Determination of Tocopherol in Oxidized Fats. Interference from Heat-Formed Reducing Substances in Highly Oxidized Fats

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A SIMPLE METHOD for tocopherol determination in oxidized fats has been reported (2) where interfering peroxides are removed by thermal destruction. This method of heating fats at 210°C. in vacuum for 10–15 min. has been successfully applied to follow the destruction of tocopherols in fats during autoxidation (1, 3). However, when highly oxidized fats were subjected to such heat treatment, the appearance of interfering reducing substances was observed. This paper presents results to indicate that the interference from these reducing substances is negligible within the range of peroxide values (0 to 100) of most interest in investigations in the edible fat field. Evidence is also presented which indicates that the reducing substances are polymeric in nature and are derived from fat peroxides.

Experimental

In studies of the oxidation of tocopherols in fats, samples taken at different oxidation times were heated at 210°C. for 15 min. in vacuum to remove peroxides. The reducing power was measured by the ferrous bipyridine color reaction of Emmerie-Engel (2). In autoxidizing lard, added tocopherol reached a minimum value toward the end of the induction period. When lard was oxidized beyond this point (at peroxide values exceeding 100 by the ferric thiocyanate method [5]) a rise in reducing power was observed which corresponded to the appearance of browning after heating the oxidized samples (Figure 1). To determine whether tocopherols were involved in the formation of these reducing substances, lard and completely stripped soybean oil (treated with carbon-black to remove natural tocopherols [2]) were autoxidized at 100° and 60°, respectively. The reducing power of different samples was determined after heating at 210°C. for 15 min. *in vacuo* to remove peroxides. The results in Figures 2 and 3 show that reducing and browning material is formed in both lard and soybean oil in the absence of tocopherols. Therefore tocopherols are not involved in the formation of these substances. Rather, the reducing substances appear to be derived from the peroxides accumulated in the fats. The concentration of the reducing material does not become significant until the peroxide value exceeds 100.

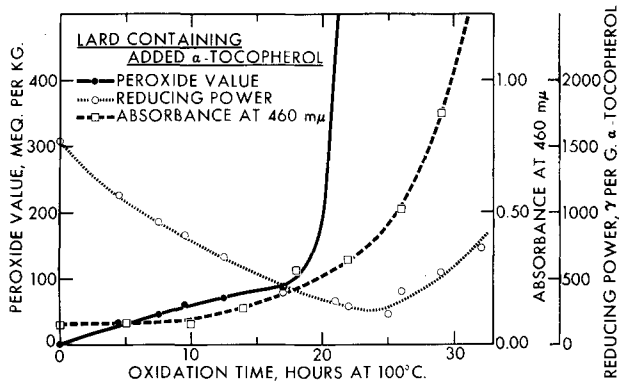

FIG. 1. Relation between reducing and browning material in autoxidizing lard containing 1540 γ/g. α-tocopherol, after heating at 210°C., *in vacuo*, for 15 min.

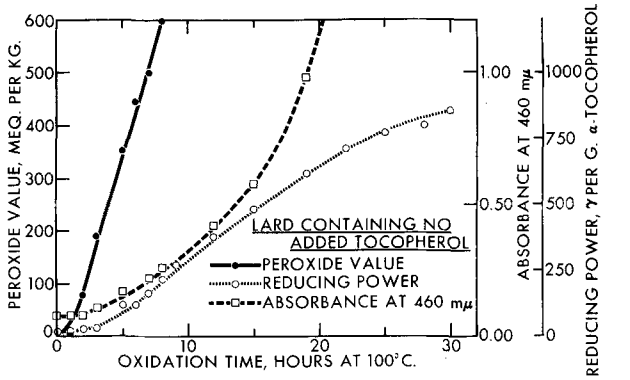

FIG. 2. Development of reducing and browning material in autoxidizing lard containing no added tocopherol after heating 210°C., *in vacuo*, for 15 min.

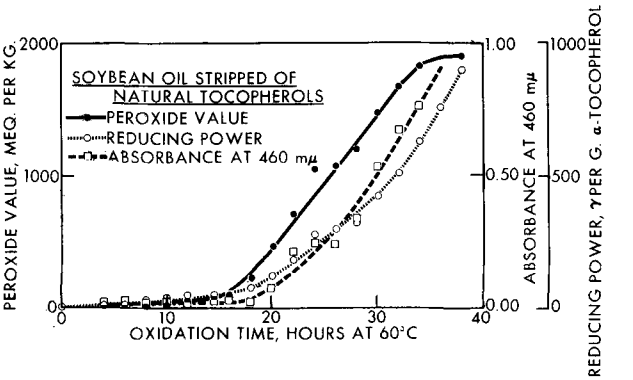

FIG. 3. Development of reducing and browning material in autoxidizing soybean oil, stripped free of natural tocopherols, after heating at 210°C., *in vacuo*, for 15 min.

The heating method (210°C., 15 min., *in vacuo*) was compared to a chromatographic method for removing peroxides prior to the tocopherol determination to verify whether these reducing substances interfere at lower peroxide values. The chromatographic procedure consisted of passing 1.0 g. of oil in 10 cc. of redistilled benzene through a column 20 mm. in diameter containing 1 g. of silicic acid (Mallinckrodt No. 2847, 100 mesh).[1] The column was washed with benzene to collect a total volume of 50.0 ml. of eluate. This procedure yielded recoveries of 96–97% of the tocopherol from fresh soybean oil. The results obtained with soybean oil that was autoxidized at 60°C. in a Warburg apparatus (Table I) show good agreement between tocopherol values obtained in the heated and in the chromatographed

[1] The mention of products does not imply endorsement by the U. S. Department of Agriculture over similar products not mentioned.

TABLE I

Comparison of Heating and Chromatographic Method to Remove Peroxides Prior to Tocopherol Determination

Oxidation[a] time at 60°C.	Peroxide value	Tocopherol		Difference
		Heated	Chromatographed	
hrs.	*me./kg.*	*μg./g.*	*μg./g.*	%
4	0.50	1480	1420	+4.1
8	0.96	1430	1420	+0.7
16	1.44	1470	1360	+7.5
20	1.51	1325	1390	−4.9
24	2.47	1385	1330	+4.0
33	11.1	1315	1370	−4.2
37	14.9	1375	1360	+1.1
41	20.2	1395	1370	+1.8
44	24.2	1350	1280	+5.2
51	25.6	1350	1265	+6.3
55	42.1	1285	1300	−1.2
59	44.3	1305	1260	+3.4
				Av. +2.0

[a] Soybean oil.

samples. The chromatographic method for removal of peroxides is applicable to the determination of tocopherol in the more highly oxidized fats (peroxide values exceeding 100). The method has also been applied satisfactorily to the determination of tocopherol in oxidized methyl esters of fatty acids which are distillable under conditions of the heating method.

A few chemical and physical properties of the reducing material developed in the oxidized fat on heating were studied. A highly oxidized lard was mixed with varying proportions of fresh lard to yield different levels of peroxides. The samples were then heated at 210°C. for 15 min. *in vacuo*. The results in Table II show a linear relation between peroxide

TABLE II

Heat-Induced Changes in Oxidized Lard Diluted with Fresh Lard

Peroxide value before heating[a]	Properties after heating[a]					
	Reducing power (as α-tocopherol)	Browning (absorbance at 460 mμ)	Relative viscosity[b]	Acid values	Iodine values	Carbonyl values[c]
me./kg.	*μg./g.*					*μM./g.*
00.0	30.5	0.088	.....	.....	70.5	5.8
22.2	27.6	0.100	1.41	2.16	68.5	12.2
66.2	54.1	0.090	1.39	2.06	66.2	20.5
133	105.7	0.140	1.41	3.12	63.8	42.7
265	186.5	0.252	1.42	4.64	58.2	75.0
400	279.3	0.380	1.47	6.16	55.8	106
626	477.0	0.798	1.56	10.2	48.7	167

[a] 210°C., *in vacuo*, 10 min.
[b] 10% solutions of fat in benzene.
[c] As crotonaldehyde, method of Henick *et al.* (4).

values of the fats before heating and the reducing power after heating. The browning (absorbance at 460 mμ), viscosity, acid, and carbonyl values of the fats increased markedly with reducing power whereas the iodine values decreased appreciably. These properties indicate that the reducing material obtained on heating oxidized fats is derived from fat peroxides, or from some of their decomposition products, and is polymeric in nature. In this series of lard samples a linear relation was obtained between the peroxide values and reducing power at peroxide levels above 22.2. However during autoxidation this relation is not observed until the peroxide value exceeds 100 (Figure 2). It appears therefore that the precursor of the reducing substances is not formed in measurable amounts in the oxidized fats until the peroxide value exceeds 100. In the series of oxidized lard samples which were diluted with fresh lard the precursor of the reducing substances was diluted correspondingly, thus giving reducing power at the lower peroxide levels.

The presence of carbonyls in the heated-oxidized lard samples is in agreement with the finding of Holm *et al.* (6) that nonvolatile aldehydes remain in fats after deodorization. These aldehydic substances appear to be polymeric in nature and affect the oxidative stability of fats. The contribution of these high-molecular weight carbonyls to flavor reversion in soybean oil and in other fats is being investigated.

Additional evidence for the polymeric nature of the reducing material was obtained by heating oxidized methyl esters of oleate, linoleate, safflower, and soybean oils. These esters were oxidized at 50°C., under ultraviolet light, by oxygen to a peroxide value of 1,000 to 2,000. After heating the oxidized esters in a nitrogen atmosphere for 15 min. at 210°C., all the peroxides were decomposed. The esters were then distilled *in vacuo* to remove monomeric material. The yields of polymeric residues ranged from 15 to 50%, depending upon the peroxide value of the original oxidized esters. The molecular weight of the polymeric residues was determined cryoscopically in solutions of benzene saturated with water. The molecular weights obtained were from 550 to 700. This range indicates that a dimeric material derived from the methyl ester hydroperoxides is chiefly being dealt with. This material may be similar to the dimers obtained by Williamson (7) upon polymerization of methyl linoleate hydroperoxide at 100°C. Chemical and physical characterization of these polymeric materials is being undertaken and will be the subject of a future communication.

Isolation and purification of the reducing material from oxidized-heated fats can be obtained by passing a benzene solution of the fat through a column of silicic acid. Elution of the free fat with benzene followed by elution with mixtures of ethyl ether in benzene gave concentration increases of 2.5 to 3 times and a yield of 76% of the reducing material. Heating of hydroperoxide concentrates, obtained by countercurrent extraction of oxidized lard and oxidized methyl esters of unsaturated fatty acids, was also used to obtain a high concentration of the reducing material.

Summary and Conclusions

By application of a heating method to determine tocopherol in oxidized fats it was shown that polymeric reducing substances were produced when fats have a peroxide value exceeding 100. By comparison with a chromatographic method to remove peroxides it was shown that the interference in determining tocopherol from these heat-produced reducing substances was negligible at peroxide levels lower than 100. It is concluded that the heating method is satisfactory within the peroxide range (0–100) most important in oxidative and flavor-stability studies of edible oils. The chromatographic method for removing peroxides can be relied upon for determining tocopherol in more highly oxidized fats as well as in methyl esters of fatty acids which are distillable under conditions of the heating method.

The appearance of reducing substances in heated oxidized fats is related to the peroxides present in the fats prior to heating. It is accompanied with an increase in browning, viscosity, acid, and carbonyl values of the fats and with a decrease in iodine val-

ues. The polymeric material from heated-oxidized methyl esters of unsaturated fatty acids has been separated by vacuum distillation and chromatography on silicic acid. It is believed to be principally dimeric in nature.

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Fat Transport Mechanism

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FATS HAVE LONG BEEN KNOWN to provide the body with rich sources of energy and to act as a protective insulating layer when they are stored in adipose tissue. For many years however the difficulties of fat chemistry largely dissuaded the biochemist from a more detailed study of body lipides. Then this luxury became too costly as the evidence mounted relating certain lipides to heart disease (1, 2).

The body transports its fat from organ to organ *via* the circulating plasma and lymph. An amazing phenomenon, present in this system of fat transport, is the ability of the body to render plasma lipides water-soluble. Although fats are notoriously insoluble in water and tend to coalesce and layer out, the fats which are present in the aqueous medium of plasma remain in solution or in a stable colloidal suspension. Obviously if the lipides in plasma were suddenly to lose this property and coalesce, the resultant fat droplets would soon plug the capillaries of vital organs, resulting in death. This fortunate but seemingly paradoxical behavior of plasma lipides is largely the result of their combination with certain proteins. These impart water-soluble properties to the lipides and by so doing preserve the single-phase aqueous system of plasma. As a result plasma and lymph are able to serve as vehicles for the transport of fat by the body.

A study of fat transport quite naturally evolves into a study of plasma lipides. Already investigation of the circulating lipides has provided a fundamental insight into the mechanism of fat transport for it is now apparent that extremely small amounts of lipide, often regarded previously as insignificant, are in reality of great importance biologically. The lipides in normal plasma total only about 5.0 g. per liter. Comprising this total are several distinct species of lipide, each apparently serving a different and probably important function.

Of the plasma lipides perhaps the most thoroughly understood are the unesterified fatty acids (UFA), which have to be measured by microtitration after being extracted from plasma. Until recently these tiny amounts of "free" acid in plasma were considered to be either a laboratory artifact or of no biological significance. Certainly their normal concentration of 0.1 to 1.0 milliequivalents per liter of plasma is not very impressive. These low amounts however belie their importance.

While precise chromatographic analyses of the plasma UFA have not been published, they appear to consist mostly of 14 to 18 carbon aliphatic fatty acids, a large part of which are normally oleic and palmitic acid (3). All are present in ionized form. In spite of their small concentration in plasma they would still precipitate out as soaps were they not bound to the water-soluble plasma protein, albumin. The ability of albumin to bind UFA increases the solubility of UFA in an aqueous medium by many fold (4). A more detailed understanding of plasma UFA largely stems from experiments in which humans and animals are given an intravenous injection of a radioactive carbon, labelled UFA.

IN A TYPICAL EXPERIMENT palmitate-1-C^{14} bound to albumin is injected intravenously into a human subject (5, 6). The radioactive palmitate mixes rapidly throughout the plasma and thereafter is presumed to behave in exactly the same manner as the nonradioactive palmitate already present. Following the injection, a quick fall of radioactivity in the plasma indicates a rapid disappearance of palmitate. However equally rapid replenishment with UFA from storage tissues, of course, occurs to maintain the constant plasma UFA level observed throughout the experiment. Of the palmitate molecules which disappear from the plasma many are rapidly metabolized by the tissues to carbon dioxide and water, as evidenced by radioactive carbon dioxide which quickly appears in the expired air. Further calculations from these and similar data reveal the startling fact that the small concentrations of plasma UFA are probably the main form in which fat becomes a major source of energy. The explanation for this phenomenon lies in the short period of time an UFA molecule stays in the plasma. The rapid removal of UFA by metabolizing tissue enables the plasma to transport from storage tissues large quantities despite a low UFA concentration.

The remaining plasma lipides are linked to proteins in macromolecules called lipoproteins. They are associated not with albumin but with different types of globulin, the other major class of plasma protein. About 8–12% of the plasma proteins are, in reality, lipoprotein. All of the plasma lipoproteins contain basically the same kinds of lipides, namely, free and esterified cholesterol, phospholipide, and triglyceride. Despite the chemical similarity, distinct species of lipoprotein exist which significantly differ in size, density, type of protein, and relative proportions of lipide. These differences have made possible a variety of methods to separate the lipoprotein types although none as yet yields a truly pure lipoprotein.

Ultracentrifugation, for example, takes advantage of differences in density to separate classes of lipoprotein. This method has been fully developed by Gofman and his colleagues (7). The more lipide a particular lipoprotein molecule contains, the larger its size and the less its density. Those lipoproteins