Further Observations on the 2-Thiobarbituric Acid Method for the Measurement of Oxidative Rancidity[1]

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Abstract

The validity of the 2-thiobarbituric acid (TBA) procedure for the measurement of oxidative rancidity by the determination of malonaldehyde (MA) as the red pigment with an absorption max at 532 mμ has been questioned [J. Am. Oil Chem. Soc. *39*, 34 (1962)]. Side reactions were reported to occur yielding degradation of TBA to products which absorb at the same wave length as the TBA-MA complex and at 450 mμ.

Results reported in the present paper stress the importance of reagent purification. Little or no decomposition of TBA to produce interfering colors was found after heating with acids, oxidizing agents or hydroperoxides.

Introduction

THE 2-thiobarbituric acid (TBA) method for the measurement of fat autoxidation has been widely used to determine the extent of lipid peroxidation in foods and animals tissues. It has been established by Sinnhuber et al. (6) and confirmed by Schmidt (4) that the red color complex responsible for the absorption max at 532–535 mμ is due to the condensation of two molecules of TBA with one of malonaldehyde (MA). MA originates from the autoxidation of certain unsaturated lipids and according to a recent paper by Dahle et al. (1) must come from the oxidation of polyunsaturated fatty acids other than linoleic. This highly reactive dicarbonyl, MA, exists to a very limited extent as the free compound even in highly oxidized fat (2,6). The decomposition of hydroperoxides or MA derivatives by the acid TBA reagent gives rise to MA which condenses with TBA.

The presence of certain materials such as sugars and certain other carbonyls may yield yellow interfering pigments with absorption max in the range of 450–490 mμ. Yu and Sinnhuber (10) recently described a procedure permitting the separation and purification of the TBA-MA complex in food samples which yields interfering pigments. A corrected MA value may then be obtained.

Tarladgis et al. (7) reported that the TBA reagent, used by many investigators, when heated in the presence of acids, decomposed to give side reactions that would interfere or give misleading results in the TBA-MA reaction.

In the interest of this procedure which has come to be widely used in lipid oxidation studies, the TBA reagents as well as procedures have been critically examined. Experimental data are presented which attempt to explain the causes of the reported side reactions (7).

Methods and Results

TBA Reagents. Tarladgis et al. (7) reported that the TBA test as conducted by various workers, which involves the heating of TBA in various acid solutions, often leads to the development of two pigments with absorption max at 450 and 532 mμ. These pigments, the authors stated, were due to the partial breakdown of TBA and cautioned that blanks were imperative to achieve quantitative results. Although we routinely run blank determinations, we have only on rare occasions found high reagent blank values, which were usually the result of contaminated reagents. For example, pyridine used in our original procedure must be carefully purified by refluxing with TBA and redistilled before use (10). This reagent is no longer used in our TBA method.

The procedures investigated by Tarladgis et al. (7) which include the following: a) HCl method; Sinnhuber and Yu (5); b) Trichloroacetic acid; Wilbur et al. (8); and c) Acetic acid; Tarladgis et al. (7) were carefully followed and the reagent blanks prepared and heated. The resultant solutions were compared against distilled water in a Beckman DK-1 recording spectrophotometer. It is evident from the results shown in Figure 1 that all three procedures gave low optical density values at 450 and 532 mμ with the slight exception that the acetic acid reagent diluted 1:1 gave a slight peak at 450 mμ. These results contradict the high values formerly reported (7).

In seeking an explanation for the lower values shown in Figure 1 than those obtained by Tarladgis et al. (7), one might assume that these investigators used undiluted TBA reagents (acids) in their work. The curve "a" in Figure 2 shows the result obtained when 0.02 mole TBA in 90% acetic acid (HOAc) was heated in a boiling water bath for 35 min. In this instance no dilution was made. Although the results (Fig. 2a) show higher max at 532 and 450 mμ, the magnitude of the absorption is considerably less than that previously reported (7).

A possible source of the color interference was first thought to be due to an impure lot of TBA. Four different lots were checked and the results were similar to that shown in Figure 2a. However, in every case, when dilute HCl was used with the various TBA sam-

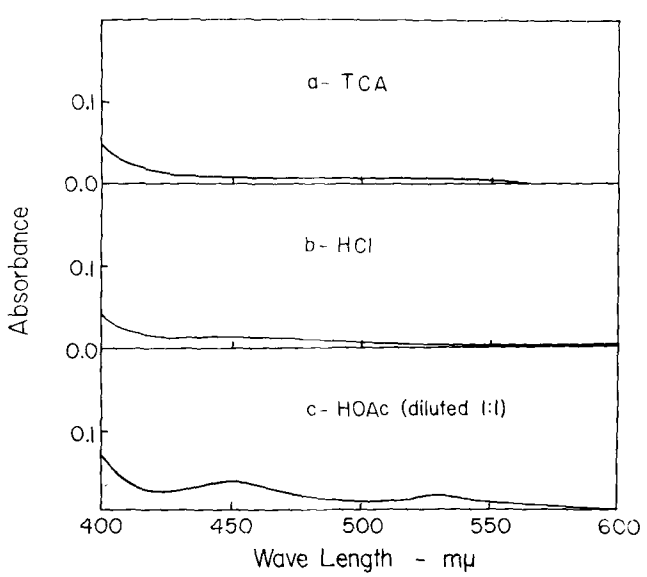

FIG. 1. Absorption spectra of various TBA reagent blanks.

[1] Technical Paper No. 1669, of the Oregon Agricultural Experiment Station. Research supported in part by the National Institutes of Health. RG 7006.

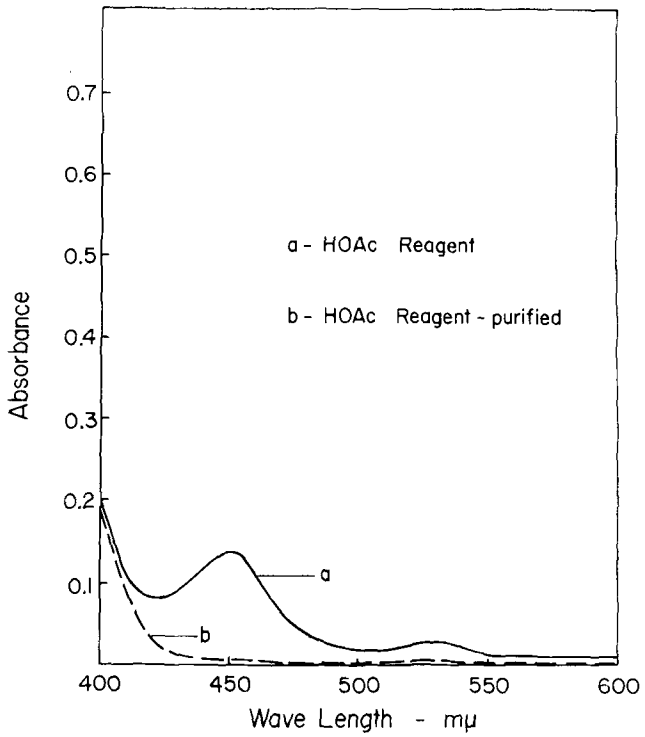

FIG. 2. Effect of purification of HOAc on absorption spectra of heated TBA reagent.

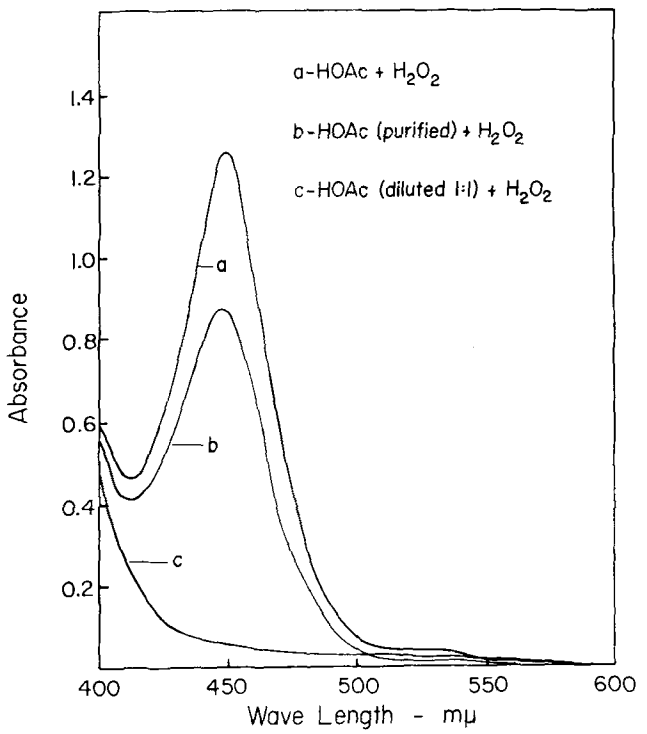

FIG. 3. Effect of H_2O_2 on absorption spectra of HOAc reagent.

ples instead of HOAc, no yellow or interfering color was obtained. It was therefore concluded that the high blanks were probably not due to the TBA, but perhaps originated with the HOAc.

The same reagent grade, glacial HOAc, used in curve "a," Figure 1, was treated by refluxing 100 ml with 2 g TBA for 3 hr. The HOAc was distilled and the purified HOAc used to prepare the Tarladgis reagent (0.02 M TBA in 90%HOAc). A heating test gave the results shown in Figure 2b, in which a very low blank was obtained with purified HOAc. Purification of HOAc was also achieved by refluxing HOAc with 2,4-dinitrophenylhydrazine or with potassium dichromate followed by distillation.

A new bottle of reagent grade, cp glacial acetic acid, was used in another test. Using the same procedure (7), an optical density of 0.450 at 450 mμ was obtained which was 3 times higher than reported in Figure 2a. Another sample of glacial acetic acid which was labeled "to conform to the dichromate test" was also used. In this case, the optical density values at 450 and 532 mμ were 0.022 and 0.005, respectively. This lot of HOAc was considered essentially free of interfering substances.

These results indicated that even reagent grade HOAc may contain unknown substances (possibly carbonyls) which produce interfering pigments when heated in a concn form with TBA. No evidence was found that the structure of TBA was altered by heating in dilute acid. Tarladgis et al. (7) used HOAc extensively throughout their work, as a solvent for the TBA reagent and as an elutant for column chromatography. It seems reasonable to assume interfering substances were introduced by the HOAc used.

Effect of Acid, Heat and Hydrogen Peroxide on TBA Reagents. Tarladgis et al. (7) reported that lower yields of TBA-MA color complex resulted when the reaction occurred in various acid solutions. For example, they obtained only an 81% yield of pigment from the tetraethoxypropane (TEP) when the reaction took place in 12% HCl. This acid concn was assumed by them to be the same concn that was used by Yu and Sinnhuber (9). The concn of HCl actually used in this work was 0.6 N and the molecular extinction coefficient reported was 1.56×10^5 (6) which was higher than the higest value given by Tarladgis et al. (7).

According to the results presented by Tarladgis et al. (7) the heating of TBA with HOAc for 35 min in the presence of 5×10^{-5} mole H_2O_2 magnified the color complexes at 450 and 532 mμ by many fold. This might then imply that in the presence of peroxides, even fat hydroperoxides, that MA and/or artifacts would be produced from the TBA reagent.

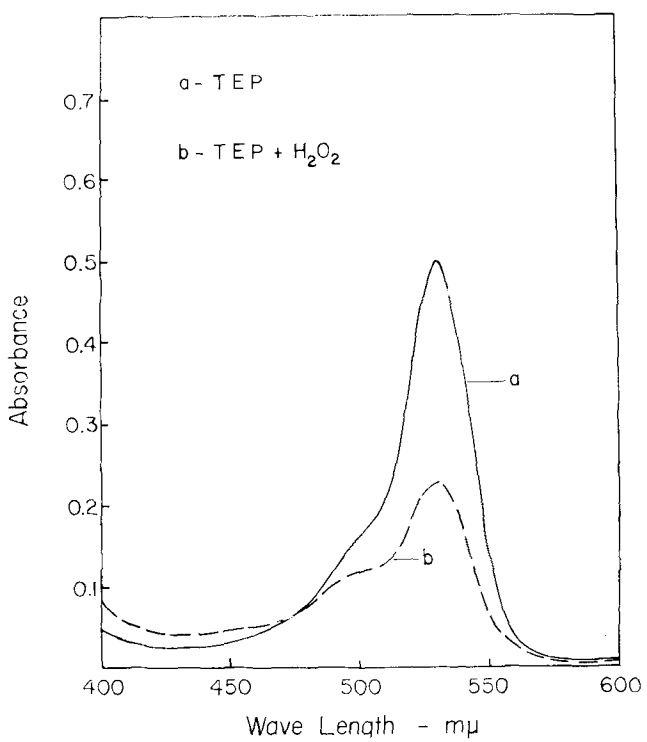

FIG. 4. Effect of H_2O_2 on the TEP-TBA reaction.

TABLE I
Effect of *t*-Butyl Hydroperoxide on the Absorption Spectra of Malonaldehyde TBA Complex

HCl—TBA reagent	Optical density	
	532 mμ	450 mμ
Reagent blank	0.004	0.007
t-Butyl hydroperoxide (5×10^{-5} M)	0.010	0.012
TEP solution[a] (3 ml)	0.490	0.018
TEP solution[a] (3 ml) + *t*-butyl hydroperoxide (5×10^{-5} M)	0.415	0.034

[a] Equivalent to 0.0001 M MA.

Both purified and unpurified HOAc were heated with 0.02 mole TBA solution in the presence of 5×10^{-5} mole H_2O_2 for 35 min. Results show in Figure 3a and 3b. It can be seen that the addition of H_2O_2 caused an increase at 450 mμ in both cases. However, the effect on MA peak at 532 mμ was relatively slight. If the TBA reagent was diluted 1:1 with distilled water before the addition of H_2O_2 and heating, both the 450 and 532 mμ peaks were substantially smaller as shown in Figure 3c. It is apparent that H_2O_2 will react with the TBA reagent if the conen of HOAc is sufficiently high, i.e. 90%, but not at lower acid conen.

Effect of Hydrogen Peroxide and t-Butyl Hydroperoxide on Malonaldehyde. An experiment was conducted to determine the effect of added H_2O_2 on MA. TEP, which on acid hydrolysis yields MA, was used to prepare a solution equivalent to approx 0.0001 mole MA. The TBA reagent was prepared by dissolving 1 g TBA in 75 ml 0.1 N NaOH (without heating). The solution was made up to 100 ml with distilled water. Four ml of this reagent, 6 ml water, 5 ml HCl (0.6 N) and 10 ml trichloroacetic acid (20%) were added to a 250-ml flask containing 3 ml of the above TEP solution. The contents were heated under reflux condenser in a boiling water bath for 30 min. Then 75 ml HCl (0.6 N) was added through the top of the condenser to make a total volume of 100 ml. The heating was continued 10 more min. The resulting solution was cooled, centrifuged and subjected to spectrophotometric analysis. Another flask containing the same quantity of TEP and reagent with added 5×10^{-5} mole H_2O_2 was refluxed in the same manner. The results in Figure 4 show that the added H_2O_2 has lowered the peak at 532 mμ to ca. 50% of the TEP sample without added H_2O_2. One might assume that H_2O_2 could oxidize malonaldehyde to malonic acid, and this could account for the lower values.

The experiment was repeated using *t*-butyl hydroperoxide as a source of organic hydroperoxide. The results in Table I show that when *t*-butyl hydroperoxide (5×10^{-5} mole) was added to TBA reagent along with the TEP solution, the peak at 532 mμ was decreased ca. 15% of that without added *t*-butyl hydroperoxide. These results revealed that added *t*-butyl hydroperoxide has much less influence on the TBA reaction system than equal moles of added H_2O_2.

Column Chromatography. Tarladgis et al. (7) reported the formation of a number of colored complexes when TBA reagents were passed through a cellulose column that had been treated with isoamyl alcohol and HOAc. Experiments reported in this paper confirm these results. The reason for the development of the yellow color is believed to originate from trace materials in the isoamyl alcohol and HOAc. These impurities could be removed by refluxing the reagents with TBA and redistilling. Cellulose powder was also found to contribute to the yellow color. Complete removal of the interfering substance from cellulose was not entirely successful and the nature of the material is unknown. It is possible that trace pentosans may be present and have reacted with TBA. A series of experiments were conducted to investigate this color formation and to seek corrective measures.

The column was prepared as indicated by Tarladgis (7); 1.50 g cellulose powder (chromatographic grade) was packed into a column. Five ml isoamyl alcohol-HOAc (98:2 v/v, reagent grade) were then added to the column. The excess alcohol was removed with the aid of suction. Two ml colorless 1% TBA in 10% HOAc were then added to the column and eluted with 10% HOAc. The elutent was collected in a 10-ml flask. The elutent formed two layers; isoamyl alcohol, the top, and HOAc, the bottom layer. The top layer was carefully transferred to another container. Both layers were yellow in color and the color became more intensified and some pink color developed after standing at room temp for 24 hr or longer as described by Tarladgis. Table II shows the color intensity of the eluted isoamyl alcohol layer and HOAc layer immediately after elution.

The above experiment was repeated, except that isoamyl alcohol and HOAc were purified by refluxing with TBA and dedistilling. The results also show in Table II. During the purification process, isoamyl alcohol developed a deep yellow color with TBA. It has been been reported (3) that certain alcohols reacted with TBA to produce yellow and red pigments.

The above experiment demonstrated that colorless TBA solution when put through cellulose powder column together with unpurified reagents had developed a yellow color in both the eluted isoamyl alcohol and HOAc layers. The cause for the development of yellow color is mainly attributed to the impurities in the isoamyl alcohol and HOAc. These impurities could be removed by refluxing the reagents with TBA and redistilling. Cellulose powder is another source of interfering substances. Washing the cellulose powder column before starting the experiment lowered the OD values. The washing process was carried out by passing through the cellulose column 20 ml 0.5% TBA in 10% HOAc, then successively 10 ml each 10% HOAc and distilled water.

TABLE II
Reaction of Chromatographic Reagents with TBA

Reagents[a]	Freshly eluted				After standing			
	450 mμ		532 mμ		450 mμ		532 mμ	
	Alc layer	HOAc layer	Alc layer	HOAc layer	Alc layer	HOAc layer	Alc layer	HOAc layer
	Optical density				Optical density (after 24 hr)			
Unpurified	0.210	0.086	0.009	0.012	1.280	0.232	0.030	0.020
						(after 68 hr)		
					<2	0.290	0.100	0.028
Purified HOAc	0.150	0.088	0.012	0.008	.....	.....	.....	.....
+ Purified isoamyl alcohol	0.093	0.098	0.010	0.008	.....	.....	.....	.....
+ Washed cellulose	0.060	0.086	0.004	0.006	.....	.....	.....	.....

[a] Reagents include: HOAc, isoamyl alcohol and cellulose powder.

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[Received July 9, 1963—Accepted April 9, 1964]

Detergent Effects of Ultrasonics

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Abstract

The effect of ultrasonic treatment using 300 and 650 kc fields, respectively, on the detergent power of five products has been investigated. Factorial experiments were devised varying the different parameters such as textile fibers or fabric, intensity of the field, nature and concn of the detergent and length of treatment. The ultrasonic treatment proved highly efficient compared to the laboratory washing machine.

Introduction

SOME EFFECTS of ultrasonic energy on detergency have been reported in the past (1–5). The object of this investigation was not so much to establish whether such treatment was commercially feasible but to shed some light on the still elusive details of detergent action.

Apparatus and Technique. In order to study dirt removal from fibers the chopped fiber technique of Powney & Feuell (6) was used, the fibers being soiled with the mixture described by Wagg (7).

For ordinary washing experiments the laboratory washing machine (6), henceforth to be called "posser" was employed. In case of subsequent ultrasonic treatment, wool and cotton, but not viscose fibers, had to be broken up in the posser (pre-possing) by treating 0.5–2.5 g fibers with 400 ml water for 10 min at room temp in the posser. This pre-possing, as will be shown, has no washing effect. For ultrasonic treatment a gentle stirring in the detergent solution, while the field is on, is necessary. As will be shown, this has only a negligible washing effect. After treatment the fibers were rinsed in water. The reflectances of the pads of washed fibers were measured by means of an EEL P.R.S. reflectometer, using a porcelain tile as standard. The reflectances are then expressed as percentage of this standard, which itself has 81% of the reflectance of MgO. It will be shown that the temp dependence of the washing effect is negligible so long as the Krafft point is exceeded. This last condition affects high titer (H.T.) soap only. For ultrasonic treatment, the initial temp was that of the room (for H.T. soap it was 42C), rising as the treatment proceeded.

The ultrasonic energy was generated by either a 300 or 650-kc quartz crystal of 5 cm diam, radiating vertically into a vessel containing the aqueous detergent solution. The total ultrasonic power has been measured calorimetrically (9,10) by radiation pressure (11–13), by the height of the fountain (24), with an ultrasonic proble, by the hot wire method (14–18) and by the heat adsorption method (8). The most reliable and most reproducible method, albeit one giving an upper value owing to some dielectric heating, was the calorimetric method.

TABLE I
Reflectances
Initial Values

Fibers	Soiled	Unsoiled
Wool	32	85
Viscose	32	102
Cotton	36	105

Experimental

Shown in Tables I and II are data for the reflectance values of the fibers, and the possing or mechanical washing of them.

Data for the necessary gentle stirring of the fibers shows in Table III, both Tables II and III showing the effect of no detergent and of mechanical action on the fibers.

Detergent solution used:

1) 0.2% sodium oleate solution by itself.
2) 0.1% H.T. soap solution containing 0.15% sodium metasilicate.
3) 0.1% Lissapol N solution containing 0.15% sodium metasilicate and 0.0015% sodium carboxymethyl cellulose (CMC).
4) 0.1% Santomerse solution containing the same admixtures as 3).
5) 0.1% Teepol solution containing the same admixtures as 3) and 4).

Percentages are calculated on the commercial product. H.T. soap contains 90% active compound. For the rest an analysis is given by Wagg (8).

The effect of temp upon soil removal from two fibers washed in the posser shows in Table IV: No temp effect was found.

Cotton Fibers. A statistical evaluation of detergency using cotton fibers was performed. Raw data show in Table V, and the analysis in Table VI.

A three factor experiment with two replications was done. The three factors were: 1) Detergent used at five levels, D1,D2,D3,D4,D5; 2) Length of treatment at two levels (five min and 20 min, respectively, T5, T20); and 3) Kind of treatment at two levels (posser and 30 kc ultrasonics at 30 w power, W_p, W_u). R_1 and R_2 are the replications.

The standard deviation of means of two is 1.36, and the difference of means is 1.92. For significance at the 0.05 level, the means of two reflectances taken at random must differ at least by 4.

TABLE II
Possing of Fibers in 400 ml Water for 10 Min at 20C

Fibers	R (Before)	R (After)
1.25 g Wool	32	33
6.5 g Viscose	32	33
1.5 g Cotton	37	37

No washing effect.