Antioxidant Losses During the Induction Period of Fat Oxidation[1]

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IT is generally agreed that rancidity in fats and fatty oils may be attributed to a series of oxidative reaction chains. Unsaturated constituents of the fat are converted by the process of oxidation to highly active peroxides. The active peroxides possess high energy levels and they are capable of transferring a portion of their energy to unactivated fat molecules, thereby instituting the reaction chains. Such reaction chains result in an accumulation of peroxides which can decompose to the secondary products which characterize rancidity (1, 2).

The role of antioxidants in the stabilization of fats is to inhibit or prevent the institution of oxidative reactions. This can be accomplished if the inhibitor has a greater tendency than the unactivated fat molecules to absorb energy from the active peroxides. Thus, instead of the normal chain of reactions which occurs in the absence of an inhibitor (F = fat substrate; FO_2 = peroxides; * represents activated molecule):

$$F + \text{energy} \longrightarrow F^* \quad (1)$$
$$F^* + O_2 \longrightarrow FO_2^* \quad (2)$$
$$FO_2^* + F \longrightarrow FO_2 + F^* \quad (3)$$
$$F^* + O_2 \longrightarrow FO_2^* \quad (4)$$

the chain is broken by the antioxidant (A) which absorbs the high energy from the activated peroxide

$$FO_2^* + A \longrightarrow FO_2 + A^* \quad (5)$$

and becomes activated itself (A*). The activated antioxidant is subsequently oxidized on collision with molecules of oxygen. Apparently, it is unable to pass on its energy to unactivated fat molecules.

It follows, then, that the concentration of effective antioxidant should show a decrease during the induction period of a fat substrate Golumbic (2) has made a study of the changes in the amount of α-tocopherol and its oxidation products in the process of oxidative rancidification. He found a rapid increase in peroxides only after the α-tocopherol had completely disappeared and the chroman-5,6-quinones had begun to diminish.

It has seemed desirable to extend such studies to other antioxidants and combination of antioxidants in order to obtain a more thorough comprehension of the mechanism of antioxidant action, and ultimately of synergistic action. The present report summarizes our findings for gallic acid and ascorbic acid, used singly and in combination.

Experimental

THE substrates used in these studies were samples of a commercially refined cottonseed oil. In a typical experiment the substrate oil and antioxidant were mixed in the desired proportion in a flask, from which individual samples (approximately 20 ml. each) were poured into 8" x 1" test tubes. An individual sample was not poured until it was time to connect the tube into the oxidation system. Before any sample was poured, the flask containing the oil and antioxidant was shaken vigorously in order to obtain maximum homogenization. As a result, fairly representative sampling was attained.

The individual tubes of oil were oxidized for varying lengths of time under the conditions of the accelerated Swift stability test (3). The tubes were immersed in an oil bath maintained at 110 ± 0.3°C. and aerated by a constant flow of 2.3 ml. of washed air per second. After each sample had been oxidized its prescribed length of time, it was removed from the oxidation system and peroxide values and antioxidant concentrations were determined on representative portions. The induction period was measured as the number of hours of aeration necessary to raise the peroxide value of the sample to 120 milliequivalents of sodium thiosulfate per kilogram of fat.

Peroxide values were measured by a modified Wheeler method (4). A 3 gm. sample was dissolved in 30 ml. of acetic acid-chloroform (3:2) solution, 0.5 ml. of saturated aqueous KI solution was added. The mixture was shaken for 30 seconds, allowed to stand in the dark for two minutes, diluted with 75 ml. of distilled water, and then titrated with standard thiosulfate.

For the determination of the concentration of ascorbic acid in a sample of oil, 5 gm. of the oil were weighed quantitatively into a flask and extracted with 50 ml. of a 3 per cent aqueous solution of metaphosphoric acid at room temperature. The mixture was filtered through a water wetted filter paper, to prevent the passage of the oil. The flask and filter paper were washed with two 25 ml. portions of the metaphosphoric acid solution. The combined extract and washings were then titrated immediately with a standardized solution of sodium 2,6-dichlorphenolindophenol (5).

In connection with the study of ascorbic acid losses, it was considered desirable to gain information on the possible oxidation products. The formation of dehydroascorbic acid by oxidation of ascorbic acid added to an oil as an antioxidant was investigated by an application of the method of Roe (6) which is dependent on the formation of the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid.

COTTONSEED OIL containing 0.03 per cent ascorbic acid was oxidized and the amount of dehydroascorbic acid in the samples was determined as follows:

A sample of fat (2 to 3 g.) was weighed into a flask and extracted with 15 ml. of 0.03 per cent metaphosphoric acid solution. The mixture was filtered through a wetted filter paper into a 25 ml.

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volumetric flask. The flask and filter paper were washed with three portions of metaphosphoric acid solution to bring the final volume of extract and washings to a total volume of 25 ml. An aliquot of this solution (4 ml.) was pipetted into a colorimeter tube and one ml. of 2,4-dinitrophenylhydrazine solution (2 g. 2,4-dinitrophenylhydrazine in 100 ml. of 9 *N* sulfuric acid) was added. One drop of thiourea solution (10 g. in 100 ml. 50 per cent ethyl alcohol) was added and the resulting solutions were incubated for three hours at 37° in an atmosphere of nitrogen. After incubation the tubes were removed and chilled in an ice bath. Concentrated sulfuric acid (5 ml.) was added dropwise while the tubes were agitated and the resulting orange color which developed was measured in the Evelyn photoelectric colorimeter using a 540 mμ filter. The K value used to calculate the amount of dehydroascorbic acid was the same as that experimentally determined by Feng (7) who standardized the method with ascorbic acid solutions oxidized to the dehydro stage by activated charcoal.

The concentrations of gallic acid in the samples of oil were determined by a method developed in this laboratory (8). The method is based on a report by Mitchell (9) of the color reaction of polyphenolic compounds with ferrous tartrate reagent. A weighed sample of oil (5 to 10 g.) was extracted with 65 ml. of distilled water. It was found that a more efficient extraction of gallic acid was obtained when the mixture was heated to boiling. The mixture was again filtered through a water wetted filter paper and the flask and paper were washed with an additional 20 ml. of water added in divided portions. The filtrate was collected in a 100 ml. volumetric flask. Color was developed in the extract by the addition of 2 ml. of an aqueous solution containing 0.1% ferrous sulfate and 0.5% sodium potassium tartrate. The solution was buffered with 10 ml. of 10% ammonium acetate solution. The intensity of the violet color which resulted was determined at 540 mμ by comparison with a blank using an Evelyn photoelectric colorimeter. The concentration of gallic acid in the extract was calculated by the following equation:

$$C = \frac{L}{K}$$

where:

C = concentration of gallic acid in mg. per 100 ml.

K = the experimentally determined constant for the gallic acid originally used (=0.325±.006).

L = 2-log G where G is the galvanometer reading of the sample obtained under conditions such that the galvanometer reading for the reagent blank was 100.

From the concentration of gallic acid in the extract, the concentration of gallic acid in the oil sample can be calculated readily.

The presence of ascorbic acid in a sample of oil interfered to some extent in the determination of other constituents. When the ascorbic acid concentration was fairly high, as in those samples which were only slightly oxidized, it was not possible to determine the peroxide values accurately. The color of the iodine liberated by the peroxide would appear in the organic solvent after the addition of potassium iodide, but when the reaction mixture was diluted with water and shaken, the ascorbic acid present reacted with the iodine. However, after the ascorbic acid concentration decreased, it was always possible to obtain peroxide value for the determination of the length of the induction period.

In the analysis of gallic acid, high concentrations of unoxidized ascorbic acid sometimes caused erratic results which interfered with the full development of the violet color. This error could be corrected by the addition of several drops of concentrated NH_4OH.

TABLE I

Disappearance of Antioxidants During the Development of Oxidative Rancidity of Cottonseed Oil

Initial concentration of antioxidant	Length of induction period*	Time for complete loss of antioxidant
per cent	*hrs.*	*hrs.*
Gallic acid		
0.019	17	12
0.040	19	18
0.046	25	21
0.062	30	25
0.068	32	30
Ascorbic acid		
0.03	10	**
0.06	15	**
Ascorbic acid + gallic acid (G.A.)		
0.03 each	24½	24(G.A.)**
0.06 each	36	36(G.A.)**

* The induction period of the cottonseed oil used was 5 to 6 hours.
** Ascorbic acid decreased, but was never completely lost.

The change of antioxidant concentration during the rancidification of the substrate oils was followed using several different initial concentration levels of the following antioxidant systems: gallic acid, ascorbic acid, and gallic acid with ascorbic acid. The results obtained are shown in Table I. Since the natural inhibitors present in the cottonseed oil were not removed the reported effects are all superimposed on their effect.

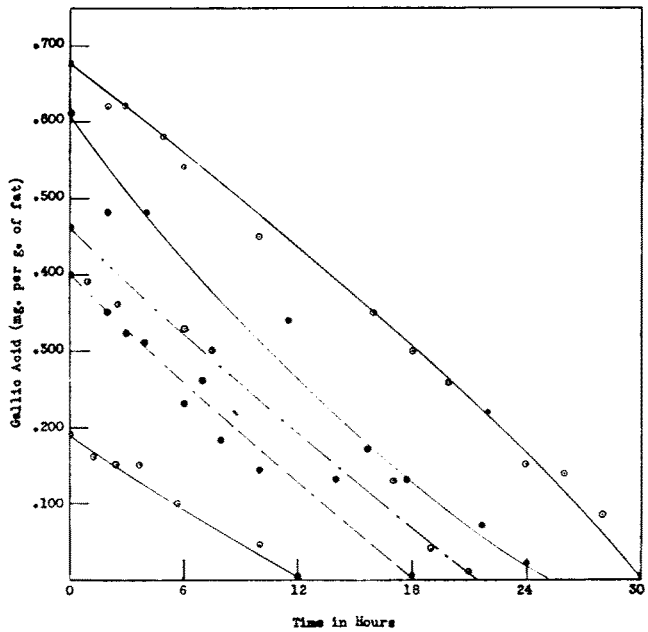

FIG. I. Rate of loss of gallic acid during accelerated autoxidation of cottonseed oil.

Discussion

IT is evident from Table I that both gallic acid and ascorbic acid are effective stabilizers for vegetable oils, as has previously been found in other laboratories (10, 11). When used together their effects appear to be somewhat less than additive.

From Fig. I it is apparent that the rate of loss of gallic acid in an oxidizing oil is fairly constant

(i.e., the slopes of the lines plotted are approximately the same). A sharp rise in peroxide concentration never occurred until some time after the gallic acid had completely disappeared. After the gallic acid was gone, the substrate seemed to proceed along its normal course of rancidification with a peroxide increase comparable to that which would have been obtained with an untreated oil of the same peroxide concentration.

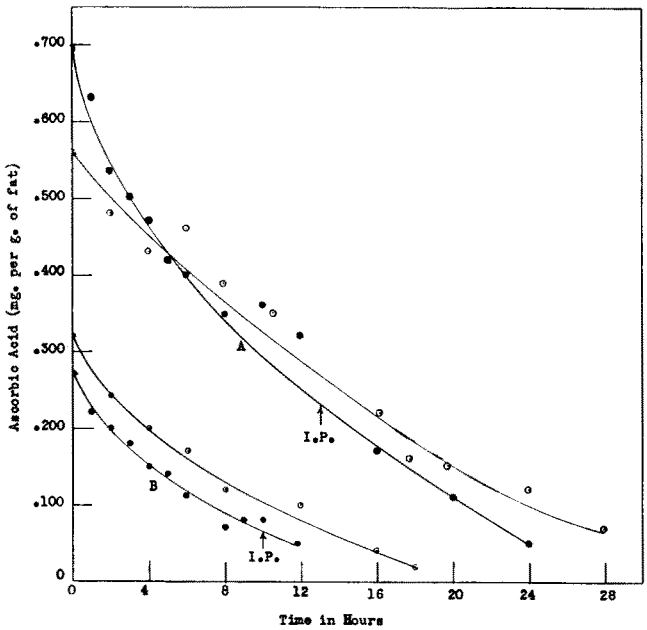

FIG. II. Comparison of the rates of loss of ascorbic acid during fat autoxidation in the presence and absence of gallic acid.
● ascorbic acid alone.
○ ascorbic acid in the presence of an equal concentration of added gallic acid.

In contrast, ascorbic acid disappeared rapidly in the first few hours, and then the loss became more gradual (Fig. II). However, ascorbic acid never completely disappeared from the oils to which it had been added. All the samples which had originally contained ascorbic acid decolorized a measurable quantity of the 2,6-dichlorphenol-indophenol.

It may be observed from Fig. II that a rapid rise in peroxide concentration of the substrate oil could occur while there was still an appreciable amount of ascorbic acid remaining in the oil. In fact, in curve A the rapid rise in peroxide value occurred when the concentration of remaining ascorbic acid was at approximately the same level as the initial concentration, which had shown effective antioxidant properties in curve B. Such apparently contradictory results might be explained if one were to assume that the end products of the oxidation of ascorbic acid

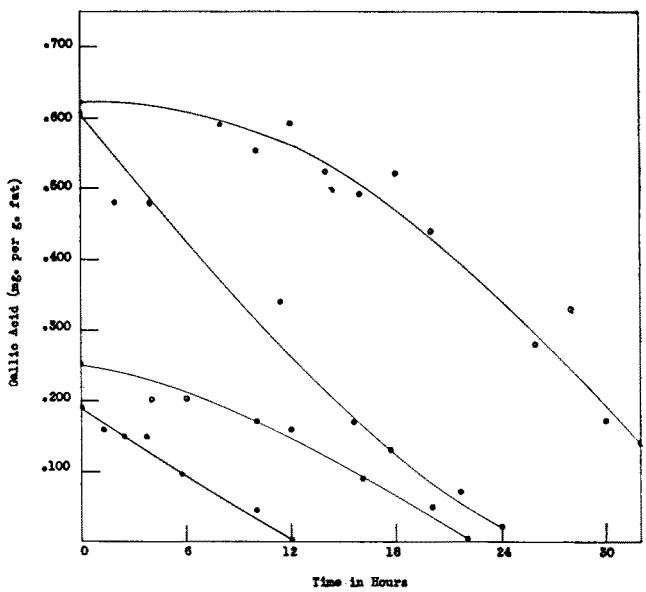

FIG. III. Comparison of the rates of loss of gallic acid during fat autoxidation in the presence and absence of ascorbic acid.
● gallic acid alone.
○ gallic acid in presence of an equal concentration of added ascorbic acid.

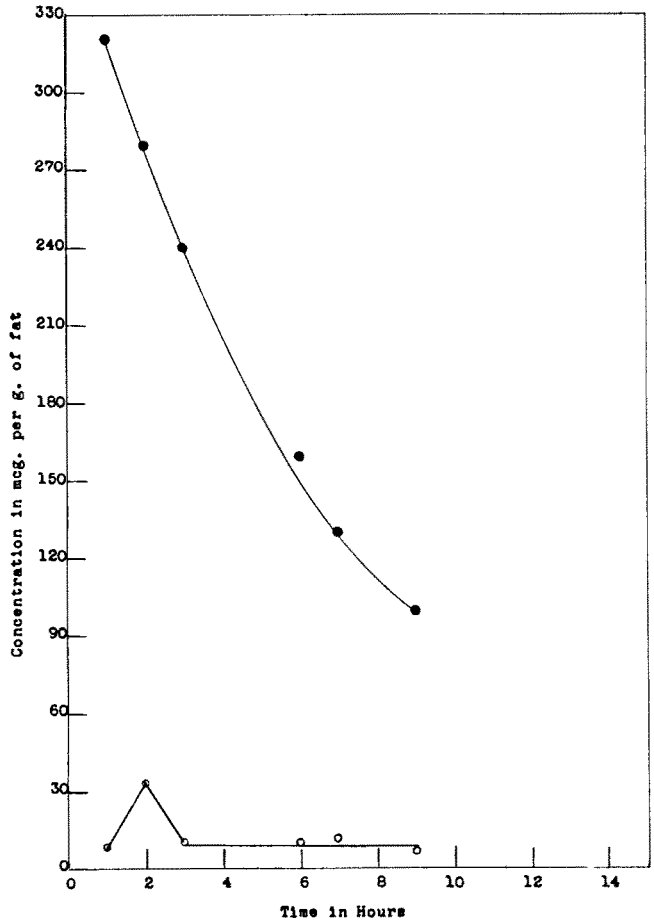

FIG. IV. Rate of disappearance of ascorbic acid and the formation of dehydroascorbic acid in an oxidizing fat.
● ascorbic acid.
○ dehydroascorbic acid.

are relatively pro-oxidant in nature. Thus, as the ratio of ascorbic acid to its oxidation end products decreased, the antioxidant effect decreased and the latter effect may be conceived to have become negative long before the ascorbic acid disappeared. Again, it is conceivable that the reactions between ascorbic acid and its oxidation products are partially reversible, thus accounting for the failure ever to observe a complete loss of ascorbic acid.

When the antioxidant effects of ascorbic acid and gallic acid were combined in a sample of oil, the rates of disappearance of each were decreased (Fig. II and III). In the early stages of the oxidation the loss of gallic acid was less than when it had been

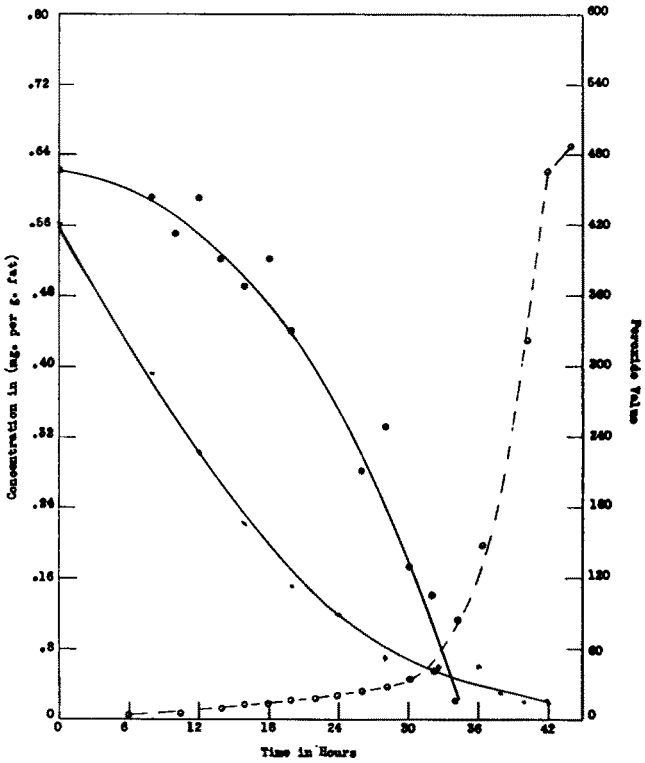

FIG. V. Correlation of antioxidant losses with peroxide formation during fat autoxidation.
× ascorbic acid.
● gallic acid.
○ peroxide value.

used alone. During that period of time ascorbic acid disappeared at approximately its predetermined rate. Later in the oxidation, the gallic acid decomposed more rapidly, while the rate of loss of ascorbic acid decreased.

A preliminary study was made of the nature of the oxidation products of ascorbic acid. After the completion of this investigation, Calkins and Mattill (12) reported similar results. To measure the amount of dehydroascorbic acid they used hydrogen sulfide reduction of dehydroascorbic acid to ascorbic acid and subsequent titration with 2,6-dichlorophenol-indophenol. Dehydroascorbic acid was formed in trace amounts. A more specific test for dehydroascorbic acid is that employed in the method of Roe (6). The latter method used in this work indicated that dehydroascorbic acid was formed in appreciable amounts at the initiation of the oxidation of ascorbic acid. The data are shown graphically in Fig. IV.

Since Rosenfeld (13) had demonstrated that dehydroascorbic acid in aqueous systems formed oxalic acid, the presence of this possible oxidation product was tested for qualitatively. No precipitate was found at pH 5 when a solution of calcium chloride was added to an aqueous extract of an oxidized oil stabilized with ascorbic acid and the solution allowed to stand overnight. Although a higher pH favors the formation of insoluble calcium oxalate the solution was held at pH 5 since a more alkaline solution would have formed oxalic acid due to the destruction of the ascorbic acid present.

The relationship of the rate of loss of the two antioxidants with respect to the length of the induction period is indicated in Fig. V. From an inspection of the curve, it may be observed that the gallic acid had disappeared before the end of the induction period and that the ascorbic acid was still present at the same time.

Summary

GALLIC ACID and ascorbic acid tested alone or in combination as antioxidants for cottonseed oil, disappeared gradually during the induction period under conditions of accelerated autoxidation. By the end of the induction period no gallic acid could be determined in the oxidizing fat substrate. However, ascorbic acid never completely disappeared under the same conditions. When the two substances were used together, no synergistic effect was noted with respect to an increased length of the induction period but the rate of disappearance of the individual compounds was altered somewhat, each substance acting apparently to delay the destruction of the other. The rate of disappearance of gallic acid alone was found to be independent of its initial concentration in the oil substrate.

A study of the possible oxidation products of ascorbic acid undergoing oxidation in an autoxidizing oil showed the formation of dehydroascorbic acid but no formation of oxalic acid.

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