THE ROLE OF DIHYDROQUERCETIN AS AN ANTIOXIDANT FOR SOME DAIRY PRODUCTS

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THE ROLE OF DIHYDROQUERCETIN AS AN ANTIOXIDANT FOR SOME DAIRY PRODUCTS

INTRODUCTION

All natural lipids are subject to oxidation by molecular oxygen. This universal phenomenon, which proceeds through numerous reactions of self-perpetuating nature, occurring more or less simultaneously, is collectively referred to as "autoxidation". Since in edible fats these processes culminate in the development of "oxidized flavor", involving a loss in nutritional value as well as organoleptic quality, the entire subject of lipid oxidation and antioxidation has attracted the attention of researchers for over one hundred years. The early work, however, involved studies of lipid material containing complex mixtures of fatty acids, and it was well nigh impossible to gain an insight into the numerous reactions taking place simultaneously.

The current theories of autoxidation in pure fat systems were reviewed by Morris (114) in 1954 and more recently by Badings (10). Most fat-containing foods, however, are polyphasic, often including an aqueous phase, and the overall mechanism of oxidation in such systems may differ considerably from the reactions which occur in pure

fat systems (80, 103, 108). As pointed out by Lea (103) the reactions leading to the deterioration of phospholipids and lipoproteins present in butter, milk and eggs indeed constitute complex phenomena.

The search for acceptable inhibitors of oxidation in edible lipids has led to the discovery of many effective processes and chemical agents, most of which have been patented, but few of which have been acted upon by the Food and Drug Administration.

The flavonoids comprise an important group of compounds found widely in nature which have been shown to possess antioxidant properties. From the physiological, pharmacological and nutritional aspects, the addition of flavones and their derivatives to foods appears acceptable and beneficial (2, 128). Among the flavonoids dihydroquercetin offers promise as a highly-suitable antioxidant in view of its water solubility and practically tasteless and colorless properties in the concentrations used for antioxidant purposes.

The purpose of this study was to investigate the relationship between certain components comprising the oxidative system in milk and the overall reaction leading to the development of oxidized flavor, and to elucidate the role of added dihydroquercetin as an inhibitor of

oxidative deterioration. It was of interest, further, to study the effectiveness of dihydroquercetin as an antioxidant in a variety of fluid milk products, as well as the carry-over properties of dihydroquercetin in spray-dried milk products.

REVIEW OF LITERATURE

Oxidation in Milk and Milk Products

More than fifty-five years ago, Golding and Feilman (58) reported the occurrence of metal-induced oxidized flavor in milk. From the results of several investigations that followed on this subject, it appears that oxidized flavor is the most important single flavor defect in dairy products and is encountered more frequently than any other type of defect. Guthrie and Bruckner (69) observed that 21 per cent of the cows in the five herds they had examined, consistently produced milk that developed oxidized flavor after three days storage. Dahlberg et al. (38) reported that 17 per cent of the fresh pasteurized milk samples, collected by them in eight cities, were scored as oxidized milks. When the samples were held for seven days at 1°C, a total of 67 per cent was found to be oxidized.

The term oxidized flavor designates a number of more or less closely-related flavors. Riel and Sommer (124), in their review, summarized a list of terms used in the literature to designate oxidized flavor. The authors further pointed out the distinction between the flavor defect encountered in milk and the defect encountered in milk

fat. They referred to the former phenomenon as "oxidized flavor", while the latter was referred to as "oxidized fat flavor".

The bulk of evidence on the origin of oxidized flavors, suggests that phospholipids associated with the fat globule "membrane" are involved. Milk products relatively rich in phospholipid material such as sweet buttermilk, cream and fluid milk, show a marked tendency to develop oxidized flavor, whereas butter oil, a product essentially free of phospholipid material, is comparatively stable to oxidation. It has been found to develop "oxidized fat flavor" under conditions of prolonged storage.

Swanson and Sommer (141) observed marked differences between the Iodine numbers of phospholipids from oxidized and non-oxidized milk, while no difference was discernable in their triglycerides. Smith and Jack (135) observed that the milk phospholipids were more unsaturated than the milk fat. Koops and Pette (91), from oxygen absorption studies of butter, observed that the phosphatides were more susceptible to oxidation than milk fat, and that the site of oxidation appeared to be the fat-water interface of butter. Tarassuk et al. (146) attributed the sensitivity of washed cream to the development of fishy flavor, to the phosphatides of the fat globule "membrane".

Considerable research has been done to clarify the concepts related to autoxidation in dairy products. The reviews of Brown and Thurston (25), Greenbank (62), Strobel et al. (139), Lea (102) and Pont (120) adequately cover the literature on this subject. The developments in chromatography and spectrophotometry over the past decade have made possible the identification of compounds responsible for oxidized flavor. One of the first attempts to identify the flavor components of oxidized milk lipids was made by van der Waarden (157) on cold storage butter. He observed that the flavor compounds were fat-soluble and could be completely removed from the fat by degassing at low pressure. From comparison of the properties of the distillate with pure compounds, it was postulated that methyl ketones and aliphatic, unsaturated, unconjugated aldehydes were largely responsible for the oxidized flavor. Tamsma (143) and Keeny and Doan (83) subsequently found evidence to support the findings of van der Waarden. Hoff et al. (81) studied the oxidation of unsaturated fatty acids from milk fats by irradiation with Co^{60} source. Linoleic acid produced fishy flavor while a mixture of fatty acids gave rise to a candle-like flavor. The blending of the two flavors yielded tallowy-oxidized flavor of irradiated milk fat. Day and Lillard (38) have identified compounds isolated from oxidized milk fat, while Riel and

Sommer (124) and van Duin (47) have investigated oxidized phospholipids. Forss et al. (54) studied the volatile compounds derived from oxidized skim milk. Quantitatively and qualitatively the carbonyl compounds arising from the triglyceride fraction and the phospholipid fraction appear to be distinctly different. At the present time, it appears that the majority of volatile mono carbonyl compounds occurring in oxidized milk lipids belong to three classes, namely the alkanals, alk-2-enals and alk-2,4-dienals.

From the accumulated evidence presented by various investigators on the contribution of carbonyls to off flavors, a brief summary is given below.

1. Saturated aldehydes. 3 carbon-10 carbon . . . . soapy, tallowy.
2. 2-enals. 5 carbon-11 carbon . . . painty, tallowy.
3. 2,4-dienals. 6 carbon-10 carbon . . . intense oily.
4. Enals with the double bond not in the 2 position. 6 carbon-10 carbon . . . . . intense cardboard.
5. Dienals with double bond in non-conjugated position to 2-enal configuration. 8 carbon-10 carbon . . . . . intense cucumber, fishy on dilution.
6. Ketones. 3 carbon-5 carbon . . . . . relatively unimportant in their contribution to oxidized flavor.

It should be borne in mind that since autoxidations which occur in natural lipids have a dynamic character, each chemical or organoleptic analysis represents a profile of the situation obtaining in this dynamic system at the moment of observation.

The Relationship of Ascorbic Acid to Oxidized Flavor

Milk as secreted by the cow has been reported to average 22.2 mg. ascorbic acid per liter (131). Ascorbic acid is an ene-diol or reductone-type acid which plays a significant role in reactions involving oxidation-reductions and the development of oxidized flavors.

Ascorbic acid is readily oxidized and the literature reveals riboflavin, oxygen, light and copper to be the components participating in the oxidative system. Bernhardt and Linden (19) reported the catalytic effect of added copper on ascorbic acid oxidation in milk, while Stribley *et al.* (138) reported similar catalytic effect of natural copper in milk. Weisberger and Lu Valle (162) proposed the following mechanism for copper-catalyzed oxidation of ascorbic acid:

$$H_2A \longrightarrow H^+ + HA^-$$

$$HA^- + Cu^{++} \longrightarrow HA + Cu^+$$

$$HA \longrightarrow H^+ + A^-$$

$$A^- + Cu^{++} \longrightarrow A + Cu^+$$

$$2Cu^+ + 2H + O_2 \longrightarrow 2Cu^{++} + H_2O_2$$

Dekker and Dickinson (44), while studying the oxidation of ascorbic acid by oxygen in the presence of copper, found hydrogen peroxide to be capable of oxidizing ascorbic acid to dehydro ascorbic acid. The reaction appeared to be self propagating. Hand et al. (71) and Hopkins (82), from their separate studies, observed riboflavin to be essential in the photochemical oxidation of ascorbic acid.

The literature pertaining to the role of ascorbic acid in the phenomenon of oxidized flavor is anomalous. Chilson et al. (31) observed that fortifying milk with ascorbic acid delayed the onset of oxidized flavor in susceptible milks. Similar results were reported by Anderson et al. (4) and Bell and Mucha (17) in frozen milk, and by Chilson et al. (31) and Hartmann and Garret (73) in fluid milk. The evidence indicates that ascorbic acid levels between 10-20 mg. per liter in milk, under pro-oxidant conditions, favor the development of oxidized flavor. When, however, under the same conditions, the milk is fortified with 50 to 100 mg. per liter of ascorbic

acid, the compound acts as an antioxidant.

Greenbank (61) explained the role of ascorbic acid in terms of proton suppliers to regenerate the natural antioxidant in milk. Partial confirmation of a similar role of ascorbic acid was shown by Scarborough and Watts (130). While studying oxidation in model systems consisting of fat dispersed in an aqueous phase, they found that ascorbic acid acted synergistically to phenolic antioxidants, but was strongly pro-oxidant in their absence. Bell (16), on the other hand, explained the same phenomenon in terms of changes in oxidation-reduction potential.

Olson and Brown (117) observed that washed cream in the presence of added copper failed to develop oxidized flavor. The addition of ascorbic acid to washed cream contaminated with copper, however, induced a strong oxidized flavor. A similar effect was noted with glutathione.

The fact that ascorbic acid may establish equilibrium with dehydro ascorbic acid, indicates its possible role as a hydrogen donor and acceptor in the overall reaction of oxidative deterioration. Krukovsky and Guthrie (90) postulated that the attainment of an equilibrium pressure between ascorbic acid and dehydro ascorbic acid (equal to or less than one) was a prerequisite condition for the development of oxidized flavor. They reported that in spontaneously oxidizable milks the oxidation of ascorbic

acid was greater than in resistant milks. They noted that when the ascorbic acid present in milk was rapidly and completely oxidized either by exposure to light or by added hydrogen peroxide, the development of oxidized flavor was prevemted. Partial oxidation of the ascorbic acid present, however, favored the development of oxidized flavor.

Henderson (75) observed that the oxidation of ascorbic acid on the one hand and the reactions leading to oxidized flavor on the other, were competing reactions for the same catalyst and oxygen in the medium. Hartman and Garret (10) observed that oxidation of ascorbic acid was accompanied by a decrease in dissolved oxygen in milk, until a constant oxygen concentration was attained. This preceded the development of oxidized flavor.

There is reason to believe that besides the postulation of critical equilibrium pressures between ascorbic acid and dehydro ascorbic acid (95) and various reactants competing for common catalysts (75), there may exist a third possible role for ascorbic acid in the overall reaction of oxidative deterioration. Chilson et al. (32) observed that the addition of propyl gallate to milk failed to prevent the oxidation of ascorbic acid in milk, but protected the milk against oxidative deterioration.

Evidence in the literature indicates that the factor responsible for the development of oxidized flavor is not necessarily responsible for the oxidation of ascorbic acid in milk. Further, the greater resistance of summer milk produced from pasture-fed cows to the development of oxidized flavor cannot be ascribed solely to ascorbic acid oxidation rates, as claimed by Trout and Gjessing (154).

The Relationship of Oxidation-Reduction Potential to Oxidized Flavor

The Eh of milk normally falls within the range of 200 to 300 millivolts. The oxidation-reduction systems that appear to contribute to the Eh of milk (74) are: ascorbic acid - dehydro ascorbic acid; glutathione - glutathione disulfide; cysteine - cystine; and riboflavin - reduced riboflavin.

Saal and Heukelom (129), in summarizing their studies on the oxidation-reduction potential of milk and butter plasma, observed that dissolved oxygen and oxidation products caused an increase in Eh, while substances with low potential such as ascorbic acid, lactoflavin, thermal decomposition products and substances derived from bacterial activity tend to lower the Eh of milk. Harland et al. (72) later confirmed that oxygen was an important

factor contributing to the Eh of fresh unheated milk. They observed a decrease in Eh of milk resulting from deaeration of milk with nitrogen.

The Eh of milk is also influenced by other factors. Cupric ions are strong electron acceptors and potent oxidizing agents. Consequently, when milk is contaminated with even small amounts of copper, there is tendency for the Eh to increase. Such a rise in Eh is accompanied by the oxidation of the ascorbic acid present. Gebhardt and Sommer (56) observed that the presence of dissolved copper caused a rise in Eh in milk. Later, Swanson and Sommer (142) observed a rapid increase in the Eh of milk after the addition of copper, but the potential did not rise until virtually all of the ascorbic acid present had been oxidized. They noted a decrease in potential when crystalline ascorbic acid was added. In conclusion, they stated that the oxidation-reduction potential of milk apparently is not related to the development of the oxidized flavor. Webb and Hileman (160) also reported that there was no correlation between the oxidation-reduction potential of milk from individual cows and the development of oxidized flavor. They found that summer milk resisted the development of oxidized flavor, even though the oxidation-reduction potential was high. Likewise Thurston

(151) found that the Eh of milk was increased by bubbling oxygen through the milk but that such an increase was not accompanied by the development of oxidized flavor.

On the other hand, Greenbank (61) observed a direct relationship between the Eh of milk and its tendency to develop oxidized flavor. He found that milks which readily developed oxidized flavor showed rapid increase in Eh when small amounts of copper were added.

While the data published in the literature on the significance of the oxidation-reduction potential appear to be conflicting, much of this could be attributed to the differences in milk in terms of poising action. The actual poise of the Eh system in milk has not been adequately studied. A poorly poised milk develops oxidized flavor spontaneously even without any added copper, while a well-poised milk resists the development of oxidized flavor even in the presence of added copper. Apparently the difficulties involved in securing reliable data on the oxidation-reduction potential of milk have discouraged investigation of the subject.

The Relation of Enzymes to Oxidized Flavor in Milk

The evidence in the literature reveals that if there is a direct relationship between enzyme activity and development of oxidized flavor, it is not an apparent one.

Kendo (85) in 1931 reported that oxidized flavor in milk was caused by oleinase. He observed metal contamination, oleinase and reductases to be factors influencing the development of oxidized flavor in milk. Webb and Hileman (160) attributed the protective effect of heating on the development of oxidized flavor to the destruction of an oxidizing enzyme. They reported that oxidation was catalyzed by "oleinase" when the potential of milk was low, and by copper when the potential was high. Brown and Olson (24), however, have reported that enzymes are not involved in reactions leading to the development of oxidized flavor.

Xanthine oxidase is a prominent enzyme in milk. Ball (11) in 1931 and more recently Avis et al. (9) have isolated xanthine oxidase and reported its properties. The enzyme has been reported to be tightly bound to the fat globules in milk as secreted by the cow, but is inactivated and released into the medium by subsequent cooling, agitation and similar treatments (126). Morton (115) found the enzyme to exist in the form of discrete particles attached

to fat globules, and variations in conditions caused the release of enzyme into the plasma phase of milk. Dorn (46) stated that a peroxide action upon the milk fat could be ascribed to the action of xanthine oxidase and ascorbic acid oxidation. Greenbank et al. (67) reported the possible significance of xanthine oxidase in the oxidative deterioration of milk and milk products.

Recently Aurand et al. (8) observed that a chemical oxidation was involved in copper-induced oxidized flavor, whereas an enzyme mechanism apparently was involved in the development of spontaneously-oxidized flavor. An enzyme inhibitor (p-chloro mercuribenzoate) effectively prevented spontaneous oxidation, but was ineffective in preventing copper-induced oxidation.

Aurand and Woods (7) later found that the occurrence of spontaneously oxidized flavor in milk was dependent on a high level of xanthine oxidase activity. Smith and Dunkley (134) failed to find any correlation between natural xanthine oxidase and spontaneous oxidation in milk. Campbell and Keur (28) reported that xanthine oxidase does not play a significant role in the reduction of resazurin by raw milk. When they added folic acid, an inhibitor for xanthine oxidase, the resazurin reduction was not slowed down.

The Effect of Oxygen

Molecular oxygen appears to be one of the components participating in the oxidative system in milk. Leeder and Herreid (107) noted that in surface-cooled milk, ascorbic acid oxidation proceeded more rapidly and more oxidized flavor developed than in milk cooled in pasteurizing vats; deaeration of milk reduced the rate of oxidation of ascorbic acid.

Guthrie (68) reported the inhibition of oxidized flavor in milks deaerated on a semi-commercial scale. Krukovsky and Guthrie (96) ascribed the inhibiting effect of added ascorbic acid in milk to the depletion of oxygen. Tarr and Cooke (149) attributed the antioxidant properties of carbonyl ene-diols in frozen fish to oxygen depletion.

While the bulk of evidence indicates the vital role of oxygen in the development of oxidized flavor, Bell and Mucha (17) claimed that since oxidative changes occur merely by transfer of electron from reductant to oxidant, free oxygen is not essential for oxidation.

The Effect of Copper

Golding and Feilman (58) first noted the relationship between copper contamination and the development of

oxidized flavor. Subsequent investigations indicate that added or natural copper in milk is an important catalyst for oxidative deterioration. Willard and Gilbert (164), from their studies on the influence of dairy equipment on the copper content of milk, observed more copper in cream than in skim milk and that the cream developed a more pronounced oxidized flavor.

A difference of opinion exists among investigators as to the distribution of added copper in milk. While Koops and Pette (91) reported that added copper was adsorbed by phosphatides, King et al. (87) reported that only 2-3 per cent of total added copper was associated with the fat globules and the rest of the added copper was uniformly distributed among the skim milk proteins. Stotz et al. (137) found that a mixture of copper and albumin behaved similarly to natural copper-ascorbic acid oxidases except in dialyzability. The copper in the latter was not dialyzable, indicating a different type of association.

Tappel (144) found copper bound to protein was a more effective catalyst than copper alone for linoleate oxidation and he attributed it to a complex of linoleate-peroxide-copper-protein. King and Dunkley (86) found a highly significant correlation between the incidence of spontaneous-oxidized flavor and the natural copper content of milk from cows in mid and late lactation. The milk of

early lactation cows with high copper content, however, appeared to be more resistant to the development of oxidized flavor. They found no difference in natural copper contents in milks obtained from cows in pasture and under dry lot feeding conditions.

Copper is an effective catalyst for oxidation of ascorbic acid (as reviewed earlier). Apparently copper, by increasing the rate of oxidation of ascorbic acid, catalyzes the reactions which culminate in the development of oxidized flavor. Allan (1) observed that the rate of oxidation of ascorbic acid in butter serum was dependent on the concentration of added copper. Tobias and Herreid (152) observed that copper catalyzed the oxidation of ascorbic acid as well as gulonic acid in milk.

The inactivation of the catalytic effect of copper in milk by direct addition of antioxidants or by heat treatment has been reported. Gjessing and Trout (57) observed a decrease in the catalytic effect of added copper on ascorbic acid oxidation in milk when the addition was done prior to heating. They attributed the effect to the lowering of oxidation-reduction potential by the reducing substances liberated by the heat treatment. Bernhardt and Linden (19) noted a similar effect when copper was added after heating milk to 121°C. They attributed this to the binding of copper by sulfhydryl groups released during

heating. Chapman and MacFarlane (30) observed that the addition of wheat germ oil (high in Vitamin E) inhibited copper-induced oxidation in whole milk powder.

Arrington and Krienke (5) observed that the addition of chelating agents to milk was effective in the inhibition of copper-induced oxidized flavor.

The Effect of Light

Studies on the influence of light on milk date back to 1907 (27). Subsequently several investigators have pursued studies on the photochemical oxidation of milk and milk products (110, 118, 136, 161). A review by Stull (140) adequately covers the literature pertaining to this subject until 1953.

The evidence from the studies completed to date indicates that at least two distinct flavor defects, namely sunlight flavor and oxidized flavor, develop in milk as a result of exposure to light. The former defect has been attributed to the breakdown of a minor protein in milk. Patton (118) postulated a possible mechanism for the development of sunlight flavor in milk. He reported that methionine in the presence of riboflavin and light formed methional. This compound is detectable in milk at a level of one part in twenty million.

On the other hand, the oxidized flavor in milk

resulting from exposure to light, is associated primarily with reactions involving the phospholipid fraction of milk. Pont (120) observed an increase in peroxide concentration of the fat phase in milk exposed to sunlight. In spite of the fact that these two flavor defects arise from distinct components of the milk system, and may develop independently of each other, there is evidence in the literature to show that sunlight flavor has been confused with oxidized flavor. Dahle (39) reported that homogenization failed to prevent the development of oxidized flavor in milk exposed to sunlight. It is now well established that homogenization renders milk less susceptible to the development of oxidized flavor, while it favors the development of sunlight flavor when the product is exposed to light.

More recently a third type of flavor defect induced by light has been reported in low-fat milks fortified with Vitamin A. Weckel and Chicoye (161) used the term hay-like flavor to denote this defect and attributed the defect to the degradation of added Vitamin A.

Light energy is capable of inducing the formation of free radicals, thus initiating the chain reaction leading to the development of flavor defects similar to the chain initiation resulting from oxidation of ascorbic acid as reviewed earlier. Weiss (163) postulated a mechanism for photochemical oxidation involving free radicals:

Light absorption: $RH + h\upsilon \longrightarrow RH^{\circ}$

Quenching by oxygen: $RH^{\circ} + O_2 \longrightarrow (RH)^{+} + O_2^{-}$

Dissociation of photoperoxide: $RH \rightleftharpoons RH + O_2$

Light catalyzes the oxidation of ascorbic acid, similar to metal catalysis. Krukovsky and Guthrie (95) observed that rapid and complete photochemical oxidation of ascorbic acid in milk prior to pasteurization enhanced the resistance of milk to oxidized flavor development while partial oxidation of ascorbic acid stimulated the development of tallowy flavor. Pont (120), however, reported that exposure of milk to light resulted in the development of oxidized flavor. Crowe (37) reported that homogenized milk was more sensitive to light than non-homogenized milk. Herreid et al. (78) observed that ruby colored bottles, followed in the descending order by amber, paper and clear glass bottles, proved most protective against the development of sunlight flavor in milk.

Effect of Temperature

Gould (60) observed that heating milk to 84°C or higher was effective in inhibiting copper-induced oxidation. He attributed this to the liberation of sulfhydryl compounds. When, however, the milk was heated to 90°C and copper added after the heat treatment, he noted that oxidized flavor developed. When copper was replaced by

ferrous ions, the heat treatment protected the milk regardless of the time of addition of the iron.

Townley and Gould (153) observed that serum proteins in milk as well as the fat globule "membrane" protein served as a source of sulfur compounds formed during heating of milk.

Larson and Jenness (100) reported beta lactoglobulin to be the principal reducing component of milk proteins. Tarassuk et al. (146) observed that blocking of sulfhydryl groups by N. ethyl maleimide nullified the protective action of sulfhydryl groups.

Saal and Heukelom (129) showed that the oxidation-reduction potential was lowered as a result of heating of milk. Twigg (156), however, observed that heat treatment decreased the reducing power of milk and attributed it to the destruction of reducing enzymes present in unheated milk.

Greenbank (62) reported that storage of milk at low temperatures was conducive to the development of oxidized flavor and ascribed it to unfavorable temperature conditions for the growth of bacteria. Collins and Dunkley (34) noted that the number of bacteria needed for retardation of oxidized flavor development in milk was considerably higher than normally encountered in market milk.

Theories Concerning Autoxidation and Antioxidation

The work of Farmer and Sutton (53) in 1943 established the significance of free radical mechanisms associated with the autoxidation of fats. From subsequent studies of Bolland and Ten Have (21) and the work of Bateman (12), the present knowledge regarding autoxidation and antioxidation processes may be summarized as follows:

1. Autoxidation proceeds through free radical chain reactions.
2. There are three distinct stages of these reactions.
   (a) Initiation: or formation of the free radicals.

$$ROOH \longrightarrow RO^{\circ} + HO^{\circ}$$

$$RO^{\circ}(HO^{\circ}, HOO^{\circ}) + RH \longrightarrow ROH(H_2O, H_2O_2) + R^{\circ}$$

   (b) Propagation: consisting of the free radical chain reaction itself:

$$R^{\circ} + O_2 \longrightarrow ROO^{\circ}$$

$$ROO^{\circ} + RH \longrightarrow ROOH + R^{\circ}$$

   (c) Termination: $2RO_2 \longrightarrow X$

3. The rate of autoxidation is dependent on the energy required for the rupture of alpha CH bond.
4. The intermediately formed alpha methylene radical is stabilized by resonance.

$$- C^{\circ}H - CH = CH - \longleftrightarrow - CH = CH - C^{\circ}H -$$

The two contributing structures to the resonance hybrid lead to the formation of isomeric hydroperoxides.

5. The reaction may be accelerated by light and radical forming products such as benzoyl peroxide, thus initiating new chains.
6. Autoxidation is inhibited by compounds which react with free radicals to form non-radical products.

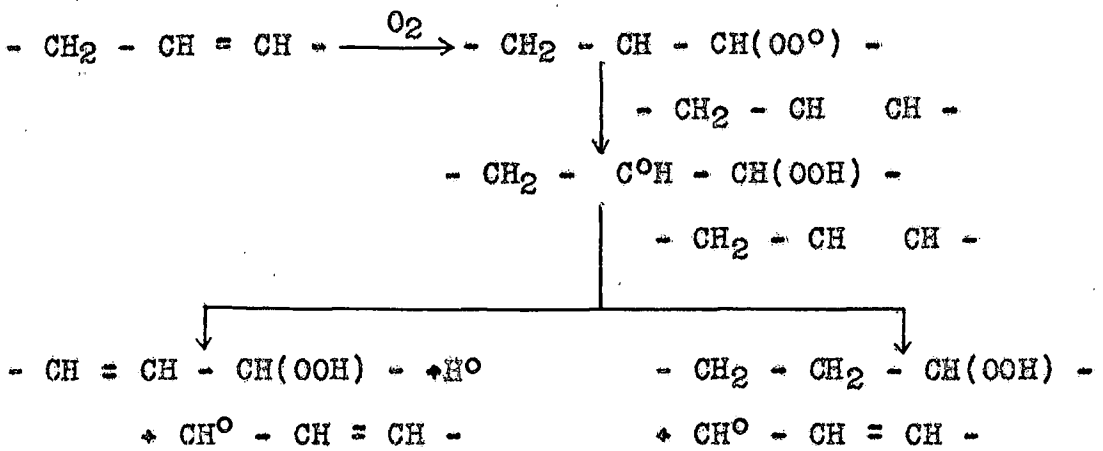
Inhibition:

$$RO_2^{\circ}(RO^{\circ},\ HO^{\circ},\ HOO^{\circ}) + AH \longrightarrow ROOH(ROH,\ H_2O_2,\ H_2O) + A^{\circ}$$

$$RO_2^{\circ}(RO^{\circ},\ HO^{\circ},\ HOO^{\circ}) + A^{\circ} \longrightarrow Y$$

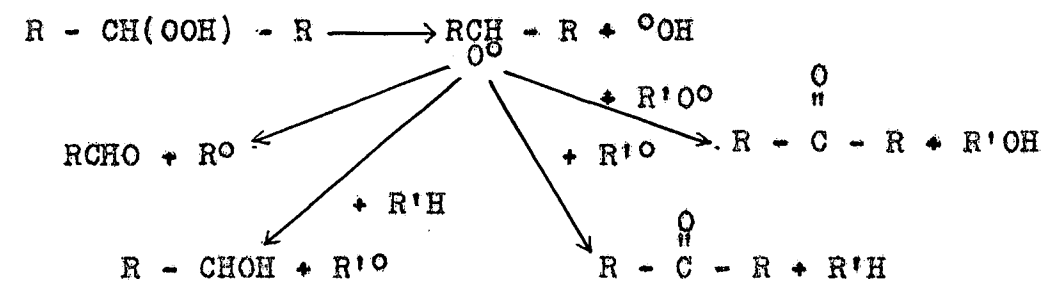
Initiation: The energy required for the separation of an alpha methylene hydrogen atom in a monoene configuration is about 80 kilocalories and it is not clear in which way this energy should be supplied.

As the energy required for a direct attack on the double bond is not as large as that required for the rupture of an alpha CH bond, Farmer (51), Bolland and Gee (20), and Gunstone and Hilditch (65) independently came to the conclusion that a direct attack on the double bond by oxygen occurred in the initial stage of autoxidation and not at the alpha methylene group. In this way the expenditure of energy required at the beginning of the reaction would be considerably diminished. A few additions are sufficient to form enough radicals to start the free radical chain reaction itself.



Dismutation of hydroperoxides:

The hydroperoxides formed in the autoxidation of olefins and unsaturated fatty acids are not stable and will decompose further. The scheme of Bell et al. (18) presents some of the possible mechanisms.



Factors which promote autoxidation:

Those factors leading to the formation of additional free radicals which initiate new chains, and those factors which enhance the rate of the free radical formation, promote autoxidation.

Formation of additional free radicals is possible by light, radiation and by proxidants such as peroxides which on decomposition give rise to free radicals. Weiss (153)

postulated a mechanism for free radical formation in photochemical oxidations. Metal ions like copper indirectly contribute to the formation of free radicals by catalyzation of the decomposition of hydroperoxides present. Bawn et al. (15) and Bawn (14) postulated the following reaction:

$$M^{n+} + ROOH \longrightarrow ROO^{\circ} + H^{+} + M^{(n-1)+}$$

$$M^{(n-1)+} + ROOH \longrightarrow RO^{\circ} + OH^{-} + M^{n+}$$

$$2ROOH \longrightarrow ROO^{\circ} + RO^{\circ} + H_2O$$

Tappel has demonstrated the strong accelerating effect of copper-protein complexes (142) and hematin compounds (145) on the rate of autoxidation of fatty acids. These compounds also catalyze the decomposition of hydroperoxides into chain-initiating free radicals. Maier and Tappel (109) studied the formation of initial and secondary products in the hematin-catalyzed decomposition of hydroperoxides. Initial products were found to be alkoxy free radicals produced by hemolytic cleavage of the O-O bond in ROOH. Lipoxidases also catalyze the oxidation of unsaturated fatty acids to hydroperoxides. Greenbank (62) observed that the presence of free fatty acids promoted autoxidation. Thus, lipolysis will indirectly accelerate autoxidation. At high temperatures autoxidation proceeds with greater velocity than at lower temperatures.

Factors which inhibit autoxidation:

In general, all those factors which lead to the interruption of the free radical chain reaction, or those which decrease the rate of these reactions, tend to inhibit the autoxidation process.

In the absence of an inhibitor, free radicals are formed by cleavage of any hydroperoxides. The free radical is capable of abstracting a hydrogen atom from the hydrocarbon to form the alkyl radical, which may react with oxygen to form a peroxy radical. Two peroxy radicals may dimerize to terminate the reaction.

Antioxidants when present in low concentration, however, are able to interrupt the chain reaction by the capture of free radicals involved. Hammond *et al.* (70) postulated the following mechanism:

$ROO^{\circ}(RO^{\circ}, HO^{\circ}, HOO^{\circ}) + AH \longrightarrow ROOH(ROH, H_2O, H_2O_2) + A^{\circ}$

This reaction proceeds with greater speed than earlier-mentioned reactions. The inhibitor radicals A° are consumed directly with another radical. One mole of inhibitor may stop two free radical chains. The more rapid the above reaction occurs, the fewer the number of moles of hydroperoxide formed.

Phenolic antioxidants exhibit an accelerating effect on peroxide decomposition and are thus capable of exerting a pro-oxidant effect. Privett (121) observed that

nordihydroguaretic acid accelerated peroxide decomposition and suggested that antioxidants function at an optimum concentration which represents a balance between reactions as a free radical acceptor and accelerator of peroxide decomposition.

The effective inhibitor would be one which could more readily donate a hydrogen atom to a free radical. If the inhibitor hydrogen atom is very loosely bound, however, it can also be removed by direct attack by oxygen to form two radicals, thus:

$$AH + O_2 \longrightarrow A^{\circ} + HOO^{\circ}$$

It is possible, therefore, for an inhibitor to act as an initiator. For these reasons, inhibitors essentially represent a compromise between the sensitivity necessary to terminate the oxidation chains and the stability to withstand direct attack by oxygen.

Mechanism of synergism:

The action of many antioxidants can be reinforced by so-called synergists which in themselves do not possess antioxidant properties. Organic acids containing hydroxyl groups such as citric, tartaric and galacturonic acids appear to be effective with polyphenolic antioxidants, whereas phosphoric acid, ascorbic acid and its fatty acid esters and lecithin appear to be effective synergists with

mono and polyphenolic antioxidants. These acidic compounds are metal deactivators and Reimenschneider (125) suggested that this property may account for their effectiveness. Golumbic (59) observed that synergists provide a reservoir of hydrogen to regenerate the antioxidant. Privett (121), however, suggested that the mechanism of synergism involved the suppression of the accelerating effect of antioxidant on peroxide decomposition. He postulated the following sequence of reactions concerning synergist action.

$$A^{o} + S \longrightarrow A \cdot S$$

$$A \cdot S + ROO^{o} \longrightarrow OxA + ROOH + S$$

where OxA represents the oxidized antioxidant and A·S the antioxidant-synergist complex. The chemical association of synergist with antioxidant impedes, but does not entirely block the accelerating effect of antioxidant on peroxide decomposition.

The Inhibition of Oxidized Flavor

The methods adopted for inhibiting the development of oxidized flavor in milk are varied and numerous. The bases of all methods involve the effective blocking or inactivation of one or more of the components participating in the oxidative system, namely oxygen, the unsaturated lipid material associated with the fat-globule "membrane",

natural and/or added copper, and ascorbic acid in the aqueous phase. The presence or absence of natural antioxidants is an important factor which influences the effect of each of the participating components.

Strobel et al. (139) adequately reviewed the composition and processing factors concerned with oxidized flavor in milk. Several investigators have shown that cows on pasture yield milk more resistant to spontaneous or copper-induced oxidation than milk produced under dry feed lot conditions, in which the incidence of spontaneous oxidation is higher. Krukovsky et al. (97) studied the tocopherol level of milk produced under different feeding conditions and observed that pasture feeding increased the tocopherol level of milk. Gamma tocopherol is reported to possess antioxidant properties.

De Luca et al. (43) reported that when N-diphenyl paraphenylene diamine was fed to cows, the milk produced was resistant to copper-induced oxidation. Likewise, Teichman et al. (150) observed that 2, 6 ditertiary, 4 methyl phenol, when included in the feed of the cow, inhibited the development of oxidized flavor in the secreted milk.

Oxygen depletion in milk resulting from bacterial activity (106) or by deaeration (68) has been reported to prevent the development of oxidized flavor. Presumably

bacteria could contribute to the inhibition of oxidation by synthesis of antioxygenic substances, depleting the oxygen content, or by decreasing the oxygen tension.

The liberation of sulfhydryl groups either by heat treatment (60) or by the addition of proteolytic enzymes to milk (116) has been shown to inhibit oxidized flavor. Foster and Sommer (55) attributed the effect of enzymes to the liberation of sulfhydryl groups which exerted a sparing action on the lipids by being preferentially oxidized. The use of colored glass containers for milk in order to minimize the effect of sunlight has been reviewed earlier.

Perhaps the most widely used processing method for inhibiting the development of oxidized flavor in milk has been the homogenization of pre-warmed milk prior to pasteurization. There have been several attempts made to explain this phenomenon, and they are not in agreement on the effects of homogenization. The process of homogenization splits the large fat-globules into smaller ones, thus creating a larger lipid-water interface. The supply of natural emulsifying agent, consisting of phospholipid-protein, remains constant. Since the emulsion resulting after homogenization is quite stable, there is reason to assume that a different type of emulsifying agent has been responsible for the re-surfacing of the greater surface

area resulting from homogenization. Greenbank and Pallansch (66) observed that homogenization at or below 2000 p.s.i. caused a migration of phosphatides away from the fat globule surface, but at higher pressures the direction of migration was reversed. They attributed the increase in oxidative stability of homogenized milk to the redistribution of phosphatides. Brunner and Thompson (26), from their sedimentation velocity studies of minor protein fractions of milk, reported that "membrane" protein exhibited more heterogeneity than minor protein fractions of skim milk, and that while the latter were rennin substrates, the former was unaffected by rennin.

Lea (102) suggested that in the process of homogenization, the phospholipid-protein complex was split, and that this was accompanied by the withdrawal of labile lipid material into the interior of the fat globule. Krukovsky (94), on the other hand, attributed the protective effect of homogenization to a redistribution of natural antioxidant (tocopherol) in the milk, coupled with the withdrawal of labile lipids into the interior of the fat globule. More recently Kleyn and Shipe (88) observed an increase in sulfhydryl groups in milk subjected to homogenization and attributed the resistance of homogenized milk to oxidation to sulfhydryl activation. It has to be borne in mind that homogenization, while

conferring added resistance to the milk toward developed oxidized flavor, renders it more susceptible to the development of sunlight flavor.

The literature pertaining to the effect of direct addition of antioxidants has been covered adequately by several reviews (25, 79, 101, 119, 124). Chilson et al. (32) observed that added propyl gallate exerted a protective effect in milk against copper-induced oxidation. They reported that the oxidation of ascorbic acid, natural or added, was not prevented by propyl gallate. Tarassuk and Henderson (148) reported that the tannins (gallates) present in tea, were effective in inhibiting oxidized flavor in milk. Corbett and Tracy (35) reported that hydroquinone, tyrosine, and pancreatic extract were effective inhibitors of oxidation. Nelson and Dahle (116) found that oat flour, gum guaiac, and crude sugar exhibited antioxidant properties. El-Rafey et al. (50) reported that added alpha tocopherol as well as phospholipids (from soy beans) were effective as inhibitors of oxidation in butter oil, and they suggested a possible synergism between the two types of compounds. The addition of salts of ethylenediamine tetra acetic acid has been reported effective in inhibiting copper-induced oxidized flavor and spontaneous oxidized flavor. The characteristics and limitations of antioxidants and synergists have

been discussed by Mattil (111) and Daubert and Longenecker (40).

Flavonoids as Antioxidants

The trend in recent years toward increasing the interval between production and consumption of milk, coupled with low storage temperatures, leads to conditions which favor the development of oxidized flavor.

The replacement of tinned copper and white metals with stainless steel and glass in dairy equipment has offered a solution to the problem of oxidized flavor caused by copper contamination. Most market milk is now homogenized, making it much less susceptible to oxidized flavor but renders the milk more susceptible to the development of sunlight flavor.

The continued high incidence of the oxidized flavor defect in milk and milk products has interested investigators in utilizing acceptable, non-toxic inhibitors of oxidative deterioration. Many processes and chemical agents have been discovered and patented, but few have been accepted by the Food and Drug Administration for application to milk products.

One group of compounds found widely distributed in nature, the flavonoids, has been investigated in a variety of fat-containing food products and offers promise as a

suitable antioxidant for the dairy industry.

Greenbank and Holm (64) reported that quercetin seemed to possess antioxidant properties for cottonseed oil. Bradway and Mattil (23) observed that quercetin was an effective inhibitor of oxidation in a mixture of lard and cod liver oil. Richardson et al. (123) found quercetin, quercitrin, rutin and certain other flavones and flavanones were effective antioxidants for milk fat, lard, and copper-sensitive milk. Kurth and Chan (99) reported that dihydroquercetin inhibited oxidative deterioration of lard, cottonseed oil and butter oil, and that the presence of citric acid markedly enhanced the antioxidant activity of dihydroquercetin. Erickson (51) reported that quercetin and dihydroquercetin, when added to milk, prior to or after pasteurization, inhibited the development of copper-induced oxidized flavor. Tarassuk et al. (146) reported quercetin to be effective in the inhibition of oxidation in washed cream. Lea (105) observed that the flavonoids gossypetin and quercetagetin possessed antioxidant properties. Mehta and Seshadri (112) tested twenty-seven flavonoid compounds for their antioxidant properties in lard and observed that flavonols having a 3', 4' dihydroxy configuration were most effective as antioxidants among the compounds tested.

The effects of flavonoids in pure fat systems have been investigated. Simpson and Uri (132) studied the

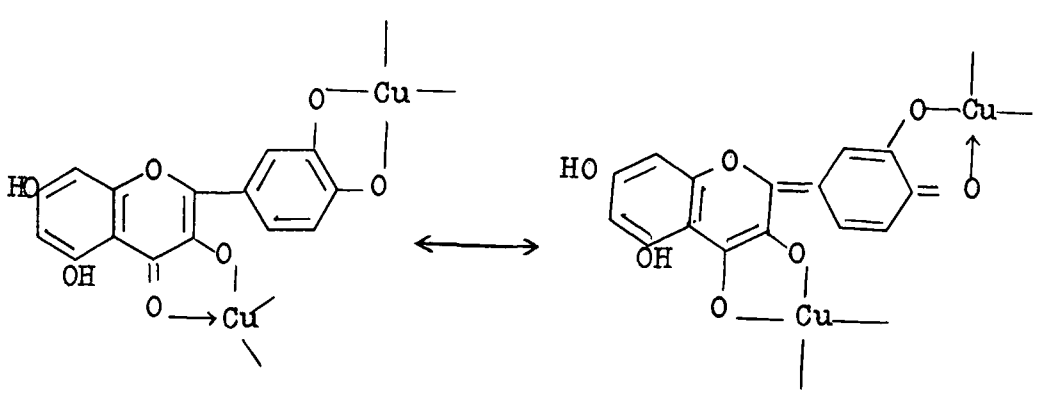
antioxidant properties of thirty hydroxy and alkoxy flavones in methyl linoleate. Heimann et al. (77) studied the antioxidant activity of quercetin in triolein, ethyl linoleate and ethyl linolenate. Kelly and Watts (84) found quercetin, quercitrin, luteolin and rutin to be effective in inhibiting the pro-oxidant effect of ascorbic acid in aqueous fat systems.

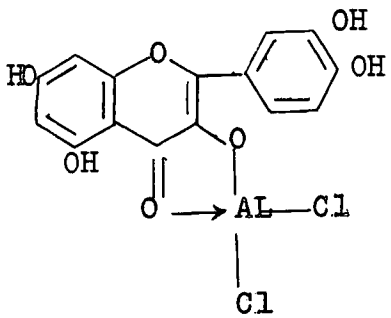
The literature reveals that flavones and flavone derivatives are capable of functioning as metal chelating agents, as well as free radical acceptors. Heimann et al. (77) have attributed the antioxidant activity of flavonoids primarily to their ability to act as free radical acceptors, while Simpson and Uri (132) and Mehta and Seshadri (112) have suggested that metal chelating properties of flavonoids may contribute in part to their antioxygenic activity. Crawford et al. (36) have also pointed out the dual role of flavonoids, but consider the function as free radical acceptors to be more important.

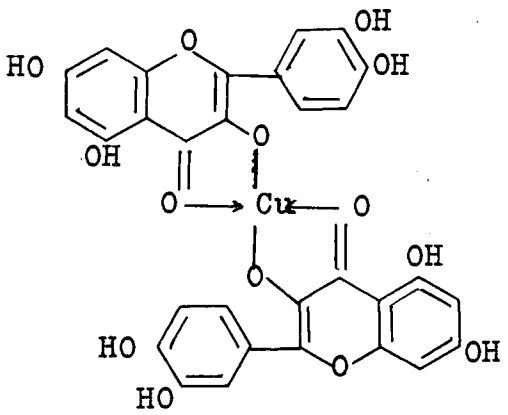
On the other hand, Kelly and Watts (84) consider the activity of flavonoids to be associated with their metal complexing abilities. Clark and Geissman (33), from their study of some seventy compounds on the relation between configuration and potentiating effects of epinephrine, attributed the chief potentiating effect of flavonoids to metal chelation. That chelation may actually take place

when cupric chloride (33) and cupric sulfate (45) were added to alcoholic and aqueous solutions of flavonoids has been shown. From the shifts in ultraviolet absorption maxima of quercetin with different molar ratios of copper ion, Crawford et al. (36) observed that complexes of quercetin and copper were being formed. Kelly and Watts (84) studied the complexing ability of several antioxidants, utilizing the time taken for colored complex development in a mixture of cuprous chloride and cuproin in the presence of the test compound, as a measure of complexing ability of the compound.

The molecular configuration of quercetin permits the formation of complexes with metal ions (33, 36 and 45). It appears that quercetin might complex with two moles of copper intramolecularly and that a third mole of copper might be complexed intermolecularly. Some of the possible metal-quercetin complexes are shown:

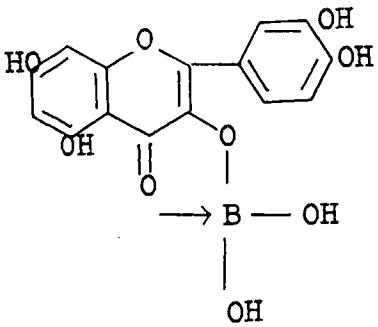


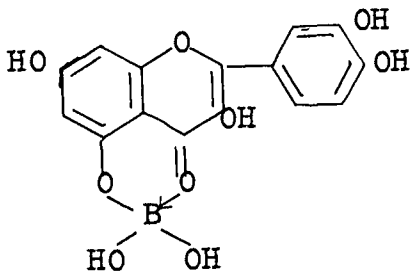




Kurth (98) observed that calcium bisulfite liquor with dihydroquercetin produced a finely divided insoluble calcium-quercetin complex and suggested this reaction as a suitable means to isolate quercetin from certain plant materials.

Wilson et al. (165) used the boric acid-quercetin complex as a standard and investigated the contents of quercetin and quercetinlike substances in various natural products. They expressed their findings in terms of "quercetin equivalents", and found that lemon peel ranked high among the products tested. The boric acid-quercetin complex are considered to represent chelation, as shown:





While the bulk of evidence indicates that the molecular configuration of flavonoids favors metal complexing, the literature reveals that several metals, when combined with flavonoids, formed only weak chelates, or failed to complex at all. Krewson and Couch (93) studied water-soluble metal complexes of rutin and related flavonols and observed that ferric ammonium sulfate, glycerophosphate, sulfate and ferrous phosphate and chloride failed to solubilize rutin, while cuprous and cobaltous chlorides solubilized it. Ferrous gluconate failed to solubilize quercetin and quercetrin. Colloidal saccharated iron oxide solubilized most of the flavonoids they studied.

The importance of molecular configuration to the antioxidant activity of flavonoids has been pointed out by several investigators. Richardson et al. (123) suggested that the antioxidant activity may be centered in the labile pyrone ring of flavonoids. In compounds lacking the unsaturated pyrone nucleus such as hesperidin, they found little or no antioxidant activity. Heimann and Reiff (76) observed that besides the α-β unsaturated

ketone structure of the pyrone ring, the free hydroxy group on C3 in the chromone ring was important to the antioxidant property of flavonoids. They pointed out that ortho hydroxy groups on the pyrocatechol residue of flavonols increased their antioxidant activity, while meta hydroxyls in the chromone ring decreased the antioxidant effect. Simpson and Uri (132) observed that p-quinol structure in the 2 phenyl ring was more important than o-quinol structure in their contribution to the activity of the flavonoid. Mehta and Seshadri (112) suggested that the keto enol tautomerism of hydroxyl group on C3 and carbonyl group on C4 to be an important factor in determining antioxygenic activity of the molecule. Crawford et al. (36) noted that selective methylation of hydroxyl groups of the quercetin molecule decreased the antioxidant activity, and thus confirmed the observations of Heimann and Reiff (76) to the same effect.

Although the precise mechanism of antioxidant action by flavonoids remains to be clarified, the literature reveals that from physiological, pharmacological and nutritional standpoint, the addition of flavones and their derivatives to foods appears to be acceptable and beneficial. The reported non-toxic property of flavonoids (2) coupled with their abundance in natural products (13, 98), and their known antioxidant properties, merits further

studies on flavone and flavone derivatives for use in a variety of food products.

Among dairy products, other than butter oil and fluid milk, the application of flavonoids has not been studied. The carry-over properties of flavonoids in products such as dried milk and milk products and baked goods has not yet been reported in the literature.

Recent investigations have shown that quercetin from feed may be deposited in the bones of rats (122). Ingested quercetin, however, does not appear to be secreted in milk (51). The lack of specific method of assaying flavonoid compounds in milk, limits the scope of such studies. It is probable that in ruminants, quercetin is so completely degraded by rumen microflora, that none of it remains intact for later absorption. Administration of quercetin, by-passing the rumen, or feeding of substituted or hindered quercetin, which is resistant to metabolic degradation, might prove an avenue of study worth pursuing. Watkins *et al.* (158) have shown that the phlorglucinol part of the quercetin molecule was built from 3 moles of acetic acid, and that the rest of the flavonoid was built from phenylalanine. This was demonstrated when buckwheat was grown in presence of methyl-labelled and carboxyl-labelled acetic acid and phenylalanine, resulting in the biosynthesis of quercetin molecules with labelled carbon

atoms on the phloroglucinol residue. Though the recovery of labelled quercetin may be inadequate for feeding experiments with ruminants, it might prove adequate for rat experiments.

EXPERIMENTAL METHODS

1. The Thiobarbituric Acid Test

Although several tests have been developed to follow the process of oxidative deterioration in lipids, few have proved to be sensitive enough to detect the onset of oxidized flavor in dairy products. Organoleptic evaluations have been shown to be reliable indices of the degree of oxidized flavor (155) and are widely used. In this study oxidative deterioration of milk and milk products was followed by using the thiobarbituric acid (TBA) test concurrently with organoleptic evaluation. The procedure adopted for TBA determinations was similar to the method of Dunkley and Jennings (49) with minor modifications.

Reagents:

TBA reagent - 0.025 M 2-thiobarbituric acid in M phosphoric acid, prepared by mixing equal volumes of 0.05 M 2- TBA and 2 M phosphoric acid.

Extraction mixture - 2:1 mixture of isoamyl alcohol and pyridine.

Procedure:

Ten ml of milk were pipetted into a stoppered, 50 ml

centrifuge tube along with 5 ml of TBA reagent and mixed thoroughly. The tube was then placed in a boiling water bath for exactly 10 minutes, and was then removed and cooled in a water bath. Fifteen ml of extraction mixture were then added to the tube and the contents carefully mixed. Precaution was taken to avoid vigorous mixing, as it resulted in the formation of a stable emulsion. The tube was centrifuged at 2000 r.p.m. for 5 minutes. The solvent layer was transferred to a test tube and anhydrous sodium sulfate was added to remove traces of moisture. The contents were filtered through Whatman No. 4 filter paper, the clear solvent collected in a cuvette and the optical density read at 535 mμ. To prevent the development of turbidity in the clear solvent, apparently caused by rapid cooling, the cuvettes were placed in a water bath at 37°C until the optical density readings were taken. A blank in which 10 ml of distilled water replaced the sample of milk was run along with the samples. A Coleman Junior Spectrophotometer, Model 6A, was used in this study. The results of the TBA determinations were expressed as μg of malonaldehyde per 10 gm sample, thus corresponding to the method used by Sinnhuber and Yu (133) with fish oil, and Day and Lillard (42) with milk fat. The preparation of the standard curve for malonaldehyde is described in Appendix 1.

2. Determination of oxidation-reduction potential

Of the two basic methods for measuring the Eh of a system, electrometric and colorimetric, the former is more reliable. In this study it was necessary to determine the rate of changes in Eh of milks under various treatments at different stages of oxidation. The procedure adopted for the determination of oxidation-reduction potentials of milks was similar to the method used by Webb and Hileman (160).

Procedure:

The oxidation-reduction potentials were determined using platinum electrodes designed after those used by Webb and Hileman (160), and a battery-operated Hellige Potentiometer. A saturated calomel half-cell (Beckman Instruments Inc.) was used as a reference. Several platinum electrodes were constructed and matched prior to use. Before and after use the electrodes were immersed in boiling detergent solution and thoroughly rinsed and stored in distilled water. Agar-KCl bridges were freshly prepared for each experiment by adding 3 gm of agar to 100 ml of saturated KCl solution and bringing it to a boil. While the agar was still warm, it was carefully drawn into a glass tubing, care being taken to avoid the introduction of air bubbles.

The milk samples were transferred to amber-colored pint bottles and the agar-KCl bridge and platinum electrodes introduced through a close-fitting rubber stopper. They were then stored at 1°C. The salt bridge and the platinum electrodes were not disturbed until the end of the experiment. Replicate samples for TBA and ascorbic acid determinations and for organoleptic evaluation were held in separate half-pint bottles. At the time of measurement of Eh, corresponding samples were taken for the other tests. To standardize the Eh measurements, each sample was kept at 1°C until ready for measurement. When the sample was connected to the potentiometer, a timer was set for 10 minutes and the reading taken after that period. The circuit was closed with the aid of two salt bridges. The salient features of the apparatus are shown in Figure A.

3. Determination of Total and Reduced Ascorbic Acid

The determination of ascorbic acid in milk is based on the use of the oxidation-reduction reaction between the reduced form of ascorbic acid and 2,6 dichloroindophenol, or a coupling of 2,4 dinitrophenylhydrazine with dehydroascorbic acid to form the derivative of bis 2,4 dinitrophenylhydrazone. In this study it was necessary to determine the ascorbic and dehydroascorbic acid contents

of milk at various stages of oxidation. The method adopted by Tobias and Herreid (152) was chosen for the determination of total ascorbic acid, and the indophenol method of determining reduced ascorbic was used.

Determination of total ascorbic acid:

Reagents:

Trichloroacetic acid solution. 4 per cent aqueous.

2,4 dinitrophenylhydrazine solution. 2 per cent in 9 N sulfuric acid.

Sulfuric acid. 85 per cent.

Thiourea.

Norit.

Procedure:

To 15 ml of a 4 per cent solution of trichloroacetic acid in a 50 ml centrifuge tube, 5 ml of milk were added with agitation. The tube was centrifuged and the supernatant whey was decanted into a beaker containing approximately 0.75 gm Norit. After thorough mixing, the suspension was filtered and 10 ml of the clear filtrate were transferred to a test tube containing 0.1 gm of reagent grade thiourea. For the determination of total ascorbic acid, 4 ml of the filtrate-thiourea mixture were transferred to a cuvette and 1 ml of a 2 per cent solution of 2,4 dinitrophenyl-hydrazine in 9 M sulfuric acid were added. The sample was

incubated at 37°C, for 3 hours and subsequently cooled in an ice bath. Five ml of 85 per cent sulfuric acid were added dropwise while the tube was still in the ice bath and the contents thoroughly mixed. The sample was then allowed to warm up to room temperature for 30 minutes and the optical density reading taken at 540 mμ.

A blank determination was performed on 4 ml of filtrate-thiourea mixture to which were added 5 ml of 85 per cent sulfuric acid, followed by 1 ml of 2,4 dinitrophenylhydrazine. The difference between the blank and the sample was that the blank was not incubated with 2,4 dinitrophenylhydrazine. A calibration curve was prepared by making up solutions of ascorbic acid at varying concentrations from 0.5 to 3.0 mg per 100 ml in 4 per cent trichloroacetic acid and proceeding the same as for total ascorbic acid (Appendix 2).

Determination of reduced ascorbic acid:

Reagents:

Indophenol dye solution. 0.1359 gm of sodium 2,6 dichloroindophenol were dissolved in 200 ml of hot water and made up to volume to 1 liter. The dye solution was standardized before using by titrating with a 5 ml aliquot of a freshly prepared solution of l-ascorbic acid (50 mg ascorbic acid per 500 ml copper-free, double-distilled

water) in 20 ml of 0.1 N sulfuric acid.

Trichloroacetic acid. 15 per cent aqueous.

Procedure:

Ten ml of milk were measured into a 50 ml centrifuge tube containing 15 ml of trichloroacetic acid, centrifuged for 3 minutes at 2000 r.p.m. and filtered through Whatman No. 4 filter paper. The clear filtrate was titrated immediately with the indophenol dye to a faint pink end-point persisting for at least 30 seconds. A blank was run with 10 ml of distilled water plus 15 ml of trichloroacetic acid titrated with indophenol dye to a faint pink end-point.

4. Amperometric Titration of Complexing Agents with Copper

The metal-complexing properties of naturally-occurring compounds have been studied by colorimetric methods, by observing shifts in the ultraviolet absorption spectra and by classical polarographical techniques. In this study it was necessary to determine the complexing abilities of substances widely differing in composition and physical make-up, and the polarographic method was chosen. The procedure followed was similar to the amperometric titration procedure described by Detty et al. (45).

Apparatus:

Sargent-Heyrovsky model XII Polarograph

Amperometric titration apparatus, consisting of saturated calomel cell, agar-KCl bridges, mercury reservoir, titration cell and nitrogen tank.

Reagents:

Sodium acetate buffer. 0.1 M adjusted to a pH of 6.6

Cupric sulfate. 0.01 M solution of $CuSO_4.5H_2O$

Complexing agents. 0.01 M solution of dihydroquercetin, propyl gallate and disodium salt of ethylenediaminetetracetic acid respectively.

Procedure:

One of the leads from the constant e.m.f. jack of the polarograph was connected to the mercury reservoir of a dropping mercury electrode and the other lead to a saturated calomel electrode. The two half-cells were connected by an agar-KCl bridge, which was introduced through a five-holed rubber stopper into a 250 ml electrolytic beaker. Figure B shows the apparatus assembled for use.

One hundred and twenty ml of 0.1 M sodium acetate buffer were transferred to the beaker and the solution was degassed with a vigorous stream of purified nitrogen. A 5 or 10 ml aliquot of complexing agent was pipetted into the cell and the resulting solution further degassed until

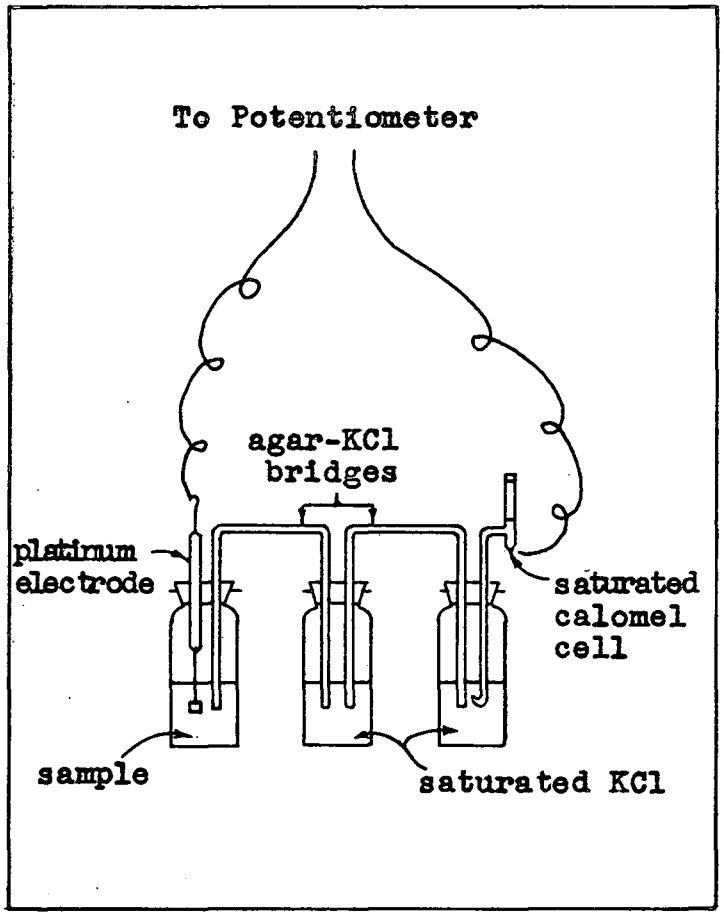


Figure A. Apparatus for determining oxidation-reduction potential

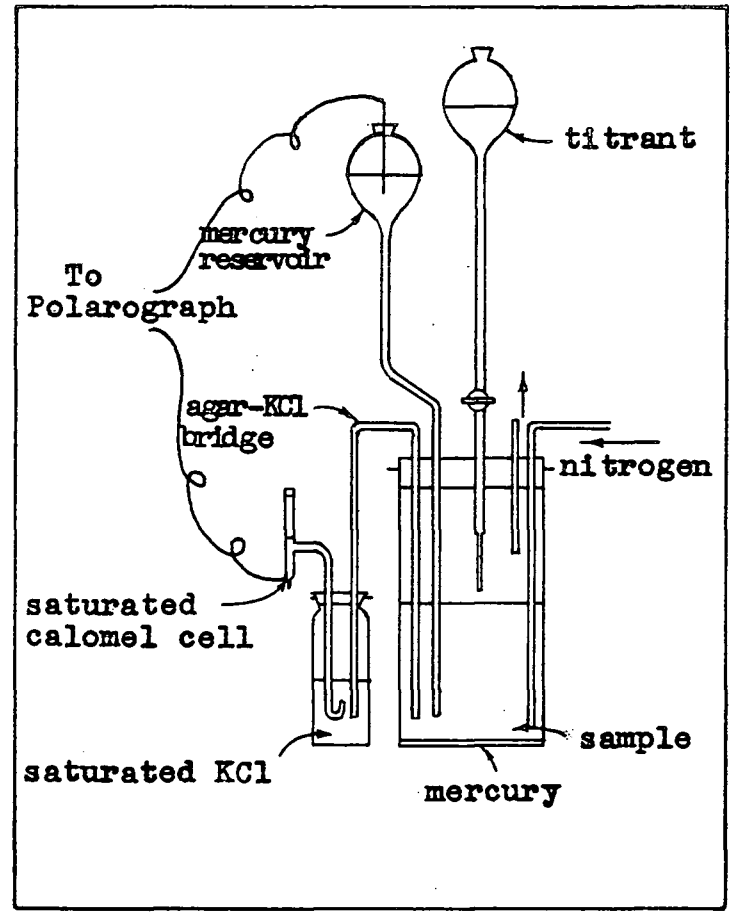


Figure B. Apparatus for amperometric titration

a constant current was again obtained at the constant voltage setting of the polarograph. The cupric sulfate solution was added in 0.1 to 0.5 ml increments from a 5 ml microburet. After each addition of the titrant, the cell solution was mixed by a 1 minute degassing period, following which the current reading of the polarograph was recorded. In order to obtain clear breaks in the current vs. volume curve for a given complexing agent, it was necessary to perform the titration at more than one galvanometer shunt ratio. In the titrations involving milk and milk fractions, it was necessary to use a larger titration cell in order to avoid the build-up of foam.

Determination of Xanthine Oxidase

The determination of xanthine oxidase involves the use of xanthine as a substrate and methylene blue as a hydrogen acceptor. The time required for the reduction of methylene blue to its colorless leuco-form serves as a measure of the enzyme concentration.

In this study the method of Rodkey and Ball (127) was adopted with minor modifications.

Reagents:

Methylene blue solution - 0.005 per cent aqueous.

Xanthine solution - 0.05 M xanthine in 0.05 M sodium

hydroxide.

Paraffin oil.

Procedure:

Two ml of milk were placed in a test tube and 0.5 ml of methylene blue added, followed by 0.2 ml of xanthine solution. Immediately after the addition of the xanthine substrate, 3 ml of paraffin oil were gently added to form a layer on top of the milk sample. The tube was carefully rotated to mix the contents and placed in a water bath maintained at 40°C. The layer of paraffin oil prevents access of air, which would re-oxidize the leuco methylene blue as it is formed. A blank was run with each sample to rule out the possibility of reduction caused by bacterial activity. The blanks differed from the samples in that they contained all the addenda except the substrate. Samples were run in duplicate and the reduction time noted by timing the reaction from the moment of addition of the substrate.

Organoleptic Examination of Milk and Milk Products

For the fluid products milk, cream and buttermilk, organoleptic evaluations were made by three trained judges. Essentially the same judges participated in the tests during the entire course of this study. The milks were allowed to warm to room temperature after removal

from cold storage and scored as unknowns.

Reconstituted Products:

Dried whole milk and dried sweet-cream buttermilk were reconstituted to 14 per cent and 9 per cent total solids respectively in distilled water previously warmed to 145°F. The powders were stirred gently to avoid foaming. As the powders reconstituted well, there was no need for filtering or clarifying. After samples were taken for TBA and ascorbic acid determinations, the milks were stored at 1°C for 4 hours prior to organoleptic evaluation in copper-free, polyethylene containers fitted with plastic lids. During this storage period the flavor of freshly-prepared powder was dissipated, facilitating the detection of other flavors.

Preparation of Fluid Milk and Milk Products

For the experiment on the incidence of spontaneous oxidation, individual samples from 14 cows were obtained through the courtesy of the Field Department, Dairy Cooperative Association, Portland, Oregon, at regular intervals between the months of February and June, 1960.

The milks were received in sterilized quart containers in the morning and were tested for fat content and xanthine oxidase activity upon arrival. Organoleptic evaluations

and TBA determinations were made initially and after 72 hours storage at 1 to 2°C.

For the study on the effect of added dihydroquercetin on the resistance of milk to spontaneous oxidation, milks from four cows which consistently developed spontaneous oxidized flavor were used for organoleptic evaluations and TBA determinations.

For the study of the effect of added dihydroquercetin on the resistance of spray-dried whole milk powder to spontaneous oxidation, two gallons of milk from one cow producing spontaneously oxidizable milk were spray dried as described under preparation of dried products.

The study of copper-resistant and copper-sensitive milk, cream, and buttermilk was made during the summers of 1960 and 1961, from milks of individual Holstein cows belonging to the Oregon State University herd which had been selected on the basis of the reaction of their milks to copper. Advantage was taken of the experimental conditions of a long-term mastitis control experiment in progress, under which a group of cows were kept on dry-lot feed on year-round basis.

Preparation of Fluid Milk Products:

The milk was received in stainless steel containers, pasteurized immediately at 145°F for 30 minutes and

separated. The cream was held overnight at 1°C, and was churned the next morning. The buttermilk, as well as a portion of cream before churning, was saved for the experiment.

For the study of dried whole milk and buttermilk the fluid products were held in stainless steel containers or amber-glass bottles at 1°C until ready for drying.

Preparation of Dried Products:

The fluid product was forewarmed to 145°F before drying. Since the capacity of the drier was one gallon per hour, forewarming was restricted to one quart at a time to prevent prolonged exposure to light or cooling.

The drying of fluid whole milk, skim milk, and buttermilk was done in the Dairy Products Laboratory at Oregon State University, using the laboratory spray drier which had been recently installed. The machine was made by Anhydro A/S, Danish Dehydrating Company Ltd.

The principle involves the dispersion of the fluid product by a rotary atomizer as a fine spray into a chamber warmed by hot, dry air. Drying takes place almost instantaneously and the powder settles to the bottom of the drying chamber, from whence it is removed along with the moist air to a cyclone filter where the powder is separated and deposited in the collecting jar.

Storage of Powders:

The powders were stored in copper-free containers. Twenty-four hours after drying, samples were taken for determination of moisture and estimation of denatured serum protein (as a measure of heat treatment). The powders were stored at room temperature in diffuse light in order to shorten the duration of the stability tests, since they showed prolonged storage stability when held in cold storage.

Moisture Determination:

The procedure followed was the method outlined in Methods of Analysis, A.O.A.C. (6). It consisted of weighing 1 to 1.5 gm of the powder sample into a stainless steel dish and drying to a constant weight (approximately 5 hours) at a pressure not exceeding 100 mm. at the temperature of boiling water. During drying, a slow current of air (about two bubbles per second) dried by passing through concentrated sulfuric acid was admitted into the oven.

Estimation of Denatured Serum Protein:

The volumetric method of estimating undenatured serum protein in milk as described by Anderson and Bell (3) was used.

Reagents:

10 per cent acetic acid in distilled water: 10 ml glacial acetic acid to 90 ml distilled water.

10 per cent phosphotungstic acid: 10 gm of phosphotungstic acid is diluted to 100 ml.

Procedure:

A 10 gm sample (non-fat dry milk) was weighed into a 300 ml Erlenmeyer flask. For dry whole milk the non-fat solids should equal those in 10 gm of non-fat dry milk. Ninety-three ml of distilled water were added. The flask was swirled gently for 10 minutes and 7 ml of a 10 per cent solution of acetic acid were added to the flask with gentle mixing. Care was taken not to break the curd to avoid difficult filtration. The precipitate was allowed to stand for 3 minutes before filtering through a Whatman No. 3 filter paper. Re-filtration was resorted to whenever the filtrate appeared turbid.

Seven ml of the filtrate were transferred to a 15 ml graduated, conical centrifuge tube and 3 ml of a 10 per cent phosphotungstic acid added. The suspension was stirred with a glass rod to insure uniform packing during subsequent centrifuging. The tube was then centrifuged for 25 minutes at 2000 r.p.m. The centrifuge was then brought to a gradual stop and the volume of precipitate was read directly and expressed as such, or as mg

undenatured whey protein nitrogen per gm non-fat dry milk, using the standard curve derived by Anderson and Bell (3).

EXPERIMENTAL RESULTS

1. Xanthine oxidase activity and fat test of individual milks from cows under dry-lot feed conditions and subsequent pasture feed, as related to the incidence of spontaneous oxidation.

A group of 14 Holstein cows at different stages of lactation was made available for this experiment through the courtesy of the Field Dept. Dairy Coop, Portland. Samples were collected soon after morning milking and delivered to the Dairy Products Laboratory at regular intervals. The xanthine oxidase activity and fat tests were determined on receipt of the samples and flavor evaluation made after 72 hours storage at 1°C.

The results of the experiment are shown in Table 1. The xanthine oxidase activity increased with the progress of lactation. There was no perceptible relationship between the xanthine oxidase activity and the susceptibility of the milks to undergo spontaneous oxidation. There appeared to be no correlation between fat test and xanthine oxidase activity. Spontaneous oxidized flavor was encountered most frequently in milks from cows in the second and third months of lactation, in samples showing considerably less xanthine oxidase activity than milks from late lactation cows.

TABLE 1

Xanthine oxidase activity[a] and fat test of individual milks as related to their susceptibility to the development of spontaneous oxidized flavor during storage at 1°C for 72 hours

Cow No.	Stage of lactation (months)	Feb. 29, 1960 Fat test (%)	Feb. 29, 1960 Xanthine oxidase activity	Feb. 29, 1960 Flavor	March 21, 1960 Fat test (%)	March 21, 1960 Xanthine oxidase activity	March 21, 1960 Flavor[b]
1	early	3.5	0.0548	-	3.5	0.0757	-
2	2	3.8	0.0800	+ +	3.7	0.0809	+ +
3	2	3.5	0.0930	+ +	3.4	0.0947	+ +
4	2	4.4	0.0881	+ +	4.5	0.0909	+ +
5	3	4.4	0.0826	+ +	4.6	0.0847	+ +
6	3	3.5	0.0869	-	3.2	0.0930	-
7	4	3.5	0.1052	-	3.3	0.1092	-
8	5	2.9	0.1081	+	3.0	0.1183	+
9	5	3.5	0.1025	-	3.1	0.1081	-
10	6	3.5	0.1204	+	3.5	0.1324	-
11	7	3.7	0.1273	+	3.5	0.1388	+
12	7	3.8	0.1369	-	3.6	0.1526	-
13	8	3.4	0.1612	+ +	3.2	0.1739	+
14	9	3.4	0.1923	-	(ceased milking)		

TABLE 1, continued

Cow No.	Stage of lactation (months)	April 23, 1960 Fat test (%)	Xanthine oxidase activity	Flavor	May 6, 1960 [c] Fat test (%)	Xanthine oxidase activity	Flavor [b]
1	2	3.1	0.0769	-	3.1	0.1069	-
2	3	3.2	0.0816	+ +	2.7	0.1379	-
3	3	3.7	0.1000	+ +	3.1	0.1587	-
4	4	4.7	0.0930	+ +	4.4	0.1666	-
5	4	4.9	0.0896	+ +	4.4	0.1626	-
6	5	3.5	0.0985	-	3.3	0.1869	-
7	6	3.5	0.1142	-	2.5	0.2000	-
8	7	3.4	0.1226	-	2.4	0.2000	-
9	7	3.3	0.1169	-	3.4	0.1818	-
10	8	3.6	0.1438	-	3.5	0.2222	-
11	9	3.8	0.1626	-	3.4	0.2409	-
12	9	3.7	0.1709	-	3.6	0.2325	-
13	9	3.3	0.2020	-	(ceased milking)		

[a] Xanthine oxidase activity expressed as the reciprocal of the time in minutes required for the reduction of methylene blue when 0.2 ml of 0.05 M xanthine solution is added as substrate to 2.0 ml sample of milk. Purified xanthine furnished by Dr. T. E. King, Science Research Institute, Oregon State University.

[b] Flavor evaluation: - no oxidized flavor detectable
++ pronounced oxidized flavor

[c] Cows on pasture since April 30, 1960

The animals were on dry lot feed until April 30, 1960, and then allowed to go on pasture. Pasture feeding was accompanied by increased activity of xanthine oxidase in all milks. The resistance of the milks to spontaneous oxidation, however, was enhanced. Figures 1 and 2 are scatter diagrams showing xanthine oxidase activity of milks produced under the two feeding conditions, and the incidence of spontaneous oxidation.

The milk samples 2, 3, 4 and 5 consistently developed oxidized flavor on storage, and these milks were used in an experiment to determine the effectiveness of dihydroquercetin to inhibit the oxidation. The results of this experiment are shown in Table 2. The thiobarbituric acid test and flavor evaluation indicate that dihydroquercetin added at 5 mg per cent level to the milks effectively inhibited the development of oxidized flavors. A change of feeding conditions from dry-lot feed to pasture was likewise accompanied by a decrease in the incidence of spontaneous oxidation. The experiment was concluded in May, 1960, after the animals were on pasture for two weeks.

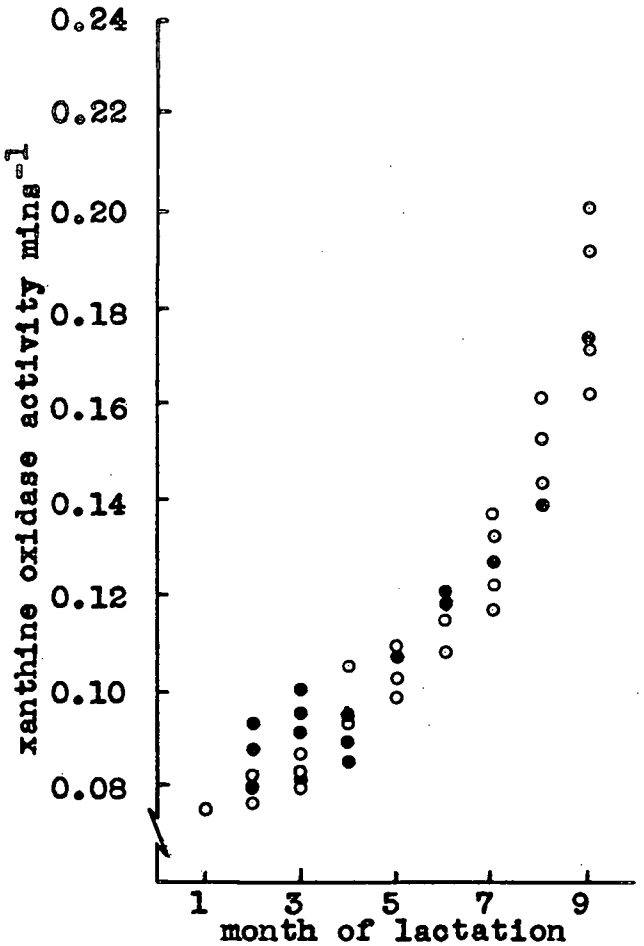


Figure 1. Xanthine oxidase activity of milks from cows on dry-lot feed, and the incidence of spontaneous oxidized flavors.

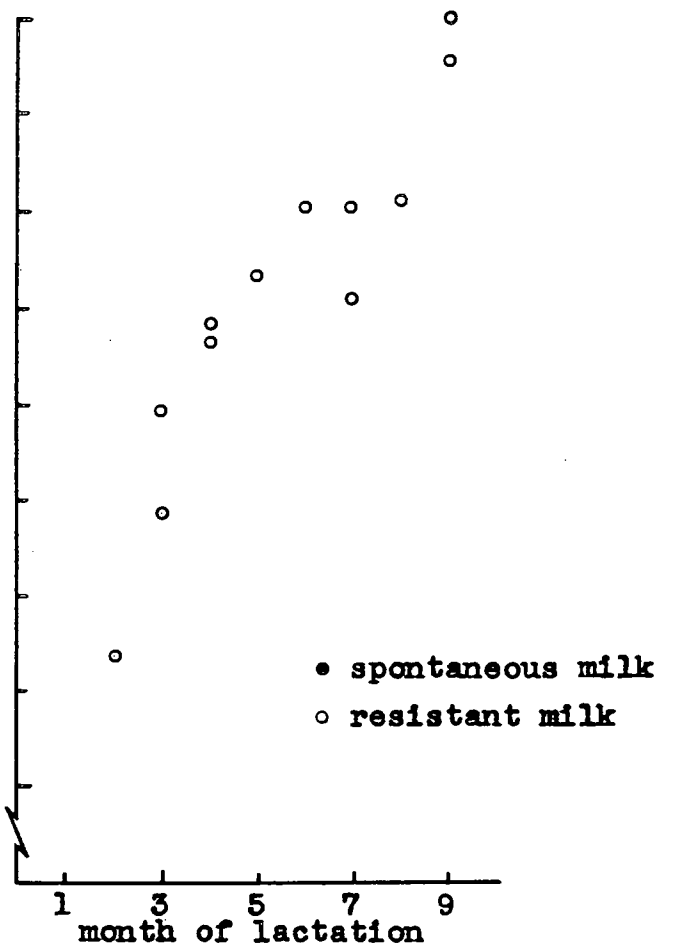


Figure 2. Xanthine oxidase activity of milks from cows on pasture, and the resistance of milks to spontaneous oxidation.

TABLE 2

The effect of added dihydroquercetin (DHQ)[a], on the resistance of individual milks to the development of spontaneous oxidized flavor during storage at 1°C for 96 hours, as measured organoleptically[b] and by the thiobarbituric acid (TBA) test[c]

Cow No.	Months of lactation	Treatment	0 hour		48 hours		96 hours	
			TBA	Flavor	TBA	Flavor	TBA	Flavor
2	2	Control	5.00 (0.018)	-	11.25 (0.041)	-	21.0 (0.076)	++
		+ DHQ	5.00 (0.018)	-	8.75 (0.031)	-	11.5 (0.042)	-
3	2	Control	7.00 (0.026)	-	15.75 (0.058)	-	22.25 (0.081)	++
		+ DHQ	7.00 (0.026)	-	11.00 (0.040)	-	13.75 (0.050)	-
4	2	Control	5.25 (0.019)	-	12.75 (0.046)	-	17.00 (0.061)	+
		+ DHQ	5.25 (0.019)	-	8.75 (0.031)	-	9.00 (0.032)	-
5	4	Control	5.85 (0.021)	-	19.75 (0.071)	++	25.25 (0.091)	++
		+ DHQ	5.85 (0.021)	-	14.00 (0.051)	-	19.00 (0.068)	+
12	8	Control	5.00 (0.018)	-	9.75 (0.035)	-	12.75 (0.046)	-
		+ DHQ	5.00 (0.018)	-	8.50 (0.030)	-	11.25 (0.041)	-

a/ DHQ dissolved in warm distilled water and added to milk at the level of 5 mg %

b/ Flavor evaluation: - oxidized flavor not detectable; ++ pronounced oxidized flavor

c/ TBA results expressed as µg malonaldehyde per 10 ml sample. Optical density readings are given in parenthesis.

2. The effect of added dihydroquercetin on the resistance of pasteurized whole milk, cream and buttermilk to copper plus ascorbic acid-induced oxidation.

This experiment was conducted in order to ascertain the effectiveness of dihydroquercetin in certain milk products. Composite milk of Holstein cows on pasture at the Dairy Barn, Oregon State University, was used in this experiment. The milk exhibited resistance to copper at the level of 2 ppm. Hence, a strongly pro-oxidant combination of copper plus ascorbic acid was employed to induce the development of oxidized flavor. Copper at the level of 0.1 ppm, with 20 mg/l of ascorbic acid, was found to be the minimum level of copper required to induce the flavor defect. The milk was pasteurized soon after receipt, a portion separated, and the cream held overnight at 1°C. The next morning the cream was churned and the buttermilk obtained. To each fraction copper and ascorbic acid were added. Tables 3, 4 and 5 show the changes in oxidation-reduction potential, TBA values and flavor of milk, cream and buttermilk, respectively, resulting from copper plus ascorbic acid-induced oxidation, and as influenced by added dihydroquercetin. In the three products examined, dihydroquercetin added at the level of 5 mg per cent, was effective in inhibiting the development of oxidized flavor. The addition of ascorbic acid

TABLE 3

The effect of added dihydroquercetin (DHQ)[a] on the resistance of pasteurized whole milk held at 1°C for 72 hours, to copper plus ascorbic acid[b] catalyzed oxidative deterioration, as measured organoleptically[c] by thiobarbituric acid (TBA) test[d] and the corresponding changes in the oxidation-reduction potentials

Treatment	0 hour			24 hours			48 hours			72 hours		
	TBA	Eh in m volt	Flavor	TBA	Eh in m volt	Flavor	TBA	Eh in m volt	Flavor	TBA	Eh in m volt	Flavor[d]
Milk control	7.00 (0.026)	281	-	7.00 (0.026)	281	-	8.25 (0.030)	286	-	8.75 (0.031)	296	-
Milk + Cu + Asc.A.	7.00 (0.026)	266	-	11.00 (0.040)	291	-	14.00 (0.051)	346	++	19.00 (0.068)	361	++
Milk + Cu + Asc.A. + DHQ	7.00 (0.026)	266	-	8.75 (0.031)	296	-	10.00 (0.037)	326	-	11.25 (0.041)	341	-
Milk + DHQ	7.00 (0.026)	276	-	7.00 (0.026)	276	-	8.25 (0.030)	281	-	8.25 (0.030)	281	-

a/ DHQ dissolved in warm distilled water and added to milk at the level of 5 mg %

b/ The milk having exhibited resistance to copper, a pro-oxidant combination of 0.1ppm copper plus 20 mg/l ascorbic acid was employed to induce oxidation.

c/ Flavor evaluation: - oxidized flavor not detectable; ++ pronounced oxidized flavor

d/ TBA results are expressed as µg malonaldehyde per 10 ml sample. The optical density readings are given in parenthesis.

TABLE 4

The effect of added dihydroquercetin (DHQ)[a] on the resistance of pasteurized sweet cream[b], held at 1°C for 72 hours, to copper plus ascorbic acid catalyzed oxidative deterioration, as measured organoleptically[c] by thiobarbituric acid (TBA) test[d] and the corresponding changes in oxidation-reduction potentials

Treatment	0 hour			24 hours			48 hours			72 hours		
	TBA	Eh in m volt	Flavor	TBA	Eh in m volt	Flavor	TBA	Eh in m volt	Flavor	TBA	Eh in m volt	Flavor[c]
Cream control	11.00 (0.040)	276	-	11.00 (0.041)	280	-	11.00 (0.041)	286	-	11.00 (0.041)	288	-
Cream + Cu + Asc.A.	11.00 (0.041)	261	-	11.45 (0.052)	298	+	18.25 (0.065)	316	++	21.25 (0.078)	340	++
Cream + Cu + Asc.A. + DHQ	11.00 (0.041)	261	-	11.25 (0.045)	286	-	15.00 (0.054)	301	-	17.00 (0.060)	320	-
Cream + DHQ	11.00 (0.041)	276	-	11.00 (0.041)	276	-	11.00 (0.041)	280	-	11.00 (0.041)	280	-

a/ DHQ dissolved in warm distilled water and added to cream at the level of 5 mg %

b/ The cream having exhibited resistance to copper, a pro-oxidant combination of 0.1 ppm copper plus 20 mg/l ascorbic acid was employed to induce oxidation.

c/ Flavor evaluation: - oxidized flavor not detectable; ++ pronounced oxidized flavor

d/ TBA results are expressed as µg malonaldehyde per 10 ml sample. The optical density readings are given in parenthesis.

TABLE 5

The effect of added dihydroquercetin (DHQ)[a] on the resistance of churned buttermilk from sweet cream, held at 1°C for 48 hours, to copper plus ascorbic acid[b] catalyzed oxidative deterioration, as measured organoleptically[c] by thiobarbituric acid (TBA) test[d] and the corresponding changes in oxidation-reduction potentials

Treatment	0 hour			24 hours			48 hours			72 hours		
	TBA	Eh in m volt	Flavor	TBA	Eh in m volt	Flavor	TBA	Eh in m volt	Flavor	TBA	Eh in m volt	Flavor
Buttermilk control	10.00 (0.037)	271	-	10.00 (0.037)	281	-	10.00 (0.037)	296	-	11.00 (0.041)	306	-
Buttermilk + Cu + Asc. A.	10.00 (0.037)	261	-	14.00 (0.051)	406	+	19.00 (0.068)	431	++	25.25 (0.091)	478	++
Buttermilk + Cu + Asc. A. + DHQ	10.00 (0.037)	249	-	10.00 (0.037)	296	-	11.00 (0.041)	376	-	14.00 (0.051)	401	+
Buttermilk + DHQ	10.00 (0.037)	271	-	10.00 (0.037)	276	-	10.00 (0.037)	276	-	10.00 (0.037)	286	-

a/ DHQ dissolved in warm distilled water and added to buttermilk at the level of 5 mg%

b/ Copper was added to buttermilk at the rate of 0.1 ppm and ascorbic acid at the level of 20 mg/l

c/ Flavor evaluation: - oxidized flavor not detectable; ++ pronounced oxidized flavor

d/ TBA results are expressed as µg malonaldehyde per 10 ml sample. The optical density readings are given in parenthesis.

was accompanied by an initial decrease in the Eh of the product as compared to the control. This decrease, however, was noted only at 0 hour. The addition of dihydroquercetin to the products containing copper plus ascorbic acid failed to prevent the increase in Eh during storage while, in the samples without added copper, the Eh remained unchanged or increased only slightly. The greatest increase in Eh was noted in samples containing only copper plus ascorbic acid. It appeared that the increase in Eh could be attributed to the oxidation of ascorbic acid. As in the previous experiment, the thiobarbituric acid values were in good agreement with the flavor evaluations.

3. The storage stability of spray-dried, spontaneously-oxidizable, copper-sensitive and copper-resistant milks and buttermilk, as influenced by added dihydroquercetin.

Having observed a marked inhibitory effect of dihydroquercetin toward spontaneous and copper-induced oxidation in milk and milk products, it was of interest to ascertain the carry-over properties of dihydroquercetin in milk products subjected to heat processing.

The products were dried in the Dairy Products Laboratory, using a laboratory spray drier. Copper was

added at the level of 0.1 ppm and dihydroquercetin at the level of 20 mg per cent to all fluid products, with the exception of spontaneous milk, which received no copper treatment. The study of spontaneous milk was conducted during the six month period commencing April, 1960, to October, 1960, and the rest of the products were studied during the period commencing March, 1961, to September, 1961. The spontaneous milk, after drying, was packed in brown bottles and stored at 1°C for 4 months, after which period they were transferred to storage at room temperature. The copper-sensitive and copper-resistant milks and buttermilk, on the other hand, were packed in polyethylene containers and stored at room temperature, exposed to diffused light.

The results in Table 6 show the stability of spontaneous milk during the period of storage at 1°C, and the rapid deterioration which ensued during the period of storage at room temperature, as measured organoleptically and by the TBA test on the reconstituted product. In all samples with added copper, a marked increase in the TBA values was observed during storage, which was accompanied by the development of oxidized flavor. Dihydroquercetin effectively inhibited the development of oxidized flavor during the period of study.

TABLE 6

The storage stability as measured organoleptically[a] and by the thiobarbituric acid (TBA) test[b] of spray dried spontaneously oxidizable, copper-sensitive and copper-resistant milks, as influenced by added dihydroquercetin (DHQ)[c]

Dried product Treatment	Initial		1 month		2 months		4 months		6 months	
	TBA	Flavor	TBA	Flavor	TBA	Flavor	TBA	Flavor	TBA	Flavor
Spontaneous milk										
Control	2.5 (0.009	-	3.0 (0.01)	-	5.0 (0.018)	-	5.5 (0.02)	-	14.1 (0.051)	++
+ DHQ	2.5 (0.009)	-	3.7 (0.014)	-	3.7 (0.014)	-	5.5 (0.02)	-	8.25 (0.029)	-
Copper-susceptible milk										
Control	2.5 (0.009)	-	3.0 (0.014)	-	3.0 (0.014)	-	5.0 (0.018)	-	5.5 (0.02)	-
+ Cu	3.5 (0.012)	-	5.5 (0.02)	-	11.25 (0.041)	+	13.25 (0.048)	++	17.2 (0.061)	++
+ Cu+DHQ	2.5 (0.009)	-	4.5 (0.016)	-	6.25 (0.022)	-	9.0 (0.031)	-	10.0 (0.036)	-
Copper-resistant milk										
Control	1.75 (0.006)	-	2.5 (0.009)	-	3.0 (0.014)	-	3.0 (0.014)	-	5.0 (0.018)	-
+ Cu	3.0 (0.01)	-	3.7 (0.014)	-	5.5 (0.02)	-	8.25 (0.029)	-	11.2 (0.04)	+
+ Cu+DHQ	1.75 (0.006)	-	3.5 (0.012)		4.5 (0.016)	-	6.25 (0.022)		9.25 (0.032)	-
Buttermilk										
Control	4.0 (0.015)	-	5.5 (0.02)	stale	6.25 (0.022)	stale				
+ Cu	7.75 (0.028)	-	13.25 (0.048	++	21.0 (0.076)	++				

TABLE 6, continued

Dried product Treatment	Initial		1 month		2 months		4 months		6 months	
	TBA	Flavor	TBA	Flavor	TBA	Flavor	TBA	Flavor	TBA	Flavor
Buttermilk + Cu+DHQ	5.5 (0.02)	-	7.0 (0.025)	stale	10.0 (0.036)	stale				

a/ Powders subjected to accelerated storage stability test at room temperature, in diffused light, except spontaneous milk, which was kept at 1°C until the fourth month. Flavor evaluation: - oxidized flavor not detectable; ++ pronounced oxidized flavor.

b/ TBA results expressed as µg malonaldehyde per 10 ml reconstituted sample.

c/ DHQ dissolved in warm distilled water and added to milk prior drying at the level of 20 mg %.

In order to ascertain if the inhibitory effect was attributable to dihydroquercetin and not to the reducing groups liberated by the heat treatment, a determination of undenatured whey protein (U.W.P.N.) was made on the powders. The powders compare favorably with the low-heat reference powder from the American Dry Milk Institute.[1]

In the case of dry buttermilk, dihydroquercetin appeared to inhibit copper-induced oxidation, as could be determined by TBA measurements, but the storage of this product was accompanied by the rapid development of a

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[1] Undenatured whey protein and moisture contents of certain spray-dried milk products.

Sample	Volume of ppt - ml	U.W.P.N. mg/gm N.F.D.M.	Moisture
1. Whole milk	1.0	8.75	2.36
2. Pre-condensed whole milk	0.3	1.50	2.88
3. Skim milk	1.1	9.0	3.46
4. Buttermilk	1.0	8.75	6.00
5. Low-heat skim milk*	-	8.12	-
6. Hi-heat skim milk**	-	0.7	-

* and ** Standard skim powders were obtained courtesy of American Dry Milk Institute. The suggested minimum for dried skim powder used for cottage cheese is 6 mg U.W.P.N./ gm N.F.D.M.

pronounced stale, but not oxidized flavor.

4. A study of the comparative effects of dihydroquercetin, propyl gallate and disodium salt of ethylenediamine-tetraacetic acid[2] on the rate of copper-catalyzed oxidation of ascorbic acid, and the changes in Eh, and flavor of individual milks.

Having established the effectiveness of DHQ in a variety of fluid and dried milk products, this part of the study was devoted to the elucidation of the role of DHQ as an inhibitor of oxidation in milk. In order to gain some insight on the phenomenon of inhibition of oxidation, PG and EDTA were included in this experiment. The individual milks were obtained from Holstein cows of the O.S.U. dairy herd, maintained either on dry-lot feed or on pasture.

Table 7 shows the rate of copper-induced oxidation of ascorbic acid, and the changes in Eh and flavor during storage of individual milks as influenced by added DHQ, PG and EDTA, and by feeding conditions. The milks from cows on pasture appeared to resist copper at the level of 0.1 ppm, while the milks from dry-lot fed cows developed oxidized flavor with the same level of added copper. The development of oxidized flavor was inhibited by the

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[2] Dihydroquercetin is henceforth denoted as DHQ, propyl gallate as PG, and disodium salt of ethylenediamine-tetraacetic acid as EDTA.

TABLE 7

The changes in oxidation-reduction potential and ascorbic acid contents of milks from individual cows on dry-lot feed and pasture with added copper[a] alone and in the presence of dihydroquercetin (DHQ), propyl gallate (PG), and disodium salt of ethylenediaminetetracetic acid (EDTA)[b] respectively during storage for 24 hours at 1°C, and the changes in flavor of the milks during storage for 72 hours at 1°C

Cow No.	Stage of lactation	Treatment	0 hour		24 hours		% increase in Eh	Flavor[c]
			Eh in m volt	Reduced AA mg/l	Eh in m volt	Reduced AA mg/l		
645	Early	Control	268	17.62	288	14.74	7.4	-
		+Cu	268	17.62	476	0.00	79.1	-
		+Cu+DHQ	268	17.62	416	0.00	55.2	-
		+Cu+PG	268	17.62	376	0.00	40.3	-
		+Cu+EDTA	268	17.62	416	0.00	55.2	-
688	Early	Control	256	19.87	326	9.61	27.3	+
		+Cu	256	19.87	486	0.00	89.8	+ +
		+Cu+DHQ	256	19.87	386	0.00	50.7	-
		+Cu+PG	256	19.87	331	0.00	29.2	-
		+Cu+EDTA	256	19.87	416	0.00	50.7	+
694	Late	Control	266	17.94	291	15.38	9.3	-
		+Cu	266	17.94	471	0.00	77.0	+ +
		+Cu+DHQ	266	17.94	411	0.00	54.5	-
		+Cu+PG	266	17.94	326	0.00	31.9	-
		+Cu+EDTA	266	17.94	416	0.00	56.3	-
700	Late	Control	266	16.34	282	14.42	6.0	-
		+Cu	266	16.34	466	0.00	75.1	+ +
		+Cu+DHQ	266	16.34	396	0.00	48.8	-
		+Cu+PG	266	16.34	346	0.00	30.0	-
		+Cu+EDTA	266	16.34	396	0.00	48.8	-

TABLE 7, continued

Cow No.[d]	Stage of lactation	Treatment	0 hour		24 hours		% increase in Eh	Flavor[c]
			Eh in m volt	Reduced AA mg/l	Eh in m volt	Reduced AA mg/l		
606	Early	Control	296	14.74	296	12.18	0.0	-
		+Cu	296	14.74	416	0.00	40.5	-
		+Cu+DHQ	296	14.74	386	0.00	30.4	-
		+Cu+PG	296	14.74	336	0.00	13.5	-
		+Cu+EDTA	296	14.74	406	0.00	37.1	-
611	Late	Control	256	12.82	286	8.83	11.7	-
		+Cu	256	12.82	396	0.00	54.6	-
		+Cu+DHQ	256	12.82	341	0.00	33.2	-
		+Cu+PG	256	12.82	316	0.00	23.4	-
		+Cu+EDTA	256	12.82	381	0.00	48.8	-
629	Late	Control	246	13.56	261	10.08	6.0	-
		+Cu	246	13.56	356	0.00	44.7	-
		+Cu+DHQ	246	13.56	336	0.00	36.5	-
		+Cu+PG	246	13.56	291	0.00	18.2	-
		+Cu+EDTA	246	13.56	341	0.00	38.6	-
670	Mid	Control	256	14.35	281	11.62	8.8	-
		+Cu	256	14.35	396	0.00	54.6	-
		+Cu+DHQ	256	14.35	351	0.00	37.1	-
		+Cu+PG	256	14.35	311	0.00	21.4	-
		+Cu+EDTA	256	14.35	366	0.00	42.9	-

[a] $CuSO_4 \cdot 5H_2O$ dissolved in distilled water and added to milk at the level of 0.1 ppm

[b] DHQ, PG and EDTA dissolved in warm distilled water and added to milk at the level of 5 mg %

[c] Flavor evaluation: - oxidized flavor not detectable
+ oxidized flavor detectable
+ + pronounced oxidized flavor

[d] Cows Nos. 606, 611, 629 and 670 were maintained on pasture.

addition of DHQ, PG or EDTA except in the case of milk from cow No. 688, where EDTA failed to inhibit the oxidation, suggesting perhaps that the amount of inhibitor was inadequate. The copper-catalyzed oxidation of ascorbic acid was rapid and complete, regardless of the feeding conditions or the presence of added inhibitors. The oxidation of ascorbic acid was accompanied by a marked rise in Eh. The initial content of ascorbic acid was lower, and its rate of oxidation slower, in milks produced by cows on pasture, than milk from cows on dry-lot feed. The latter milk appeared to be more poorly poised with respect to copper-induced changes in Eh, than milk from cows on pasture. Copper added at the level of 0.1 ppm to milks from dry-lot cows resulted in a 75 per cent increase of Eh during 24 hours storage over the initial value observed, while under the same conditions milks from cows on pasture showed increases in Eh after 24 hours storage of less than 54 per cent of the initial value. Similar results were obtained when the amount of copper added was reduced to 0.01 ppm with milks from dry-lot fed cows.

In this experiment, the amount of copper added apparently was far in excess of the amount needed to catalyze the oxidation of ascorbic acid, and the amount of EDTA added appeared to be inadequate for the inhibition of

copper-catalyzed oxidation of ascorbic acid. Hence, in the subsequent experiment, milks from cows on dry-lot feed were chosen and copper was added at the level of 0.01 ppm. DHQ and PG were added at the level of 30 mg per cent, while EDTA was added at the level of 200 mg per cent. The results of this experiment are shown in Table 8. The rate of copper-catalyzed oxidation of ascorbic acid was markedly decreased by added EDTA, while DHQ and PG failed to alter the rate of copper-catalyzed oxidation of ascorbic acid. Organoleptic examination and pH determinations were made after 72 hours storage. The added inhibitors effectively prevented the onset of oxidized flavor. The samples with added PG, however, were scored as bitter. The pH of milks containing added copper, DHQ or PG were essentially the same as control samples. The milks with added EDTA, however, showed a marked decrease in pH over the other samples. The significance of the results shown in Tables 7 and 8 to the overall phenomenon of inhibition will be treated in the next section.

5. The changes in flavor and total and reduced ascorbic acid contents of spontaneously-oxidizable and copper-susceptible milks as influenced by added dihydroquercetin.

This experiment was conducted to ascertain the

TABLE 8

The changes in oxidation-reduction potential, total and reduced ascorbic acid contents of milks from individual cows on dry-lot feed with added copper[a], alone and in the presence of DHQ, PG and EDTA[b] respectively during storage for 24 hours at 1°C, and the pH and flavor[c] of the milks after 72 hours storage at 1°C

Cow No.	Treatment	0 hour			24 hours			72 hours	
		Eh in m volt	Total AA mg/l	Reduced AA mg/l	Eh in m volt	Total AA mg/l	Reduced AA mg/l	pH	Flavor
645	Control	266	20.50	17.65	281	20.00	13.84	6.60	-
	+Cu	266	20.50	17.65	436	20.50	0.00	6.60	-
	+Cu+DHQ	266	20.50	17.65	401	20.50	2.80	6.65	-
	+Cu+PG	266	20.50	17.65	276	20.50	3.00	6.70	-(bitter)
	+Cu+EDTA	266	20.50	17.65	406	20.50	11.45	5.80	-
688	Control	276	22.00	18.10	316	21.50	10.40	6.75	+
	+Cu	276	22.00	18.10	491	21.50	0.00	6.70	++
	+Cu+DHQ	276	22.00	18.10	426	22.00	0.00	6.70	-
	+Cu+PG	276	22.00	18.10	311	22.00	0.00	6.80	-(bitter)
	+Cu+EDTA	276	22.00	18.10	431	22.00	8.14	5.95	-
694	Control	276	20.15	17.30	291	19.50	14.12	6.65	-
	+Cu	276	20.15	17.30	441	20.00	0.00	6.65	+
	+Cu+DHQ	276	20.15	17.30	411	20.00	1.90	6.65	-
	+Cu+PG	276	20.15	17.30	286	20.00	2.20	6.70	-(bitter)
	+Cu+EDTA	276	20.15	17.30	406	20.00	12.28	5.90	-
700	Control	261	24.80	18.45	286	24.00	15.02	6.80	-
	+Cu	261	24.80	18.45	456	24.50	0.00	6.75	++
	+Cu+DHQ	261	24.80	18.45	421	24.50	0.00	6.80	-
	+Cu+PG	261	24.80	18.45	276	24.50	0.00	6.85	-(bitter)
	+Cu+EDTA	261	24.80	18.45	411	24.50	11.85	5.75	-

a/ $CuSO_4 \cdot 5H_2O$ dissolved in distilled water and added to milk at the level of 0.01 ppm

b/ DHQ and PG dissolved in warm distilled water and added to milk at the level of 30 mg %, and EDTA added to milk at the level of 200 mg %

c/ Flavor evaluation: - oxidized flavor not detectable; + oxidized flavor detectable
++ pronounced oxidized flavor

relationship between reduced ascorbic acid and dehydroascorbic acid levels of the two types of milks. Total and reduced ascorbic acid determinations were made initially and at 24 hour intervals. Dehydroascorbic acid values were obtained by difference. The results of this experiment are shown in Table 9. The total ascorbic acid level appears to be essentially constant in both types of milk during storage for 72 hours, regardless of the presence of copper and/or added dihydroquercetin. The two types of milks, however, showed marked differences in reduced ascorbic and dehydroascorbic acid levels. The oxidation of reduced ascorbic acid in spontaneous milk was slower than in copper-sensitive milk, and the slow oxidation was accompanied by a gradual increase of dehydroascorbic acid. The addition of DHQ to spontaneous milk altered this relationship between the two forms of ascorbic acid. The copper-catalyzed oxidation of reduced ascorbic acid, on the other hand, was rapid and complete, regardless of the presence of added DHQ.

The changes in reduced ascorbic and dehydroascorbic acid levels of the two milks are shown graphically in Figures 3 and 4. The spontaneous milk showed clearly the ratio of reduced ascorbic acid to dehydroascorbic acid close to or slightly less than 1:1, existing over a prolonged period, which condition appeared to be

TABLE 9

The changes in flavor, total and reduced ascorbic acid contents of spontaneously oxidizable and copper-sensitive milks, during storage at 1°C for 72 hours, as influenced by added dihydroquercetin[a]

Cow No.	Treatment	Total Ascorbic Acid Hours 0	24	48	72	Reduced Ascorbic Acid Hours 0	24	48	72	Flavor[b]
688	Control	21.0	21.0	20.0	19.5	17.9	10.4	8.0	7.5	++
	- DHQ	21.0	21.0	20.0	20.0	17.9	9.9	4.0	1.8	-
700	Control	20.5	20.2	20.0	18.7	18.5	9.6	4.0	1.2	-
	- Cu[c]	20.5	20.1	20.0	20.0	18.5	0.0	0.0	0.0	++
	- Cu-DHQ	20.5	20.0	20.0	20.0	18.5	0.9	0.0	0.0	-

[a] DHQ dissolved in warm distilled water and added to milks at the level of 20 mg %

[b] Flavor evaluation made after 72 hours storage
- \- oxidized flavor not detectable
- ++ pronounced oxidized flavor

[c] Copper in the form of $CuSO_4 \cdot 5H_2O$ dissolved in warm distilled water and added to milk at the level of 0.01 ppm

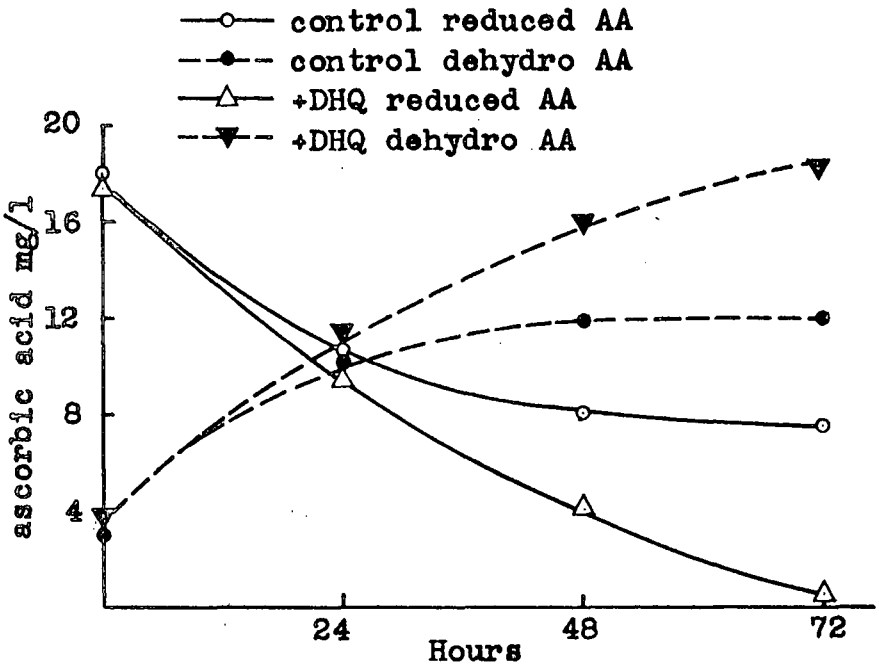


Figure 3. The changes in reduced ascorbic acid and dehydroascorbic contents in spontaneously oxidizable milk, as influenced by added dihydroquercetin

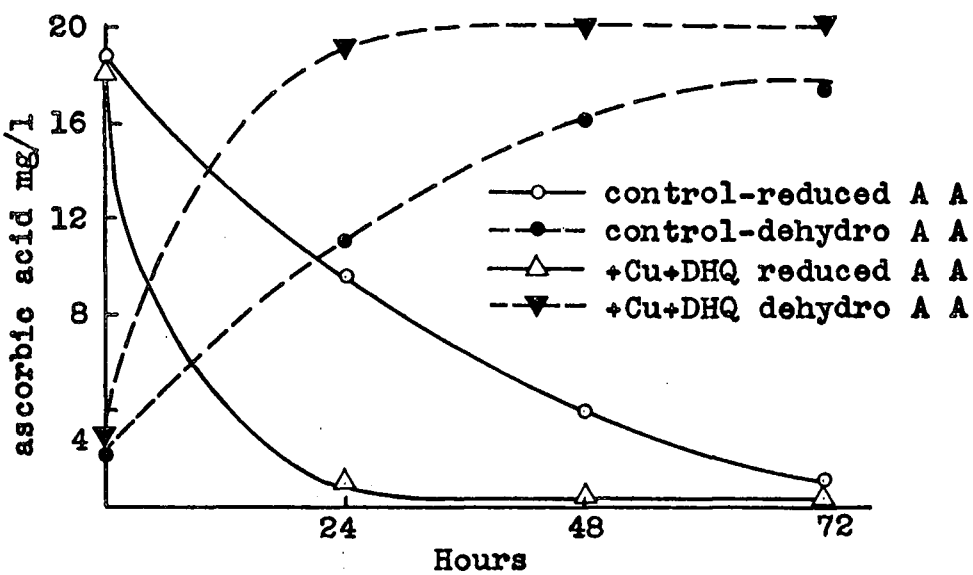


Figure 4. The changes in reduced ascorbic acid and dehydroascorbic acid contents of copper-sensitive milk, as influenced by added dihydroquercetin.

prerequisite for the development of spontaneous oxidized flavor. Such a relationship between the two forms of ascorbic acid did not exist when spontaneous oxidation was inhibited by DHQ or in the case of copper-catalyzed oxidation of reduced ascorbic acid.

6. Amperometric titration of copper with DHQ, PG and EDTA in acetate buffer and milk systems.

The third and final phase of this study was devoted to the amperometric determination of the metal complexing abilities of DHQ, PG and EDTA. Tables 10 and 11 show the results of titration of 0.01 M solution of cupric sulfate, with the complexing agents in acetate buffer and in milk systems, respectively. The acetate buffer was adjusted to a pH of 6.6, the pH of milk. Table 10 shows that 1 mole of copper appeared to be complexed by 2 moles of DHQ or PG. In the case of EDTA, 1 mole of copper was complexed by 5 moles of EDTA. Figure 5 shows graphically the results in Table 10. The gradual decrease in the diffusion current of copper denotes complexing and further addition of copper results in the emergence of a copper wave at a more negative half-wave potential. The molar ratio of copper to complexing agent is plotted on the abcissa and the changes in current on the ordinate. Table 11 and Figure 6 show the results of similar titration

TABLE 10

Amperometric titration of 0.01 M cupric sulfate with 0.01 M solutions of DHQ, PG and EDTA respectively in 0.1 M acetate buffer at pH 6.6

Complexing agent	Mole copper per mole of complexing agent	Current in μ amps[a]	Molar ratio copper: complexing agent
Dihydroquercetin	0.00	6.78	0.5:1.0
	0.10	6.35	
	0.20	5.95	
	0.50	4.65	
	0.60	4.90	
	1.00	5.97	
	1.50	7.30	
	2.00	8.65	
Propyl gallate	0.00	7.80	0.5:1.0
	0.20	7.65	
	0.40	7.50	
	0.50	7.45	
	0.60	7.60	
	1.00	8.05	
	1.50	8.70	
	2.00	9.30	
Disodium salt of ethylenediamine - tetracetic acid	0.00	6.80	0.2:1.0
	0.04	6.75	
	0.08	6.65	
	0.20	6.45	
	0.30	6.60	
	0.40	6.80	
	0.60	7.15	
	1.00	7.90	
	1.50	8.90	
	2.00	9.80	

[a] Voltage applied 1.75 volt

TABLE 11

Amperometric titration of copper with dihydroquercetin in whole milk, cream, skim milk and dialyzed skim milk respectively

Product	mole copper added/mole DHQ	Current[a] in μ amps	mM copper complexed per mM DHQ
Skim milk[b]	0.00	6.75	
	0.20	6.50	
	0.50	6.30	
	0.75	6.05	
	1.00	5.85	1 : 1
	1.25	6.10	
	1.50	6.40	
	1.75	6.80	
	2.00	7.05	
Whole milk[c]	0.00	6.00	
	0.20	5.70	
	0.50	5.05	
	0.75	4.65	
	1.00	4.20	1 : 1
	1.25	4.50	
	1.50	4.90	
	1.75	5.05	
	2.00	5.40	
Dialyzed skim[d]	0.00	2.25	
	0.20	2.25	
	0.50	2.10	
	0.75	2.00	
	1.00	1.90	1 : 1
	1.25	2.20	
	1.50	2.70	
	1.75	2.95	
	2.00	3.25	
Cream[e]	0.00	1.60	
	0.20	1.35	
	0.50	1.10	
	0.75	0.80	
	1.00	0.60	1 : 1
	1.25	0.90	
	1.50	1.20	
	1.75	1.45	
	2.00	1.80	

a/ Voltage applied 1.75 volt
b/ Skim milk: Protein % 3.5 (Kjeldahl)
c/ Whole milk: " 3.3 "
d/ Dialyzed skim: " 3.4 "
e/ Cream: " 2.7 "

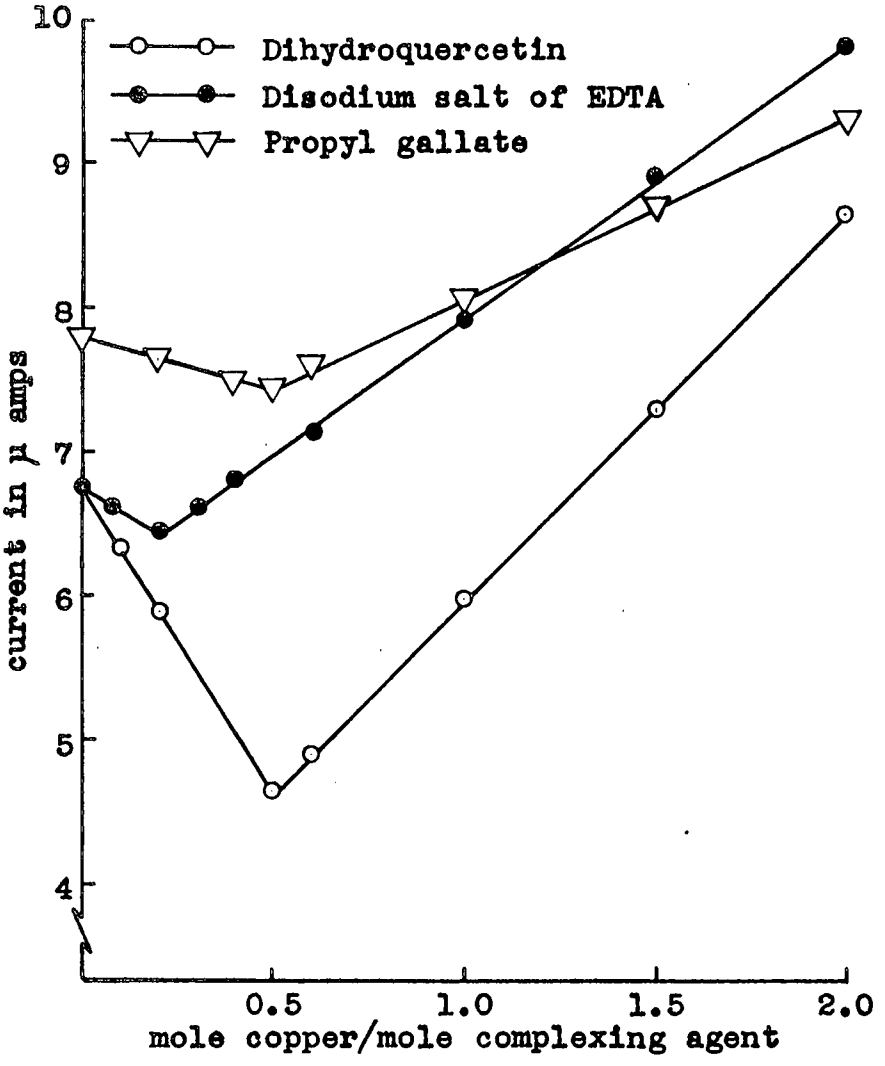


Figure 5. Amperometric titration of 0.01 M cupric sulfate solution with 0.01 M solutions of DHQ, PG and EDTA respectively in 0.1 M acetate buffer, at pH 6.6.

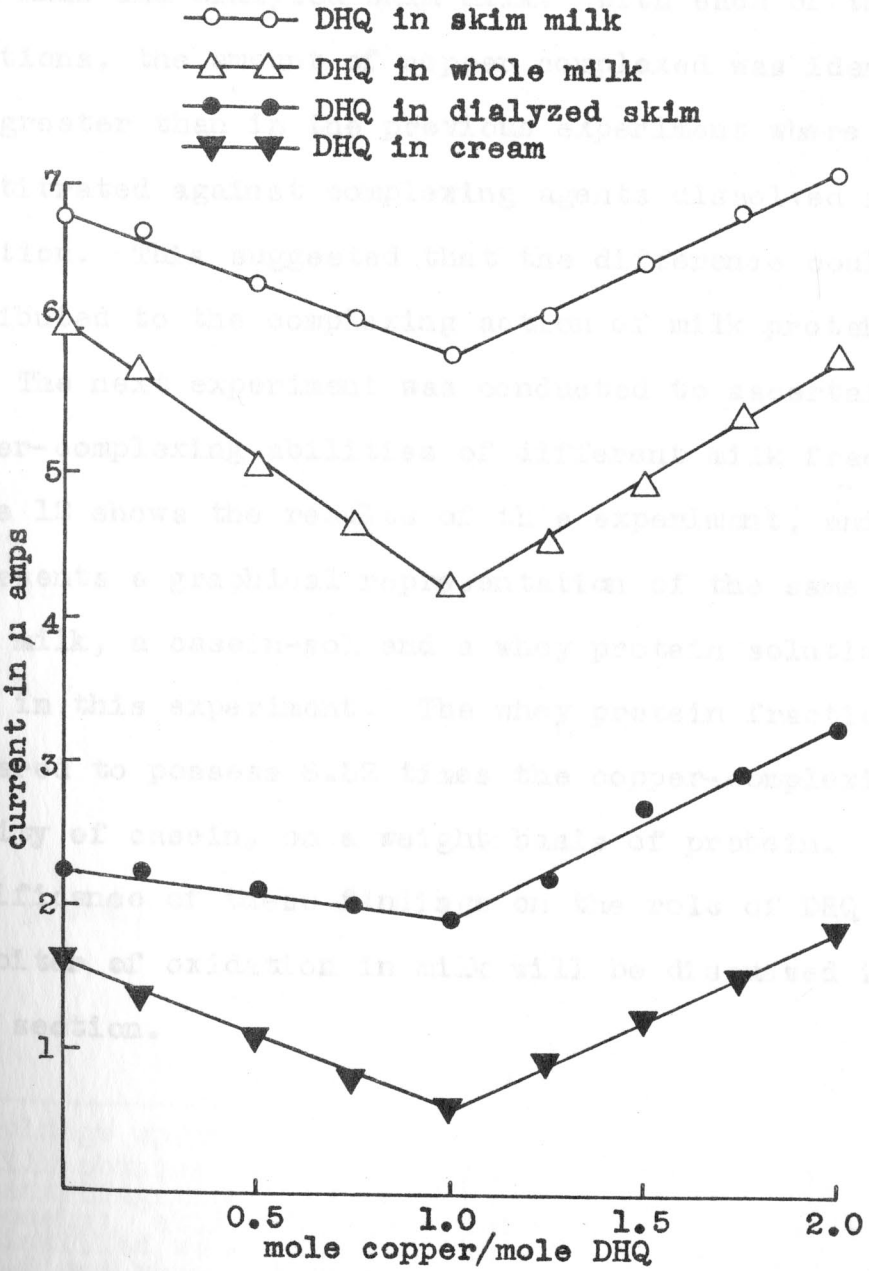


Figure 6. Amperometric titration of 0.01 M cupric sulfate solution with 0.01 M solution of dihydroquercetin in milk and some milk products.

of copper with complexing agents dissolved in milk, cream, skim milk and dialyzed skim milk. With each of the milk fractions, the amount of copper complexed was identical, but greater than in the previous experiment where copper was titrated against complexing agents dissolved in buffer solution. This suggested that the difference could be attributed to the complexing action of milk proteins.

The next experiment was conducted to ascertain the copper-complexing abilities of different milk fractions. Table 12 shows the results of this experiment, and Figure 7 presents a graphical representation of the same data. Skim milk, a casein-sol and a whey protein solution were used in this experiment. The whey protein fraction appeared to possess 8.32 times the copper-complexing ability of casein, on a weight basis of protein. The significance of these findings on the role of DHQ as inhibitor of oxidation in milk will be discussed in the next section.

TABLE 12

Amperometric titration of copper with skim milk, whey protein and casein

Milk fraction	mM copper added	Current[a] in μ amps	mM copper complexed per gm protein[e]
	0.00	6.55	
	0.01	6.35	
	0.04	5.45	
	0.05	5.10	
Skim milk[b]	0.10	5.30	0.153
	0.15	5.40	
	0.20	5.65	
	0.25	5.85	
	0.30	6.00	
	0.00	6.70	
	0.01	6.50	
	0.02	6.30	
	0.04	5.85	
Casein[c]	0.10	5.80	0.137
	0.15	5.90	
	0.20	5.90	
	0.25	5.95	
	0.30	6.00	
	0.00	3.45	
	0.01	3.25	
	0.02	3.00	
	0.04	2.60	
Whey protein[d]	0.05	2.70	1.142
	0.10	3.15	
	0.15	3.60	
	0.20	4.00	
	0.25	4.50	
	0.30	4.95	

a/ Voltage applied 1.75 volt

b/ Milk obtained raw, and skim milk recovered by centrifugation.

c/ Casein: skim milk was diluted with an equal volume of distilled water and acidified with 0.1 N HCl to a pH of 4.6 and then filtered. The precipitate was washed with distilled water and brought to a pH of 6.6 with NaOH, to constitute approximately a 3% solution.

d/ Whey protein: the filtrate was brought to a pH of 6.6 with sodium acetate

e/ Protein determination made by Kjeldahl method.

	Protein %
Skim milk	3.4
Casein	2.9
Whey protein	0.35

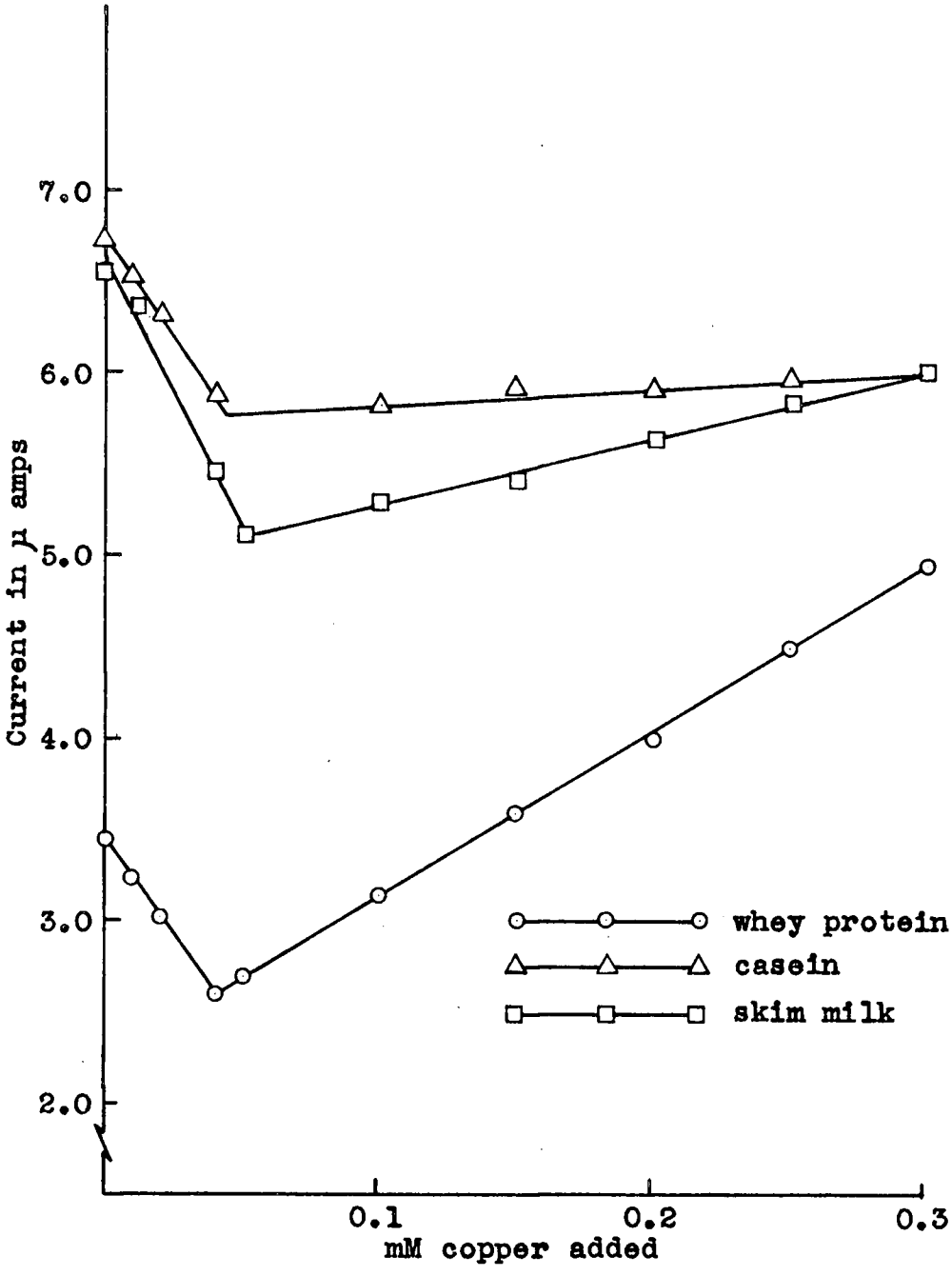


Figure 7. Amperometric titration of copper with casein, whey protein and skim milk.

DISCUSSION

The initial phase of this study was concerned with the determination of the effectiveness of DHQ as an inhibitor of spontaneous, copper-induced and copper plus ascorbic acid-induced oxidation in milk and milk products. The first experiment was more or less of an exploratory nature to gain insight into the pro-oxidative systems in milk. The results of this experiment are in agreement with the findings of Smith and Dunkley (127) and contrary to those reported by Aurand and Woods (7). If there is a direct relationship between xanthine oxidase activity of milks and their susceptibility to spontaneous oxidation, it is indeed not apparent under the experimental conditions of this study.

The results in this study on the effectiveness of DHQ in fluid milk and milk products are similar to the observations made by other researchers (51, 123). The water solubility of DHQ lends itself particularly to incorporation into fluid milk and milk products. The carry-over properties of DHQ to heat treatment has not been previously reported. The results of this study indicate that DHQ in milk possesses excellent carry-over properties in the spray drying process. Dugan *et al.* (48) studied the carry-over properties of butylated hydroxy anisole

(BHA) in several baked and fried foods. Koch (90) reported that the carry-over property of an inhibitor was related to its water solubility. He attributed the greater carry-over property of BHA and butylated hydroxytoluene (BHT), when compared to PG and nordihydroguaretic acid (NDGA), partly to the lower water solubility of the former. BHA and BHT have fewer OH groups attached to their ring structures than PG or NDGA. Lundberg and Chipault (108) suggested that the carry-over property was associated with the so-called "hindered" phenols. While DHQ shows greater water solubility than quercetin, it is considerably less soluble in water than PG or EDTA. This may account for its effectiveness in the dried powders.

The second phase of this study was devoted to the experiments related to the changes in some of the components participating in the oxidative system in milk, as influenced by added inhibitors. The influence of added DHQ, PG and EDTA on the changes in flavor, Eh and ascorbic acid contents of milks subject to copper-induced oxidation show a striking similarity between the inhibitors in certain respects. Ascorbic acid oxidation proceeded and was accompanied by a rise in Eh in milks containing added copper, regardless of the presence of added inhibitors. The development of oxidized flavor, however, was inhibited by the added inhibitors. Chilson *et al.* (32) observed

that added PG in milk failed to prevent the oxidation of ascorbic acid although it inhibited the development of oxidized flavor. Erickson (51) reported an increase in the Eh of milks with added copper alone and in presence of added DHQ, but did not present evidence to show that this increase in Eh was attributable to the oxidation of ascorbic acid. Campbell et al. (29) observed that ascorbic acid exerted a role in milk as a major determinant of Eh. Their study involved oxidation of ascorbic acid during storage of milks with no added copper. The results in this study confirm the results of Campbell et al. (29) and show an even more striking influence of copper-induced oxidation of ascorbic acid on the Eh of milks. It was also noted in this study (Tables 3, 4 and 5) that the addition of ascorbic acid to milk and milk products was accompanied by a greater decrease in Eh than samples without added ascorbic acid and that added copper caused a marked rise in Eh.

The experiment conducted with reduced levels of added copper, and increased levels of inhibitors on the changes in ascorbic acid contents and Eh of milks during storage (Table 8) showed that EDTA, at the level of 200 mg per cent, was effective in decreasing the rate of copper-catalyzed oxidation of ascorbic acid. This suggested that the complexing of added copper and the removal

of copper from a key site in milk nullified its pro-oxidant effect. DHQ and PG, on the other hand, failed to alter the rate of copper-catalyzed oxidation of ascorbic acid, even though they inhibited the development of oxidized flavor. The effect of added PG on the copper-induced changes of Eh of milk was distinct from DHQ or EDTA. In the former case, the Eh was maintained essentially at the initial level during 72 hours storage. In milks with added DHQ and EDTA, the Eh progressively increased during storage. The samples containing the inhibitors were not oxidized at the end of 72 hours, as determined organoleptically. The samples containing added PG were scored as bitter tasting. These results indicated that the inhibitory effect of EDTA was primarily attributable to its copper-complexing ability; this was not the case with PG or DHQ. PG exerted its effect by preventing a rise in Eh and maintaining the oxidation-reduction potential at sufficiently low levels to arrest the onset of oxidized flavor. DHQ, on the other hand, appeared to allow the initiation of oxidation, but exerted its protective action at the later stage of oxidation, namely the propagation step. This was confirmed subsequently by studying polarographically the complexing ability of DHQ in milk and milk fractions. A more detailed discussion on the probable role of DHQ in milk is given later in the

form of a postulation for oxidation and inhibition of oxidation. The experiment on the changes in total and reduced ascorbic acid contents of spontaneously-oxidizable and copper-sensitive milks, as influenced by added DHQ, revealed that in spontaneous milk, the ratio of reduced to dehydroascorbic acid was close to or less than 1:1 and this condition persisted for an extended period. This state of equilibrium appeared to be prerequisite for the development of oxidized flavor. These findings are in excellent agreement with the observations made by Krukovsky and Guthrie (90), that the establishment of an equilibrium pressure between the two forms of ascorbic acid was a prerequisite condition for the development of oxidized flavor. In spontaneous milk treated with DHQ, and in milks subject to copper-induced oxidation, this relationship between reduced and dehydroascorbic acid did not prevail. This pointed to the distinction between the two types of oxidation encountered in milks. The precise nature of the differences could not be ascertained. Aurand and Woods (8), from their studies on spontaneous oxidation and copper-induced oxidation of milks, offered some evidence to show that the former was an enzymatically catalyzed phenomenon, while the latter was primarily a chemically catalyzed reaction. There appears to be considerable agreement on the reactions involving

copper-induced oxidation, but the actual sequence of reactions and conditions leading to the spontaneous development of oxidized flavor is yet to be proved. The amperometric titration of copper with DHQ, PG and EDTA in acetate buffer at pH 6.6, revealed the differences in their metal-complexing abilities. Detty et al. (45) observed that the metal-complexing abilities of flavonoids were dependent on configuration as well as pH. They found that at pH 6.6 and 10, one mole of DHQ complexed 0.5 and 1.0 moles of copper respectively. The molar ratio of copper: DHQ in this study is in agreement with the result of the above workers. In the case of EDTA, the molar ratio of copper: EDTA was 0.2 : 1, and this result is in agreement with the observations of Arrington et al. (5) who found that EDTA, when added at a molar concentration five times that of added copper, effectively inhibited the development of copper-induced oxidation in milk. In this study, PG exhibited identical metal-complexing ability to DHQ in buffer system. Kelly and Watts (84) reported that PG failed to show metal-complexing property. This discrepancy could perhaps be attributable to the conditions of pH employed by these workers. They used trishydroxyaminomethane buffer at pH 5.8 in their colorimetric determination of metal-complexing abilities of different compounds.

The titration of copper with DHQ in milk, skim milk, dialyzed skim and cream, yielded identical results and the amount of copper complexed appeared to be twice that of the amount complexed by DHQ when dissolved in buffer. This suggested the possibility that milk proteins were complexing copper. The ability of proteins to complex copper has been observed by several researchers, as mentioned earlier in the review of literature. That the protein-copper complex is actually a more effective catalyst than added copper in linoleate oxidation has been reported by Tappel (138).

The titration of copper with skim milk, whey proteins and casein-sol, respectively, revealed that the whey protein fraction possessed over eight times the metal-complexing ability of casein fraction on a protein basis. Menger and Mulder (113) reported that added copper became associated primarily with the skim milk proteins. King and Dunkley (86) observed that most of the added copper was uniformly distributed among the skim milk proteins. They used Cu^{64} as cupric nitrate and fractionated the skim milk proteins after the addition of copper and determined the radioactivity of the different fractions. In this study the copper addition was made after the fractionation step; the differences in the results may be attributed to

the variation in the experimental procedure adopted. Davies (41) reported that added copper and iron were uniformly distributed in gravity-separated milk but the copper was concentrated in the cream after the milk had been subjected to centrifugal separation. The results of the amperometric titration experiments indicate that DHQ when added to milk, complexes part of the added copper and that the rest of the added copper combines primarily with whey protein fraction of the milk. The copper-protein complex is capable of exerting as strong if not a stronger pro-oxidant effect as free copper on the oxidative system existing in milk. Under these conditions it does not appear that the inhibitory effect of DHQ could be attributed to its metal-complexing ability. On the other hand, it suggests that the role of DHQ in milk as an inhibitor of oxidation is primarily that of a free radical acceptor, and that in the conditions existing in milk, ascorbic acid may act synergistically with the inhibitor. In the absence of DHQ in milk, under identical conditions, ascorbic acid present in milk acts as a strong pro-oxidant. Similar observations have been made in model systems (84).

The following postulation of the sequence of reactions leading to copper-induced oxidation in milk and the inhibition of oxidation by DHQ is offered. Milk is a complex biological fluid. All milks

contain the components involved in the reactions leading to the development of oxidized flavor. The oxidative system of milk comprises: (1) the substrate for oxidation --primarily the unsaturated lipid material in the fat-globule-membrane, (2) copper in the form of added copper, copper-protein complex, or natural copper, (3) ascorbic acid dispersed in the serum phase and (4) dissolved oxygen. The oxidative system can be effectively blocked by removing or inactivating one or more of the participating components.

Ascorbic acid incorporated in the aqueous phase of fat emulsions exerts a strong pro-oxidant effect, this effect being confined to the unsaturated lipid molecule with an active methylene group. The first step in the sequence of reactions leading to oxidation is believed to be a dehydrogenation of this active methylene group to yield a free radical (52). The energy required for the separation of an alpha methylene hydrogen in a monene configuration is of the order of 80 k calories, and it is not clear how this energy is supplied to the system. Farmer proposed a direct attack by oxygen on the double bonds during the initial stages of oxidation.

It appears very likely that at this point copper and ascorbic acid play a significant role in the chain of events leading to oxidation. Copper catalyzes the

oxidation of ascorbic acid in the serum phase in close proximity to the unsaturated lipid material of the fat-globule-membrane. The rapid formation of free radicals ensuing from this reaction can be represented thus (162).

$$AH_2 \longrightarrow AH^{\bullet} + H^{\bullet}$$

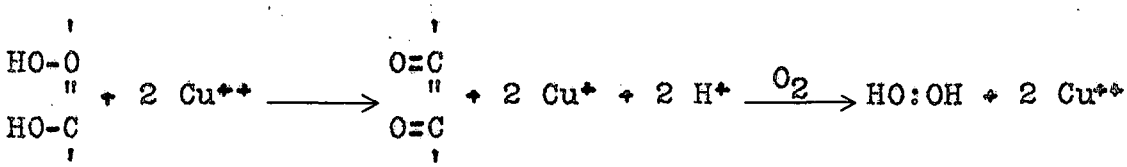
$$AH^{\bullet} + Cu^{++} \longrightarrow AH^{o} + Cu^{+}$$

The AH^{o} may well cause the dehydrogenation of the activated methylene group of the unsaturated lipid, thus constituting the initiation step of the peroxidation cycle. The lipid free radical in turn reacts with oxygen and another lipid molecule forming a hydroperoxide and another lipid free radical (84). As mentioned in the review of literature, the rate of ascorbic acid oxidation appears to be critical in determining its role in milk. It has been shown that extremely slow oxidation of ascorbic acid, induced by deaeration of milk, or its rapid oxidation by added H_2O_2 stabilizes the oxidative system in milk.

Copper in trace amounts is sufficient to catalyze the reaction, since the cuprous ion causes the scission of the peroxide formed and is in turn oxidized to the cupric form as shown below (162).

$$-CH=CH-\underset{\underset{\underset{H}{|}}{O}}{\overset{\overset{H}{|}}{C}}-CH=CH- \xrightarrow{Cu^{+}} -CH=CH-\underset{\underline{O}}{\overset{H}{C}}-CH=CH + {}^{o}OH + Cu^{++}$$

An alternate pathway for the copper-catalyzed reactions terminates with the formation of H_2O_2, as shown below.



H_2O_2 formed at the proximity of lipid material is in a position to oxidize the lipid, by dissociating into two hydroxy radicals, which remove labile hydrogen from the lipid. The role of copper in this reaction is that of an electron acceptor, and of a bridge between ascorbic acid in the plasma and the fat-globule-membrane material. The ability of metals to form bridges between protein and small organic molecules, which otherwise show no affinity to each other, was demonstrated by Klotz *et al.* (89).

Individual milks vary greatly with respect to their ability to resist copper-catalyzed oxidative deterioration. In this study it was found that the change of the cows from dry-lot feed to pasture was accompanied by a corresponding change in the stability of milk toward spontaneous or copper-induced oxidation. Copper-sensitive milk was characterized by rapid increase in Eh over 75 per cent of initial value during storage, resulting from trace amounts of added copper; while in copper-resistant milks the rise in Eh under identical conditions was only 54 per cent or less. Greenbank (61) observed that the response

of milks to trace amounts of added copper, in terms of changes in Eh, could indeed be used as an index of its stability toward oxidation. The changes in Eh, however, do not portray the conditions of the oxidative system in its entirety. Hitherto unidentified substances, which possess marked inhibitory properties, appear to be concentrated in the serum phase of milk of cows on pasture. There is a striking similarity in the effects between phenolic-type inhibitors and such substances. Watts (159) observed that the cooking water from 40 different vegetables exhibited antioxidant properties when used as dips for meat slices held in storage. Ascorbic acid under these conditions assumes the role of a synergist. Further, the changes in the relative proportion of the two ascorbic acids rather than changes in Eh distinctly characterize spontaneous milks. These factors have to be taken into consideration when interpreting results of any particular test in terms of the stability of the oxidative system in milk as whole. The oxidation of ascorbic acid in milk apparently proceeds independently of the lipid oxidation. This has been demonstrated by several workers by comparing the stability of washed cream to its instability when ascorbic acid was added to the aqueous phase. Fortification of milk by ascorbic acid results in the shifting of the oxygen availability away from the lipid system and

stabilizes the milk against oxidation. As mentioned earlier, ascorbic acid is also capable of acting synergistically in presence of phenolic-type inhibitors. Thus it is apparent that, as in the case of oxidation-reduction potentials, the role of ascorbic acid specifically as an antioxidant or pro-oxidant depends on several factors.

In terms of dynamic equilibria of the milk system, a spontaneous milk is one in which the reaction rate of the oxidative system is greater than the rates of competing or inhibiting reactions. A copper-sensitive milk is one in which the reactiom rates of the oxidative system prior the addition of copper is slower than competing or inhibiting reactions. The addition of copper, however, results in the creation of reactive sites on the fat-globule-membrane so that the balance is shifted in favor of the oxidative system. On the other hand, a copper-resistant milk is one in which the reaction rates of the competing or inhibiting reactions are so favored that even additions of copper fail to shift the direction of the reactions.

Although not classified as a typical phenolic type of inhibitor, DHQ appears to be similar in its mode of action to phenolic inhibitors. The most effective phenolic antioxidants possess two hydroxyl groups in ortho or para position to each other. The ortho

relationship of two OH groups in 3' and 4' position are found in DHQ. Richardson et al. (123) considered the antioxidant property of flavonoid compounds to be centered in the labile pyrone ring. Mehta and Seshadri (112) suggested that the keto-enol tautomerism of OH group on C_3 and carbonyl group on C_4 was responsible for antioxidant property of flavonoid compounds. Regardless of the contribution of substituents and/or configuration of the flavonoid molecules, a few conclusions concerning the role of DHQ in milk appear to be valid. DHQ interrupts the chain propagation by providing a hydrogen to the free radical formed via the copper-catalyzed oxidation of ascorbic acid and the subsequent dehydrogenation of the activated methylene group of the lipid molecule. The antioxidant may be reactivated by receiving an electron from the ascorbic acid which would be converted in turn to dehydroascorbic acid. This was illustrated clearly in the study of spontaneous milk with and without the addition of DHQ (Table 9). The presence of DHQ effectively prevented the development of oxidation. The reduced ascorbic, however, was gradually oxidized to dehydroascorbic acid. This indicates that synergism of ascorbic acid in presence of DHQ is centered around its function as a furnisher of electrons for the regeneration of the antioxidant. The rise in Eh of milks containing added copper alone and in

the presence of DHQ, shows that ascorbic acid oxidation proceeds in both cases but inhibition of oxidized flavor is noted with samples containing added DHQ. Koch (90) attributed the synergistic action of ascorbic acid to the furnishing of electrons for the regeneration of antioxidant or to the complexing of trace metals, while Golumbic and Mattel (59) considered ascorbic acid as primarily a furnisher of electrons for the regeneration of antioxidants. Kelly and Watts (84), however, observed that the copper-ascorbic acid complex was the causative agent for the pro-oxidant effect of ascorbic acid in aqueous fat systems. They found that compounds which were antioxidants in the presence of ascorbic acid were capable of forming more stable copper complexes.

Inhibitors primarily involved with metal complexing function in milks containing copper by removing the metal from the key site mentioned earlier, namely ascorbic acid and fat-globule-membrane protein. Koops et al. (92) observed this to be the case when they added tetraethylthiuramdisulfide (TETD), a fat-soluble, copper-binding inhibitor, to copper-contaminated washed cream. In this study, EDTA added at the level of 200 mg per cent to milk with 0.01 ppm added copper appreciably slowed the rate of copper-catalyzed oxidation of ascorbic acid, while DHQ and

PG under the same conditions failed to alter the rate of oxidation of ascorbic acid. This clearly indicates the differences in the mode of action of EDTA from either DHQ or PG.

The role of DHQ, in the sequence of reactions leading to the inhibition of oxidation, appears to be primarily concerned with the interruption of the free radical chain reaction by capture of the free radicals. The apparent unhindered progress of copper-catalyzed oxidation of ascorbic acid in the presence of DHQ, indicates that at this stage the chain reaction has set in. The interruption of free radicals by an inhibitor can be represented thus (70):

$$ROO^{\circ}(RO^{\circ}, HO^{\circ}, HOO^{\circ}) + AH \longrightarrow ROOH\ (ROH,\ H_2O,\ H_2O_2) + A^{\circ}$$

This reaction proceeds at a velocity equal to or greater than the initiation reactions, which yield free radicals. The inhibitor radicals A° are consumed directly with another radical. One mole of inhibitor may stop two free radical chains, and the more rapid this reaction occurs, the fewer the number of moles of hydroperoxide formed. The importance of configuration of the molecule as a determinant of the antioxidant efficiency of flavonoids has been reported by many workers. The pH dependency of the metal-complexing ability of flavonoid compounds has

also been observed. Studies have been made to determine the relationship between these two properties of flavonoids. The findings of this study show that the antioxidant role of DHQ is independent of its function as a metal-complexing agent. It appears that ascorbic acid in the presence of DHQ exerts its synergistic effect through the furnishing of electrons to the antioxidant. A similar situation is noted with tocopherol in milk in the presence of ascorbic acid. The tocopherol molecule, by changing to tocopheryl-quinone radical, provides a hydrogen for the interruption of the free radical chain reaction and is in turn regenerated by the synergistic action of ascorbic acid present. In the inhibition of oxidation by DHQ and phenolic-type antioxidants, the metal-complexing abilities of these compounds are undoubtedly evidenced, but this phenomenon is not related to the role of the compound as an antioxidant.

SUMMARY AND CONCLUSIONS

1. From over 49 duplicate analyses of milks from individual cows representing different stages of lactation, it was found that:

   (a) there was no correlation between the fat tests of milks and their xanthine oxidase activity, or between xanthine oxidase activity of milks and their susceptibility to spontaneous oxidation.

   (b) a high proportion of spontaneously oxidizable milks were obtained from cows in the second and third months of lactation.

   (c) the xanthine oxidase activity increased with the progress of lactation and with change of feeding conditions of the cows from dry-lot feed to pasture.

   (d) the change of cows from dry-lot feed to pasture was accompanied by a marked resistance of the milks toward the development of spontaneous oxidation.

   (e) the addition of dihydroquercetin to spontaneously oxidizable milks likewise markedly enhanced their resistance to the development of oxidation.

2. Dihydroquercetin was found to be effective in the inhibition of spontaneous, copper-induced and copper plus ascorbic acid-induced oxidation of milk, cream and buttermilk.

3. Dihydroquercetin was effective in the inhibition of oxidation in dried spontaneous, copper-sensitive and copper-resistant milks and buttermilk as measured organoleptically and by the thiobarbituric acid test.

4. The thiobarbituric acid values and organoleptic evaluations of milks and milk products during storage were in excellent agreement.

5. The limited studies on the milks from cows on dry-lot feed and pasture feed showed that the resistance to copper of milks from pasture-fed cows was greater than milks from dry-lot fed cows.

6. Copper-sensitive milks were characterized by a higher initial reduced ascorbic acid content, a more rapid rate of copper-catalyzed oxidation of ascorbic acid, and more rapid copper-induced increase of Eh during storage, than copper-resistant milks.

7. Spontaneous milk was characterized by a slow oxidation of its reduced ascorbic acid, coupled with the attainment of an equilibrium with dehydroascorbic acid.

8. The addition of dihydroquercetin to milks with added copper failed to alter the rate of oxidation of ascorbic acid or the rise in Eh, although it protected

the milk from the development of oxidized flavor.

9. The addition of dihydroquercetin to spontaneous milk, on the other hand, resulted in an increase in the rate of oxidation of ascorbic acid, was accompanied by a correspondingly rapid increase in the dehydro-ascorbic acid level and no equilibrium was attained between the two forms of ascorbic acid.

10. Partial evidence is submitted to show that spontaneous oxidation and copper-induced oxidation are distinctly different phenomena.

11. The inhibition of copper-induced oxidation of milk by DHQ, PG and EDTA showed distinct differences. EDTA in milk appreciably decreased the rate of copper-catalyzed oxidation of ascorbic acid, while PG and DHQ failed to alter the rate of copper-catalyzed oxidation of ascorbic acid.

12. Based on the differences in the effects of the three inhibitors in milks with added copper, a hypothesis concerning their respective roles as antioxidants is furnished.

13. The amperometric titration of copper with DHQ, PG and EDTA in acetate buffer and milk systems revealed

marked differences in the copper-complexing abilities of these compounds. The molar ratio of copper: DHQ or copper:PG in buffer was 0.5:1, while copper:EDTA was 0.2:1. When DHQ was dispersed in milk, cream, skim milk or dialyzed skim, a greater amount of copper appeared to be complexed than when DHQ was dissolved in buffer.

14. The amperometric titration of copper with skim milk, casein and whey proteins revealed that whey proteins possessed a complexing ability with copper 8.32 times that of casein on a protein weight basis.

15. Based on the findings of this study, a postulation for the sequence of reactions leading to the copper-induced oxidation and its inhibition in milk is offered.

16. The evidence indicates that the role of dihydroquercetin in milk as an inhibitor of oxidation is primarily concerned with its function as a free radical acceptor, and that while the complexing ability of dihydroquercetin does indeed manifest itself in copper contaminated milk, it is unrelated to the phenomenon of inhibition of oxidized flavor development.

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A P P E N D I X

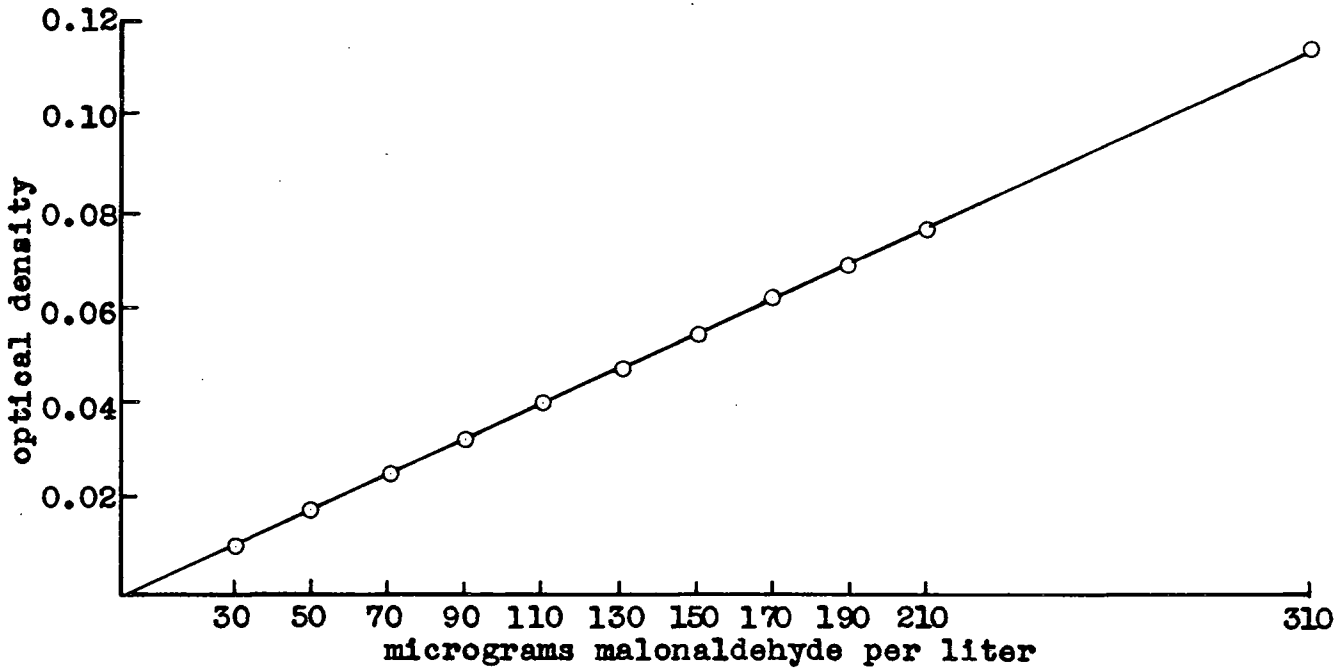
Preparation of standard curve for malonaldehyde

One mole of tetraethoxypropane (M.Wt. 220) when hydrolyzed in acid solutions, yields one mole of malonaldehyde (M.Wt. 62). Therefore, 3.5484 gm of tetraethoxypropane yields 1 gm of malonaldehyde. Using different aliquots of a solution of tetraethoxypropane to correspond from 0.001 to 0.0051 mg malonaldehyde per 10 ml sample, the procedure described earlier for TBA determinations was followed. A blank, consisting of 10 ml of distilled water was run along with the samples. The optical density readings were plotted against µg malonaldehyde per 10 gm sample.

Appendix Table 1

Standard curve for malonaldehyde

Tube No.	mg. malonaldehyde per 10 ml.	ml. solution B	ml. H_2O	% T	O.D.
1	0	0	10	100	0.000
2	0.0003	0.3	9.7	97.7	0.010
3	0.0005	0.5	9.5	96.0	0.018
4	0.0007	0.7	9.3	94.3	0.025
5	0.0009	0.9	9.1	92.8	0.032
6	0.0011	1.1	8.9	91.1	0.040
7	0.0013	1.3	8.7	89.7	0.047
8	0.0015	1.5	8.5	88.3	0.054
9	0.0017	1.7	8.3	86.8	0.061
10	0.0019	1.9	8.1	85.6	0.068
11	0.0021	2.1	7.9	84.0	0.076
12	0.0031	3.1	6.9	77.1	0.113

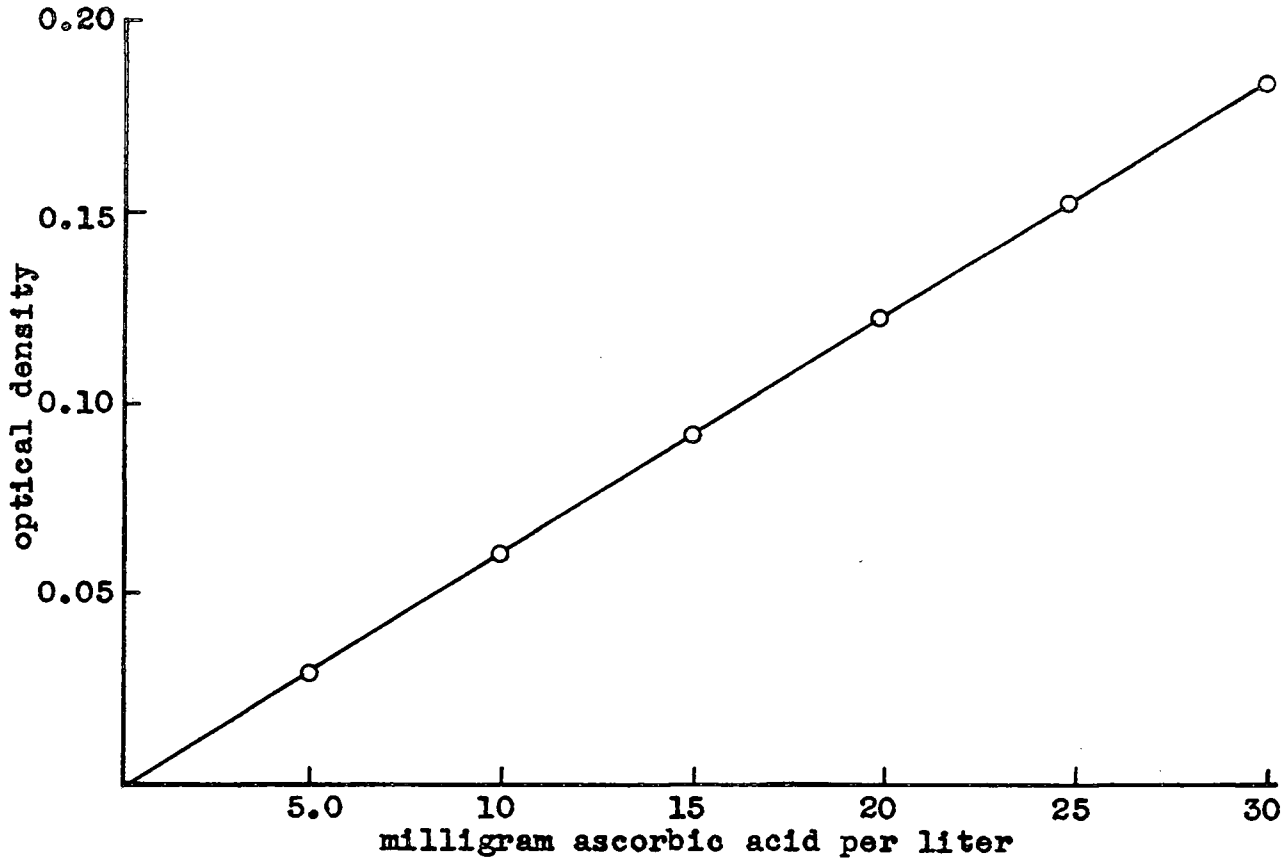


Appendix Figure 1. Standard curve for malonaldehyde.

Appendix Table 2

Standard curve for ascorbic acid

Tube No.	mg. ascorbic acid per 5 ml.	mg. ascorbic acid per 100 ml.	% T	O. D.
1	0.0	0.0	100.0	0.000
2	0.025	0.5	92.8	0.032
3	0.05	1.0	87.0	0.060
4	0.075	1.5	81.0	0.091
5	0.010	2.0	75.9	0.120
6	0.125	2.5	71.0	0.149
7	0.15	3.0	65.9	0.181



Appendix Figure 2. Standard curve for ascorbic acid.