples

concn of additive (mg/g of sample)	fresh mackerel		frozen cod	
	MDA concn[a] (ppb ± SD)	RSD (%)	MDA concn[a] (ppb ± SD)	RSD (%)
0	2289 ± 322.7	14.1	2066 ± 264.4	12.8
0[b]	2722 ± 862.9	31.7	2250 ± 751.5	33.4
AA				
1	2843 ± 272.9	9.6	2616 ± 217.1	8.3
3	2995 ± 212.6	7.1	2544 ± 198.4	7.8
BHT				
5	1207 ± 42.2	3.5	1478 ± 29.6	2.0
10	1142 ± 18.3	1.6	1333 ± 29.3	2.2
20	1114 ± 18.8	1.7	1304 ± 5.2	0.4
30	1126 ± 22.5	2.0	1313 ± 14.4	1.1
50	1117 ± 12.3	1.1	1315 ± 11.7	0.9
EDTA				
5	1571 ± 110.0	7.0	1688 ± 96.2	5.7
10	1603 ± 113.8	7.1	1616 ± 132.5	8.2
EDTA and BHT				
10, 20	1127 ± 21.4	1.9	1318 ± 22.4	1.7

[a] Mean of three replicates. [b] Processing of these samples differs in that hexane addition was carried out after initial blending with TCA solution.

To evaluate the precision and accuracy of the method, cow and mackerel muscle tissues and milk samples, fortified with standard MDA solution at different levels, were analyzed according to the procedure. The results based on five independent determinations at each fortification level are summarized in Table 5. The precision was found to be comparable to that reported by Pikul et al. (1989), and the accuracy was quite acceptable. Previously reported aqueous extraction methods state recovery values of 62% (Newburg and Concon, 1980), 69% and 74.7% (Raharjo et al., 1993), 76% and 78% (Raharjo et al., 1992), 83% (Siu and Draper, 1978), 93% (Salih et al., 1987), 94% (Witte et al., 1970; Pikul et al., 1989), 94.8% (Squires, 1990), and 98–100% (Csallany et al., 1984). Different recovery values may be the result of different types of samples or analytical methods, provided that the procedures

Table 5. Precision and Accuracy Data for the Determination of Malondialdehyde in Cow Muscle, Mackerel Tissue, and Milk Samples by Derivative Spectrophotometry

sample	concn of MDA added (ppb)	concn found[a] (ppb ± SD)	RSD (%)	mean recovery (%)
cow muscle	0	88 ± 4.0	4.5	
	647	613 ± 7.3	1.2	81.1 (98.3)[b]
	998	890 ± 23.1	2.6	80.4 (99.5)
	1285	1161 ± 19.9	1.7	83.5 (96.7)
mackerel tissue	0	2093 ± 39.3	1.9	
	364	2373 ± 49.8	2.1	76.9 (95.4)
	715	2620 ± 21.0	0.8	73.5 (97.5)
	1079	2894 ± 33.5	1.1	74.2 (97.9)
milk	0	0		
	349	330 ± 8.6	2.6	94.6 (99.2)
	699	654 ± 12.4	1.9	93.6 (95.6)
	1048	969 ± 17.4	1.8	92.5 (97.4)

[a] Average of five replicates. [b] Values in parentheses represent the recoveries found when unhydrolyzed standard solution (TEP) was used for sample spiking.

used for recovery determination are performed in the same way. In most aqueous extraction methods, however, recovery experiments have been performed by using TEP, the tetraethylacetal of MDA, instead of pure MDA for sample spiking (Witte et al., 1970; Siu and Draper, 1978; Csallany et al., 1984; Pikul et al., 1989; Salih et al., 1989), whereas Squires (1990) used pure MDA, as we also did, but added it to an extract of the sample, not to the sample itself (Raharjo et al., 1992, 1993). Considering the high reactivity of pure MDA toward proteins and amino acids (Buttkus, 1967; Crawford et al., 1967; Chio and Tappel, 1969), it seems reasonable that these recovery procedures may not adequately represent the actual status of endogenous MDA in real samples.

To determine whether the loss of the pure MDA added to samples was due to partial binding with sample constituents or to incomplete extraction, all samples were further fortified with TEP and resubmitted to analysis. The recovery values found were all higher than 95.4% (Table 5, values in parentheses). Similar results were obtained when standard MDA solutions were submitted to analysis through the whole procedure, a finding suggesting that incomplete extraction does not occur.

Since Kwon et al. (1965) have specified that heating is needed to release MDA from its bound forms, additional research was also directed toward examining whether heating could eliminate the binding of MDA to sample proteins. In this experiment, BSA samples fortified with pure MDA were either vortexed with 5% TCA and then directly heated with TBA at 100 °C for 15 min or analyzed according to the proposed procedure. The recoveries found were in the ranges 47.2–55.3% for the former samples and 86.4–89.1% for the latter ones. These results indicate that heating does not promote the release of MDA from its bound forms but may cause further binding, a process which may account for the

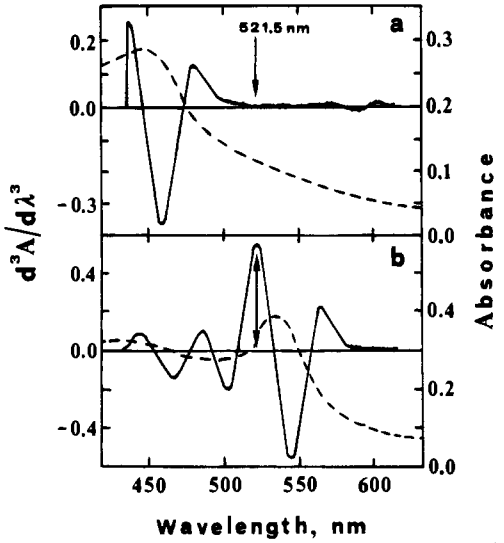

Figure 2. Representative normal (- - -, right *Y*-axis) and third-derivative (—, left *Y*-axis) spectra of sample extracts: (a) milk not containing MDA; (b) fish meal containing 623 ppb of MDA.

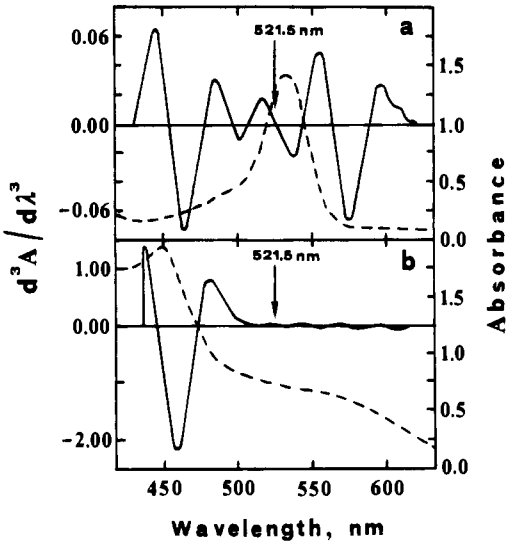

Figure 3. Normal (- - -, right *Y*-axis) and third-derivative (—, left *Y*-axis) spectra of reaction mixtures containing 15 μg of bilirubin (a) and 11 mg of sucrose (b).

lower recoveries reported for the direct heating (Raharjo et al., 1993) and distillation methods (Siu and Draper, 1978) in comparison to our method.

Figure 2 illustrates normal and third-derivative spectra of milk and fish meal samples. A secondary absorption band at around 451 nm appears in the normal spectra of both samples, with different intensities. The compound producing this absorbance was at such a high concentration that the initially colorless milk extracts turned deep yellow after the TBA reaction. Appearance of such an absorption band has been previously reported for many products (Tarladgis et al., 1964; Crackel, 1986). Several compounds may cause this secondary peak and include carbohydrates, furfural, (hydroxymethyl)furfural, alkenals, alkadienals and other aldehydes and ketones (Marcuse and Johansson, 1973; Sinnhuber and Yu, 1977; Pryor, 1980). The appearance of this interfering absorption band substantiates the thesis, advanced by Igene et al. (1985) and supported by Salih et al. (1987), that all spectrophotometric acid extraction TBA methods should only be used if the compounds producing this secondary band are absent or present only in small quantities that do not overlap the main MDA–TBA absorption band at 532 nm. Figure 2 shows, however, that such a limitation does not exist when normal spectra are submitted to derivative processing. Any substance that produces a constant background absorption or a gradual background variation can be normalized by the third-derivative function, which takes zero value.

Since there may have been additional compounds interfering with the TBA reaction, an interference test was also carried out. Various compounds with known interfering action on the spectrophotometric assay such as sucrose, fructose, ribose, cystine, formaldehyde, acetaldehyde, glyceraldehyde, and *N*-acetylneuraminic acid (Shin et al., 1972; Patton, 1973; Cordis et al., 1993) were tested and found to have no interfering action on the derivative assay. Bilirubin could react with TBA to produce a red pigment (Figure 3), but this interference was practically eliminated by the hexane partitioning and derivative processing. Red pigments with absorption spectrum and HPLC retention time similar to those of the MDA–TBA complex may also be produced by the reaction of TBA with alkanals, alkenals, and alkadienals (Marcuse and Johansson, 1973; Pryor, 1980; Esterbauer, 1982; Yu et al., 1986; Kosugi et al., 1987, 1989). However, the identity of these red pigments has not been verified. The extent to which these aldehydes contribute to the red color formation in the TBA test is also not known, but the red color yielded from these aldehydes is generally much lower than that from MDA (Kosugi et al., 1987).

The applicability of the method in various tissue, food, and feedstuff samples is shown in Table 6. Values of MDA as determined by the derivative method were far below the values found when samples were reanalyzed according to the method of Witte et al. (1970). To assess this inconsistency, all final reaction mixtures taken by the method of Witte et al. (1970) were further submitted to derivative processing and evaluation. The difference between these results and those of our method does point out that the protective action of BHT is against lipid peroxidation for the various types of products. Thus, for animal tissues the antioxidant effect of BHT was rather low, while for fish tissues it markedly increased due, obviously, to the high levels of polyunsaturated fatty acids in these samples. On the other hand, the high difference between the derivative and absorbance measurements of all samples processed according to the method of Witte et al. (1970) cannot be considered unexpected when one examines both the coloration and the normal spectra of sample extracts before and after the TBA reaction. Serious interferences due to the presence of colored endogenous compounds were noted in the case of fish tissue and feedstuff samples, whereas interferences due to yellow pigment formation after the TBA reaction also appeared in dairy products.

In conclusion, the results of the present study suggest that the derivative method can be a rapid and efficient alternative to existing methods for estimating the extent of lipid peroxidation in various products. Increased sensitivity and specificity can be readily attained through the use of derivative processing. Specially trained staff are not required, and the equipment needed is easily accessible, as most modern spectrophotometers allow instant generation of derivative spectra. These advantages make the new method particularly useful for routine control. To maximize the safety of using hexane

Table 6. Malondialdehyde Content of Animal Tissue, Food, and Feedstuff Samples As Determined by Different Procedures

	found malondialdehyde concn (ppb)		
	extraction method (Witte et al., 1970)		
sample	absorbance measurement	derivative measurement	derivative method
rat			
muscle	529	64	37
kidney	1416	141	108
cow			
muscle	749	190	138
liver	2081	494	480
pork			
muscle	621	209	201
liver	971	173	138
kidney	870	138	106
chicken			
muscle	726	291	254
liver	687	133	85
lamb			
kidney	638	68	166
fish			
frozen cod	14303	3624	2424
mackerel	19247	6357	2289
sardine	13325	4329	1565
dairy products			
unsweetened condensed milk	782	53	0
infant milk formula	2633	101	0
blue cheese	–[a]	68	27
kasseri cheese	–[a]	40	40
feta cheese	875	146	110
processed cheese	2191	1408	884
coffee cream powder	4578	56	30
butter	–[b]	–[b]	1173
feedstuffs			
fish (herring) meal	33293	2019	1209
fish meal	29475	3268	1012
meat meal	10251	4899	4182
soybean meal	11356	1842	1087

[a] Measurement could not be made because of high turbidity. [b] Butter cannot be analyzed by this method.

in sample treatment, use of an explosion-proof blender and screw-capped centrifuge tubes is highly recommended.

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