An Improved 2-Thiobarbituric Acid (TBA) Procedure for the Measurement of Autoxidation in Fish Oils[1,2]

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Abstract

An improved 2-thiobarbituric acid (TBA) method suitable for routine testing of autoxidative changes of fish oil and other polyunsaturated lipids has been developed. Air oxidation of the lipid during the TBA reaction was found to produce misleading results. The air oxidation may be controlled by addition of antioxidants to the reaction system. Other factors causing inconsistent TBA results and methods of prevention are also described. A comparison of peroxide and TBA values in autoxidized menhaden oil is presented.

Introduction

THE 2-THIOBARBITURIC ACID (TBA) reactive material in autoxidized fat responsible for the red color at 532 mμ has been found to be malonaldehyde (MA) (6,8). The TBA number, or mg of MA per 1000 g of sample, has been proposed by Sinnhuber and Yu (7) to be a measurement of the degree of autoxidation of lipids and of fat-containing foods.

The precision of the method, particularly with oils, has been subjected to question. Schmidt (6) pointed out that for oils the two-phase system of the TBA reaction could lead to errors. Biggs and Bryant (1) suggested the use of emulsifying agents to bring about better mixing of the lipid sample and the TBA reagents. They also emphasized the importance of carrying out the TBA reaction in an atmosphere of nitrogen. DeKoning and Silk (3) reported that reproducible results have been difficult to obtain when various TBA procedures were applied to fish oils and proposed a monophase reaction procedure using ethanol as reaction medium. However, this method required careful and lengthy procedures to prevent interfering color formation from the ethanol.

This paper describes the causes of inconsistent results when the TBA determination is applied to fish oils, the air oxidation of the lipid samples during the reaction, and methods for its prevention. A simplified TBA procedure suitable for routine testing of fish oils and other lipids is also presented.

Experimental

Apparatus

The TBA reaction was carried out in a constant temperature bath using glycerin as the heat transfer medium. The temperature of the bath was maintained at 100 ± 1C. An Active Oxygen Method bath or another of similar design equipped with a rack to hold 20 mm O.D. × 200 mm length test tubes fitted with cold finger condensers would be satisfactory. The reaction may also be carried out, as described previously (9), in a boiling water bath using a 250 ml round-bottom flask fitted with a standard taper West-type condenser.

A Beckman spectrophotometer, DU or DK-1, was used for all absorbancy determinations. Test tubes, 20 × 200 mm, were silicone-coated by the following method: Clean test tubes are filled with about 100 ml of warm dilute Siliclad (Clay-Adams, Inc., New York, N.Y.) solution (prepared by diluting 1 ml of Siliclad concentrate to 100 ml with warm tap water); allowed to stand for 10 sec.; emptied; rinsed immediately with tap water, and then with distilled water. The tubes were dried at room temperature for 24 hr or at 100C for 2 hr.

Reagents

TBA solution: 1 g of TBA is dissolved in 75 ml of 0.1 N NaOH and diluted to 100 ml with distilled water.

Trichloroacetic acid-HCl reagent: 25 ml TCA solution (20%) and 90 ml HCl (0.6 N) are mixed with 105 ml of distilled water.

Hydrochloric acid: 0.6 N.

Antioxidant mixture: 100 mg of trihydroxybutyrophenone (Tennessee Eastman Chemical Products, Inc., Kingsport, Tenn.) is dissolved in 1.800 g of propylene glycol. 100 mg of Tenox VI (Tennessee Eastman) is then added and mixed.

Siliclad: a water-soluble silicone concentrate.

Procedure

TBA Test. About 80–150 mg of fish oil sample is accurately weighed into a coated test tube. The sample is placed in the bottom of the tube so that it will be in contact with the TBA reagent. A medicine dropper has been used to introduce the oil into the test tube (about 4-8 drops) without causing the oil to adhere to the sides of the tube. Five drops of antioxidant mixture (about 0.06-0.08 g, exact weight is not necessary) and 1 ml of HCl (0.6 N) are added and the contents mixed gently. Five ml TBA reagent is then added and the tube is placed without shaking in the constant temperature bath. The cold finger condenser is inserted into the tube immediately. After 30 min, 44 ml of TCA-HCl reagent is added to make the final volume to 50 ml, and heating is continued for an additional 10 min. The tube is then cooled in a water bath, and the solution mixed by inverting once (with a rubber stopper on the tube). Twenty milliliters of the solution is transferred to a glass-stoppered 50 ml conical centrifuge tube. About 5 ml of chloroform is added to the centrifuge tube and the contents shaken a few times so that the residual oil is dissolved in the chloroform. The tube is then centrifuged for 5 min, at 2,000–3,000 rpm. Three milliliters of the clear aqueous acid solution is drawn into a 1 cm cuvet and the absorbance determined in a DU spectrophotometer at 535 mμ. The absorbance of a 1 g sample (in 100 ml reagent) multiplied by the factor 46 is the TBA number (7). A reagent blank is run whenever new supplies of TBA, TCA, antioxidants, etc., are used.

Cleaning of the silicone-coated glassware. After the TBA test, the test tube (or flask) is rinsed with acetone and water. The tube is then placed in a boiling detergent bath containing 100 g Oakite 63 (Oakite

[1] Technical Paper No. 2196, of the Oregon Agricultural Experiment Station.
[2] Presented at the AOCS Meeting in Los Angeles, April, 1966.

Products, Inc., 22 Thames St., New York, N.Y.) and 20 g NaOH (tech. grade) dissolved in 2 liters of water. The tube must be completely immersed in the detergent solution, without any air bubbles trapped inside. After 20 min, the tube is taken out and soaked in dilute acid solution (10 ml concentrated HCl diluted to 1000 ml with water) for 10 min. After being thoroughly rinsed with water, the tube is ready to be recoated with the silicone solution.

Peroxide Value (PV) Determination. The peroxide value of fish oil was determined by the AOCS (5) method with the exception that for highly oxidized oil, the sample size was reduced to about 1 g.

Results and Discussion

The formation of a thin oil film rising on the sides of the reaction vessel may be frequently observed when the TBA reagents are added to an oil sample or after the mixture is heated. This phenomenon was not a uniform occurrence, but is believed to be a major cause of inconsistent TBA results with oil samples, possibly because that part of the oil was not in contact with the reagents; or because, as a thin film, it would be subjected to further autoxidation.

The formation of oil film has been completely prevented by coating the glass surface with silicone. The coated glass surface becomes water repellent. Using coated test tubes or flasks, precise TBA results were obtained on oil samples of different rancidity levels. The results are shown in Table I. The standard deviation from the mean for each oil is generally small.

Air Oxidation During TBA Test

Yu and Sinnhuber (8) have shown that the color density resulting from the reaction of rancid fish meal with TBA reagents was varied with the reflux time (or reaction time). The color increase was greatest during the first 30 min. From then on, the increase was slow but continuous.

The results from the reaction of the TBA reagent with fish oil are definitely different from that with fish meal. Fig. 1, A, shows the effect of reaction time on the TBA number when oxidized salmon oil was reacted with the TBA reagent. The increase in TBA number is almost a linear relation with reaction time, and the curve does not level off after 40 min of reaction. It is believed that air oxidation of the oil sample is taking place during the reaction.

Using salmon oil as a sample, the TBA tests were performed in silicone-coated flasks. Five milliliters of TBA solution and 1 ml of 0.6 N HCl were heated with the oil sample. To flask F-1, N_2 was passed through the top of the condenser for 5 min to displace the air in the flask. The condenser top was then tightly covered with a thin rubber film. To flask F-2, five drops of antioxidant mixture were added

TABLE I

Reproducibility of the TBA Method on Various Oils Using Coated Test Tubes

	TBA Number			
	Soybean oil	Salmon oil	Tuna oil	Menhaden oil
	2.90	191	111	495
	2.16	208	108	485
	2.44	214	130	496
	2.25	203	118	425
	2.30	193	122	505
	2.44	200	114	450
	2.30	191	120	454
	2.16	190		
Mean ± Std. Dev.	2.37 ± 0.224	199 ± 8.42	118 ± 6.86	477 ± 22.09

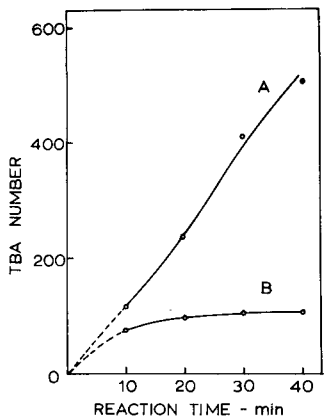

FIG. 1. The effect of antioxidants on TBA reaction time in autoxidized salmon oil.

together with the TBA reagent. Flask F-3 was a control, without N_2 or antioxidants. The results obtained are shown in Table II. It is evident that in the control flask, oxidation of the oil sample occurred. The oxidation of the oil samples was prevented by using either N_2 or antioxidants. Antioxidants appeared to be more effective than N_2.

The experiment was repeated using slightly different and more careful techniques. Menhaden oil was used as the sample. The dissolved oxygen in the oil was removed by subjecting the menhaden oil to a vacuum in a suction flask while a stream of N_2 was bubbled through the oil. After 5 min, the vacuum was released by filling the suction flask with N_2. The oil sample was then accurately weighed into a standard taper three-neck 100 ml RB flask, F-4, fitted with a cooling condenser. Purified N_2 was passed into the flask by means of a glass tubing curved at the tip, and held in place with a rubber stopper. Five milliliters of TBA reagent and 1 ml of 0.6 N HCl were added through the other neck, which was then stoppered with a glass stopper. The tip of glass tubing was then adjusted to about one cm above the reagent. The N_2 was purified by passing it through a wash bottle containing 100 ml alkaline pyrogallate solution and then through a second wash bottle containing distilled water, and finally through a cooling condenser to remove water vapor. After flushing with N_2 for 10 min, the flask was placed, without shaking, in a boiling water bath. After 30 min, 44 ml of TCA-HCl reagent were added through the condenser and the reaction was continued for 10 more minutes. The glass tubing was again adjusted to about 1 cm above the reagents. N_2 was allowed to pass through the flask during the entire course of the reaction. After cooling and clarification of the solution, the TBA number was determined. Flask F-5 was treated exactly the same as F-4 with the exception that five drops of antioxidant mixture were included in the reagents. Flask F-6 served as a control. The results

TABLE II

Air Oxidation of Oil Sample During the TBA Reaction

Experiment no.	Sample	Description of method	TBA number
F-1	Salmon oil	N_2	169
F-2	Salmon oil	Antioxidants	88
F-3	Salmon oil	Control	285
F-4	Menhaden oil	N_2	214
F-5	Menhaden oil	N_2 + antioxidants	202
F-6	Menhaden oil	Control	590
F-7	TEP	Control	10.2
F-8	TEP	Antioxidants	10.3
F-9	Blank	Containing antioxidants	0.14

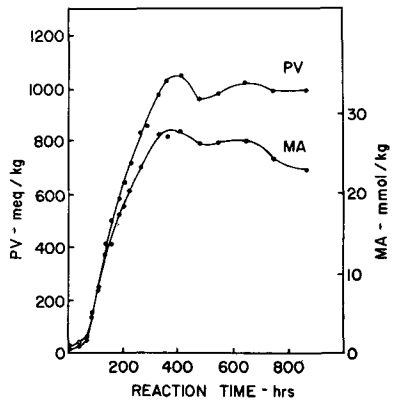

FIG. 2. PV and MA values during autoxidation of menhaden oil.

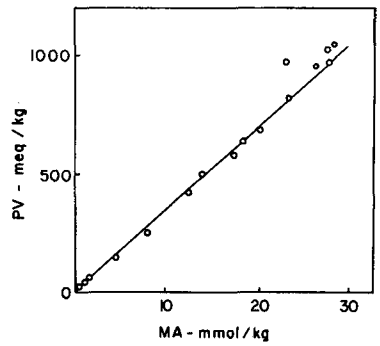

FIG. 3. Relationship of PV to MA values during the autoxidation of menhaden oil.

are also shown in Table II. The high TBA number from the control flask F-6 indicates oxidation of the oil sample. In flask F-4, air oxidation was prevented by careful removal of the air from the reaction flask with purified N_2. The TBA numbers from F-4 and F-5 are very similar. Also included in Table II are the results from the reaction of 1,1,3,3-tetraethoxypropane (TEP) and TBA reagents. To the control flask, F-7, was added 1 ml of TEP solution (approximately 0.0001 M solution) with the TBA reagent. Flask F-8 contained the same quantity of TEP but 5 drops of the antioxidant mixture were added to the TBA reagent. The results show that the addition of the antioxidants to the reaction system does not alter the absorbancy from that of the control TEP sample, which indicates that no reaction between MA and the added antioxidants has occurred. A colorless reagent blank further suggests that antioxidants do not interfere with the TBA reaction.

In the succeeding experiments the antioxidant mixture was chosen in preference to N_2 for preventing air oxidation of the oil samples during TBA reaction. The use of antioxidant is simple and more practical for routine TBA tests.

Based on the above observations, the reaction time experiment with salmon oil as the sample was repeated. Five drops of antioxidant mixture were included in the TBA reagent. The results are shown in Fig. 1, B. The curve starts to level off after 30 min of reaction. Clearly, the reaction is essentially complete after 30 min, and no further oxidation of the oil takes place.

Effect of Reagent pH and Reagent Volume

Other factors influencing the TBA results were investigated using oxidized salmon oil as a sample. When other conditions, such as heating time and reagent volume, were kept constant, changes in reagent pH would alter the results of the TBA tests. These results are shown in Table III. The effect of pH is greatly enhanced if no antioxidants are included in the reaction system. It appears that at certain pH values, air oxidation of the oil sample takes place during the reaction. With added antioxidants in the reagent, the influence of pH is minimized.

TABLE III

Effect of Reagent pH and Reagent Volume on the TBA Results of Salmon Oil

pH	Reagent volume (ml)	TBA number	
		With antioxidant	Without antioxidant
0.40	6	60.6	91.5
0.85	6	69.4	174.0
1.50	6	67.9	189.0
2.20	6	67.3	136.0
1.50	12	48.8	225.0
1.50	25	48.3	353.0
1.50	50	29.0	226.0

Table III also shows the effect of reagent volume on the TBA results. Five milliliters of TBA solution were used in each test and diluted to proper volume with distilled water and HCl. The reagents were adjusted to pH 1.5 by addition of HCl. Without antioxidants, the TBA numbers increased with increasing reagent volume up to 25 ml. With added antioxidants, air oxidation is prevented, and the smallest volume, 6 ml, gives the highest TBA number.

Peroxide Value and Malonaldehyde Content

One liter of fresh menhaden oil was autoxidized in a 2.5 liter bottle by passing air through the oil at room temperature (70–75F). Oil samples were taken out at frequent intervals for PV and TBA tests. It is desirable to compare the changes of the PV and the TBA results in molar basis. TBA values, in this case, are expressed as mmole of MA per kg of oil. The results are shown in Fig. 2. Both PV and MA showed a steady increase up to 350 hr of oxidation, and then both started to level off. The PV and MA are in linear relationship up to PV of 800 (Fig. 3). Kenaston et al., (4) reported that TBA reaction paralleled PV during autoxidation of linolenate. Dahle et al. (2) confirmed Kenaston's finding by demonstrating the linear relationship between TBA and oxygen uptake for triene, tetraene, pentaene and hexaene esters. It is of interest to notice that linearity also exists between the PV and MA in a complex system like fish oil.

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