method avoids this drudgery, and makes it feasible to prepare large amounts of the pure acid in good yields from poor starting materials.

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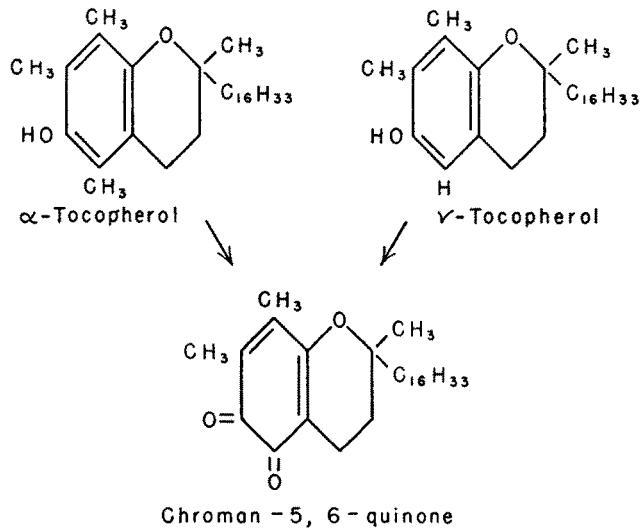
Gamma-Tocopherol as a Precursor of a Red Quinoid Substance Developed in Cottonseed Oil During Oxidation[1]

C. E. SWIFT,[2] G. E. MANN, and G. S. FISHER
Southern Regional Research Laboratory[3]
New Orleans, Louisiana

Introduction

THE development of a red color in cottonseed oil during oxidation was observed by Wheeler (10) and Freyer (3). Golumbic (5,6) reported the recovery, from fresh and incipiently rancid cottonseed oil, of a red, oily, antioxygenic substance, exhibiting the behavior of chroman-5,6-quinones. He concluded that the quinoid substances observed in oxidized fats were products of oxidation of colorless precursors. He stated that "the colorless precursors of these quinoid compounds are not the tocopherols" since "the quinoid compounds were never detected in autoxidizing animal fats or in purified fat substrates containing only added tocopherol." This and supplementary evidence were interpreted as indicating the occurrence of a newly discovered "tocol" constituent in cottonseed oil.

Smith (9) has shown that red chroman-5,6-quinones are formed when 6-hydroxychromans are oxidized with nitric acid. On the basis of the similarity of absorption spectra (1,8), α- and γ-tocopherols presumably form identical chroman-5,6-quinones when oxidized by nitric acid, as indicated in the following formulas:



If the hydrogen occupying the 5-position in γ-tocopherol is more readily removed by oxidation than the methyl group occupying the same position in α-tocopherol, it might be assumed that under milder conditions of oxidation, such as oxidation with air in the presence of a fat, γ-tocopherol but not α-tocopherol, might be oxidized to form a chroman-5,6-quinone. This possibility has apparently not been investigated, or at least has not been reported by Golumbic (5,6). Consequently, an investigation was undertaken to determine the possible role which γ-tocopherol plays in the development of red quinoid color of oxidizing fats. It had been previously observed in connection with other investigations of the mechanism of fat oxidation that cottonseed oil and other fats, when subjected to accelerated oxidation, developed a red color having an absorption maximum at 470 mμ. It was further observed that when γ-tocopherol was added to a fat relatively free of natural antioxidants and aerated at elevated temperature, a similar red color was developed which was also found to have an absorption maximum at 470 mμ. The following experimental work amply substantiates the assumption that γ-tocopherol is actually a precursor in the development of the red color of oxidizing fats in which this substance is present.

Preparation of Reactants and Methods Used

(a) Synthesis of chroman-5,6-quinone: Chroman-5,6-quinone [2,7,8-trimethyl-2-(4',8',12'-trimethyl-tridecyl-1)-chroman-5,6-quinone] was synthesized as follows: To synthetic *d,l*-α-tocopherol [1 gm., Merck, $E^{1\%}_{1\,cm.}$ (292 mμ) = 70] in absolute ethanol (900 ml.), concentrated nitric acid (100 ml.) was added. The mixture was heated at 70° C. until measurements of the spectral absorption at 470 mμ indicated that the concentration of the red product had reached a maximum, which occurred in about 50 minutes. The alcoholic solution of the reaction mixture was poured into 2.5 liters of ice water and the reaction product

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[2] National Cottonseed Products Association Fellow.
[3] This is one of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

was extracted with a mixture of ethyl ether and petroleum ether (b.p. 35-60° C.).

After removal of the mixed solvent, the reaction product was dissolved in petroleum ether and adsorbed on a chromatographic column consisting of silicic acid and Hyflo Supercel (2:1). The chromatogram was developed with petroleum ether and the band containing the red reaction product was extracted with methyl alcohol and acetone. The solvent was removed under vacuum and the residue, a clear red oil (0.26 g.) was used in the following experiments.

A portion of the red, oily reaction product was dissolved in 95 percent ethanol and examined spectrophotometrically using a Beckman photoelectric spectrophotometer. The results of this examination are shown graphically in Figure 1. Maxima occur

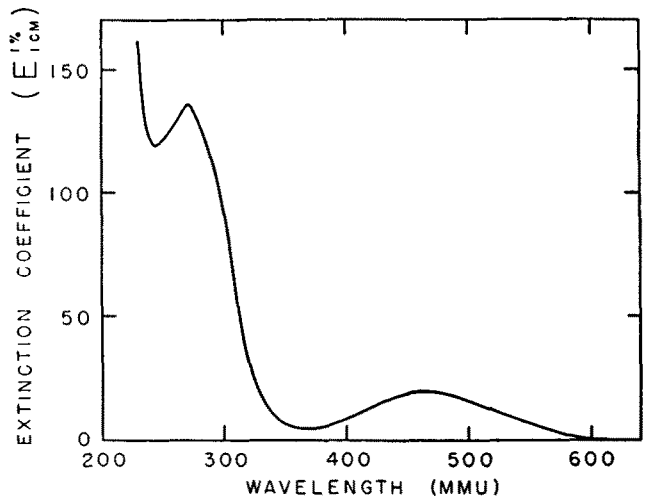

FIG. 1. Spectral absorption of 2,7,8-trimethyl-2-(4',8',12'-trimethyl-tridecyl-1)-chroman-5,6-quinone.

at 270 and 467 mμ and minima at 245 and 366 mμ, the extinction coefficients at the maxima are $E^{1\%}_{1\,cm.}$ (270 mμ) = 136.5 and $E^{1\%}_{1\,cm.}$ (467 mμ) = 20.0. John (7) obtained $E^{1\%}_{1\,cm.}$ (270 mμ) = 96.5 for a similar, though apparently less pure, preparation; the extinction coefficient for the maximum at 467 mμ, $E^{1\%}_{1\,cm.}$ = 20.0, is in good agreement with that reported by Furter (4).

(b) Cottonseed oil substrate: A modified cottonseed oil substrate was prepared using the following procedure: Refined cottonseed oil (750 g.), dissolved in petroleum ether (2,000 ml., 35-60° C.), was percolated through a column of highly activated alumina (1,200 g., Alorco, "chromatographic grade A, minus 80 mesh"). After removing the solvent, the oil was bleached with activated carbon and bleaching earth (Darco and Super Filtrol) and steam deodorized (0.5 hour at 200° C.). As a result of these treatments the characteristics of the cottonseed oil were modified as follows: The content of unsaponifiable matter was reduced from 0.63 to 0.38 percent; the color was reduced (spectral transmission at 470 mμ was increased from 76.8 percent to 100 percent compared with carbon tetrachloride as reference); the tocopherol content was reduced from 0.08 to 0.00 percent and the accelerated keeping time (Swift stability method at 97.8° C.) was decreased from 7.5 hours to 1.5 hours. The iodine value (103.8) and thiocyanogen value (65.6) of the original oil were unaffected by the above-mentioned treatment. The peroxide number of the freshly treated cottonseed oil was found to be 2 milliequivalents per kg. of fat.

(c) Tocopherols: The tocopherols, synthetic *d,l-α*-tocopherol [Merck, $E^{1\%}_{1\,cm.}$ (292 mμ) = 70], synthetic *d,l-β*-tocopherol [Merck, $E^{1\%}_{1\,cm.}$ (296 mμ) = 83.5], and γ-tocopherol [Distillation Products, Inc., $E^{1\%}_{1\,cm.}$ (296 mμ) = 92] were used without further purification since the extinction coefficients of these compounds were found to be in good agreement with those reported by Baxter *et al.* (1) for their highly purified products.

(d) Accelerated oxidation: The general method employed in the experiments to be described was as follows: Twenty-gram portions of fat were aerated in the Swift stability apparatus (oil bath temperature 97.5 ± 0.5° C.) and periodically 2 g. samples were removed for measurement of spectral absorption and determination of peroxide number. The spectral absorption value was first determined on the sample after which the same sample was used for determining the peroxide number.

(e) Spectral absorption: The spectral absorption measurement on the aerated sample of oil was determined with a Coleman DMS spectrophotometer, Model 10-S, using 2 g. of oil dissolved in 5 ml. of a mixture of acetic acid and chloroform (3:2 by volume). The comparison liquid consisted of a similar solution of the unaerated oil. The absorption was measured at 470 mμ, which corresponds to the maximum obtained with purified chroman-5,6-quinone dissolved in 2 g. of unaerated, purified, cottonseed oil substrate made up to volume with acetic acid and chloroform (3:2 by volume).

The concentration of chroman-5,6-quinone was calculated using Beer's Law and the value, $E^{1\%}_{1.27\,cm.}$ (460-470 mμ) = 25.3, obtained from measurements of the purified chroman-5,6-quinone with the Coleman spectrophotometer. The value, $E^{1\%}_{1.27\,cm.}$ (460-470 mμ) = 25.7, obtained with a solution of the purified chroman-5,6-quinone in 95 percent alcohol, is equivalent to $E^{1\%}_{1\,cm.}$ (460-470 mμ) = 20.2, assuming the validity of a conversion using a factor equal to the ratio of the cell lengths (1 to 1.27 cm.). This computed value is in good agreement with the value, $E^{1\%}_{1\,cm.}$ (467 mμ) = 20.0, which was obtained for a similar solution of the chroman-5,6-quinone with a Beckman spectrophotometer.

(f) Peroxide value determination: Following the determination of the absorption at 470 mμ on a sample of oil removed during aeration, the same sample was transferred from the adsorption cell to an Erlenmeyer flask (125 ml. cap.) with the aid of 7 ml. of the same acetic acid-chloroform solvent. A solution of saturated potassium iodide (1.0 ml.) was added to the sample, followed in 2 minutes by 10 ml. of distilled water. The sample was then titrated with N/10 sodium thiosulfate. The peroxide values determined in this manner are expressed in milliequivalents of peroxide per kilogram of fat.

Experimental

THE experimental work comprised a series of five experiments. In the first experiment, α-, β-, and γ-tocopherols were added separately to three portions of the modified cottonseed oil substrate, to the extent of 0.1 percent, after which the mixtures were oxidized by aeration in the Swift stability apparatus, as described above. Samples were removed periodically and their content of chroman-5,6-quinone and peroxide number were determined as previously described. The results are shown graphically in Figure 2.

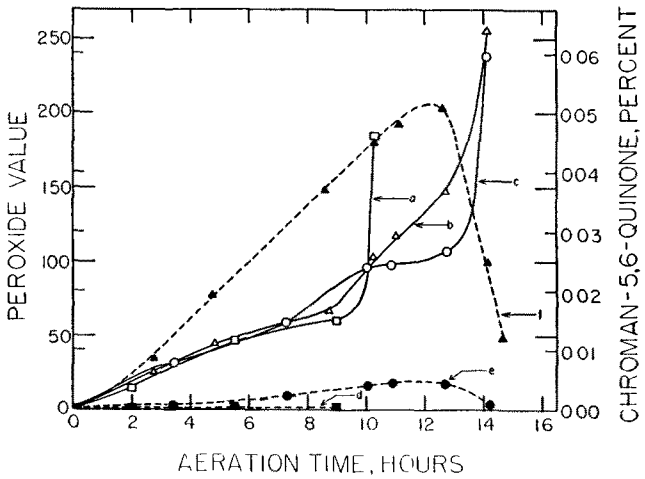

FIG. 2. Peroxide accumulation (curves a, c, and b) and corresponding percentages of chroman-5,6-quinone (curves d, e, and f) produced by aeration of special cottonseed oil substrate containing 0.1 percent of α², β-, or γ-tocopherol, respectively.

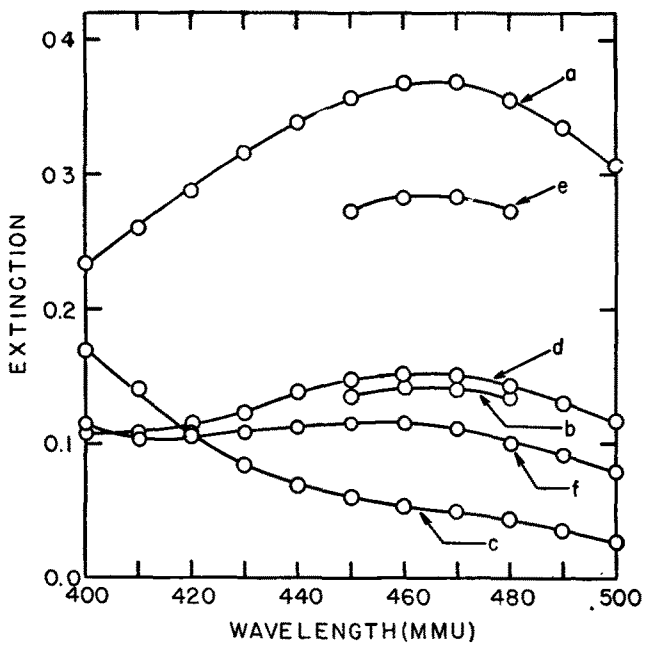

FIG. 3. Spectral absorption curves of various fat substrates.
(a) Cottonseed oil substrate + 0.038% chroman-5,6-quinone.
(b) Same substrate + 0.025% γ-tocopherol, aerated for 9.4 hours.
(c) Same substrate + 0.1% β-tocopherol, aerated for 12 hours.
(d) Refined cottonseed oil, aerated for 6 hours.
(e) Same cottonseed oil + 0.025% γ-tocopherol, aerated for 6 hours.
(f) Prime steam lard + 0.025% γ-tocopherol, aerated for 35.5 hours.

In the second experiment, the spectral absorption maxima were determined in the range 400 to 500 mμ for a series of fat substrates as follows: (a) Specially treated cottonseed oil substrate plus 0.038 percent of chroman-5,6-quinone prepared as described above; (b) same substrate plus 0.025 percent of γ-tocopherol aerated for 9.4 hours; (c) same substrate plus 0.1 percent β-tocopherol, aerated for 12 hours; (d) refined cottonseed oil aerated for 6 hours; (e) same cottonseed oil plus 0.025 percent of γ-tocopherol aerated for 6.6 hours; (f) prime steam lard plus 0.025 percent of γ-tocopherol aerated for 35.5 hours. The variation in aeration times corresponds to the maximum development of red color in each instance. The spectral absorption data for each of the samples are shown graphically in Figure 3.

In the third experiment, a portion of the modified cottonseed oil substrate containing 0.025 percent of γ-tocopherol and a portion of the refined cottonseed oil were oxidized by aeration in the Swift stability apparatus. Samples were withdrawn periodically for determination of spectral absorption and peroxide values. The results are shown graphically in Figure 4.

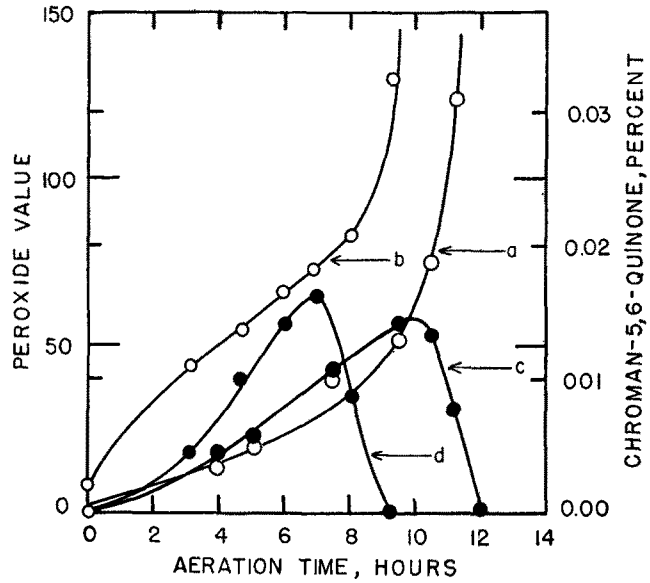

FIG. 4. Peroxide accumulation (curves a and b) and corresponding percentages of chroman-5,6-quinone (curves c and d) produced by aeration at 97.8°C., respectively, of special cottonseed oil substrate containing 0.025 percent of added γ-tocopherol, and of refined cottonseed oil containing 0.024 percent of γ-tocopherol.

In the fourth experiment, a sample of prime steam lard containing 0.025 percent of γ-tocopherol was oxidized and examined as described above. The results are shown graphically in Figure 5.

In the fifth and final experiment, the accelerated keeping times were determined for a series of substrates to which chroman-5,6-quinone had been added in various concentrations. The substrates were as follows: (a) Specially prepared cottonseed oil; (b) refined cottonseed oil; (c) prime steam lard; (d) methyl esters of cottonseed oil fatty acids. The keeping times of the various substrates alone, with added γ- or α-tocopherol (in the case of special cottonseed oil), and with added chroman-5,6-quinone are shown in Table 1.

TABLE 1

Accelerated keeping time in hours of various substrates containing added chroman-5,6-quinone.

Sample No.	Substrate	Added antioxidant	Added chroman-5,6-quinone	Keeping time
			percent	*hours*
1	Special cottonseed oil	None	0	1.5
2	Special cottonseed oil	None	0.025	2.0
3	Special cottonseed oil	γ-Tocopherol, 0.025%	0	8.5
4	Special cottonseed oil	γ-Tocopherol, 0.025%	0.025	9.5
5	Special cottonseed oil	α-Tocopherol, 0.025%	0	7.8
6	Special cottonseed oil	α-Tocopherol, 0.025%	0.025	7.0
7	Refined cottonseed oil	None	0	7.5
8	Refined cottonseed oil	None	0.025	6.0
9	Refined cottonseed oil	None	0.100	6.0
10	Prime steam lard	None	0	2.0
11	Prime steam lard	None	0.025	4.5
12	Methyl esters	None	0	0.75
13	Methyl esters	None	0.25	2.0

Discussion of Results

The results of the aeration experiments shown in Figure 2 indicate that only γ-tocopherol produced an appreciable absorption corresponding to chroman-5,6-quinone when the specially prepared cottonseed oil substrates containing α-, β-, or γ-tocopherol were subjected to aeration and spectral analysis. A relatively small amount of chroman-5,6-quinone appears to have been formed from β-tocopherol, but the spectral absorption (Figures 2e and 3c) of the substrate to which this substance was added is not characteristic of the spectral absorptions obtained with the substrates containing γ-tocopherol (Figures 2f and 3b).

As judged by the spectral absorption data shown in Figure 3, the chroman-5,6-quinone occurring in oxidized, refined cottonseed oil (Figure 3d) and that resulting from the oxidation of γ-tocopherol in the special cottonseed oil substrate (Figure 3b) are indistinguishable, each exhibiting a maximum at 460-470 mμ.

It is apparent from the increased absorption observed with oxidized cottonseed oil containing γ-tocopherol (Figure 3e), compared with oxidized cottonseed oil without addition of γ-tocopherol (Figure 3d), that γ-tocopherol must have been converted in the former case into chroman-5,6-quinone. That the absorption observed in these cases is actually due to chroman-5,6-quinone is evident from the fact that addition of this substance to the unoxidized cottonseed oil substrate gives a spectral curve having an absorption maximum at 460-470 mμ which is identical with that obtained by oxidation of a fat containing γ-tocopherol.

Contrary to the observations and conclusions of Golumbic (5) that "the quinoid compounds were never detected in autoxidizing animal fats or in purified fat substrates containing only added tocopherol," a red colored substance was produced in oxidizing prime steam lard containing added γ-tocopherol (Figure 5). The spectral absorption maximum at 460 mμ (Figure 3f) which was observed in the case of the oxidized lard substrate containing γ-tocopherol is characteristic of the chroman-5,6-quinone observed in all the other related substrates.

Further evidence of the identity and origin of the chroman-5,6-quinone becomes apparent upon consideration of the quantitative aspects of the data reported here. Calculated on the basis of the apparent amount of chroman-5,6-quinone determined spectrophotometrically as shown in Figures 4c, 4d, and 5b, using the ratio of percent chroman-5,6-quinone to percent γ-tocopherol in the fat, 67 percent of the

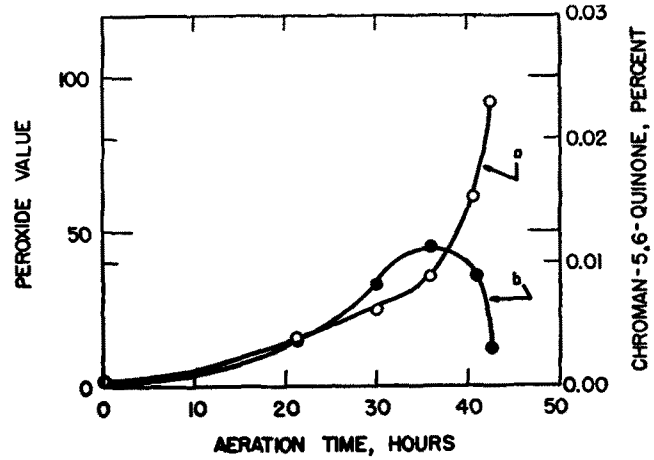

FIG. 5. Peroxide accumulation (curve a) and corresponding percentages of chroman-5,6-quinone produced (curve b) by aeration at 97.8° C. of prime steam lard containing 0.025 percent added γ-tocopherol.

γ-tocopherol existing in the refined cottonseed oil (0.024 percent),[4] 57 percent of the added γ-tocopherol (0.025 percent) in the special cottonseed oil substrate, and 45 percent of the added γ-tocopherol (0.025 percent) in lard was converted to chroman-5,6-quinone.

Since bleaching of the red color occurs on continued aeration of oxidizing fats, it appears logical to assume that decomposition or further oxidation of the chroman-5,6-quinone occurs concurrently with its formation. Therefore, the calculated percentages of conversion of γ-tocopherol to chroman-5,6-quinone may represent minimum values, whereas the conversion may have actually been quantitative.

It is evident from the data in Table 1 that the addition of chroman-5,6-quinone imparts little antioxidant activity to substrates of low stability and is ineffective in relatively stable substrates (cf. ref. 5 and 6).

Summary

On the basis of spectrophotometric data on the characteristic spectral absorptions observed in oxidizing substrates with and without added γ-tocopherol, it has been shown that the red color which develops on accelerated oxidation of fat substrates containing this tocopherol results from its conversion to chroman-5,6-quinone. The chroman-5,6-quinone thus produced possesses relatively little antioxidant activity.

Acknowledgments

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[4] The percentage of γ-tocopherol in the sample of refined cottonseed oil was determined by the recently developed method of Fisher (2).

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