EFFICIENCY OF CERTAIN FLAVONE-TYPE COMPOUNDS AS ANTIOXIDANTS FOR DAIRY PRODUCTS

by

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### TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF LITERATURE</td>
<td>3</td>
</tr>
<tr>
<td>Description of oxidized flavor</td>
<td>3</td>
</tr>
<tr>
<td>Factors affecting the development of an oxidized flavor in milk</td>
<td>4</td>
</tr>
<tr>
<td>Constituents of milk responsible for the oxidized flavor</td>
<td>8</td>
</tr>
<tr>
<td>Theories of lipid oxidation</td>
<td>10</td>
</tr>
<tr>
<td>Methods of detecting oxidation in milk</td>
<td>14</td>
</tr>
<tr>
<td>Flavonoids as antioxidants in dairy products</td>
<td>14</td>
</tr>
<tr>
<td>EXPERIMENTAL</td>
<td>16</td>
</tr>
<tr>
<td>Organoleptic examinations</td>
<td>17</td>
</tr>
<tr>
<td>2-Thiobarbituric acid test</td>
<td>17</td>
</tr>
<tr>
<td>Oxidation-reduction potential determinations</td>
<td>19</td>
</tr>
<tr>
<td>Feeding of quercetin</td>
<td>21</td>
</tr>
<tr>
<td>Standard plate count</td>
<td>21</td>
</tr>
<tr>
<td>RESULTS</td>
<td>22</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>39</td>
</tr>
<tr>
<td>SUMMARY AND CONCLUSIONS</td>
<td>45</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>47</td>
</tr>
</tbody>
</table>
EFFICIENCY OF CERTAIN FLAVONE-TYPE COMPOUNDS AS ANTIOXIDANTS FOR DAIRY PRODUCTS

INTRODUCTION

In the last 30 years the problem of oxidized flavor in dairy products has received extensive study. Despite this emphasis the problem of oxidized flavor in dairy products remains prominent and perplexing.

In 1953, Dahlberg and associates (6, p. 178) reported that 17% of 169 samples of fresh pasteurized milk, collected in 8 cities with populations of 100,000 or over, were found to have an oxidized flavor. When these 169 samples were held for 7 days at 33°F., 67% were found to be oxidized. In February, 1958, at the Oregon Dairy Industries contest at Oregon State College, Corvallis, Oregon, 17% of the fluid milk products examined were criticized for oxidized flavor (34).

These fluid products included: pasteurized milk, homogenized pasteurized milk, homogenized pasteurized 10%, and pasteurized whipping cream.

Because of the apparent high incidence of oxidized flavors in dairy products, a non-toxic antioxidant that is effective at low concentrations would be desirable. Previous investigations with flavone-type compounds as antioxidants in dairy products have indicated that these compounds might be effective in fluid milk products (23, p. 433-436 and 29, p. 397-413).
This study was undertaken to determine the efficiency of certain flavone-type compounds as antioxidants in fluid milk. The two compounds studied were quercetin and dihydroquercetin, both of which are now being obtained in commercial quantities from Douglas fir bark (11, p. 58).
Oxidized flavors have been studied probably more extensively than any other flavor defect encountered in milk. Many reviews and discussions have been published but the most recent review is that of Riel and Sommer (30), in which the authors assembled and correlated the literature in a comprehensive manner. A total of 509 separate titles is cited.

Description of Oxidized Flavor

As early as 1905, Golding and Feilman (15, p. 1285-1286) reported an alkaline, mealy off-flavor in milk contaminated with copper. Since that time the oxidized flavor has been variously described as "cappy", "metallic", "cardboard", "emery", "oily", "oxidized", "papery", and "tallowy" (30, p. 1). This plethora of descriptive terms used to describe the same defect has led to confusion. Riel and Sommer (30, p. 1-2) made a distinction between the mild oxidation of milk phospholipids and the oxidation of the milk fat itself. To the former they gave the term "oxidized flavor" and to the latter the term "oxidized-fat flavor." They also called the oxidative-like flavor resulting from solar radiation "sunshine flavor."

In 1939, Nelson and Dahle (26, p. 62-66) stated that spontaneous and copper-induced flavors were similar and that judges could distinguish no differences in the two flavors. Because of this fact, studies on the development of an oxidized flavor in milk have almost always entailed the use of minute (0.1 to 5 ppm) quantities of copper as a catalyst.
Factors Affecting the Development of an Oxidized Flavor in Milk

Many factors exert an influence on the development of an oxidized flavor in milk. Some are associated with milk production and others with milk processing.

In milk production, feed and the cow herself can influence the development of the flavor in milk. According to Sommer (33, p. 139-140) the feeding of a dry ration during late fall and winter months makes the milk more susceptible to oxidation than the feeding of a green succulent feed in the spring and summer months. In the majority of cows this will hold true, but there are individual cows that will differ from this pattern. Milk from cows on sub-maintenance rations is very susceptible to oxidation, because, the iodine value of the milk fat increases. When cows go on pasture the iodine value of the fat also increases and it oxidizes more readily but the whole milk itself is less susceptible to the development of an oxidized flavor.

The increase in iodine value is probably offset by reducing substances or antioxidants produced by the cows on green feeds. The exact nature of these antioxidants or reducing substances has not been defined (33, p. 139-140).

Thurston (38, p. 143-153) classified milk from individual cows as resistant, susceptible, and spontaneous. In resistant milk oxidized flavor does not develop in the presence of copper, in susceptible milk an oxidized flavor develops in the presence of copper, and in spontaneous milk the flavor develops without the presence of copper. The quantities of copper necessary to satisfy these categories were not given.
Corbett and Tracy (5, p. 1095-1106) found that milks from individual cows varied in susceptibility to the oxidized flavor development. This variation correlated with the age of the animal and stage of lactation but only slightly correlated with the season (March through September) and the breed of the cow. No relation was found between the quantity of milk or fat produced and oxidized flavor development. Ayrshire milk was found to be least susceptible.

In studies on 59 cows on winter rations, Riel and Sommer (31, p. 36) classified the cows according to the method of Thurston (38, p. 143-153) and found that 5 cows gave spontaneous milk and 43 and 11 gave susceptible and resistant milk, respectively, in the presence of 1 ppm copper. Exploratory work with these cows indicated that enzyme treatment ("Lactivase") of the milk and pasture feeding inhibited the development of the flavor. These workers felt that the most significant finding was that a high proteoses-peptone nitrogen content is typical of resistant milks produced naturally on a winter ration or on pasture, or by enzyme treatment of the milk.

In milk processing, the most common cause of oxidized flavor is metallic contamination. Copper and iron are the two most effective catalysts commonly encountered (30, p. 7-9).

It has been established that copper added before pasteurization or higher heat treatment is not as effective as after pasteurization and cooling (30, p. 8); however, the activity of iron was not decreased even when the milk was heated to 95°C. (35, p. 537-584). Gjessing and Trout (14, p. 373-384) reported that copper does not
affect the stability of ascorbic acid in milk if added before heat treatment. This effect was attributed to the liberation of reducing substances, namely sulfhydryl compounds, liberated by high heat treatment. This effect was thought to be due either to a lowering of the oxidation-reduction potential or to the formation of inactive copper complexes.

Homogenization retards the development of oxidized flavor (30, p. 11). Dahle (7, p. 68-76) postulated that homogenization provides a protective film around the fat globule which prevents oxidation. Thurston and co-workers (40, p. 671-682) stated that homogenization may dislodge lecithin from around the fat globule and that this dislodged lecithin acts as an antioxidant. Although homogenization is said to retard the development of metallic-induced oxidized flavor, it makes the milk more susceptible to a solar-activated flavor, usually termed "sunshine flavor" (42, p. 559-564). In 1952, Krukovsky (22, p. 21-29) pointed out that the antioxidant factor in homogenized milk might be due to the migration of the unstable lipid material from the fat globule membrane into the interior of the fat globule where it would be protected by the more stable triglycerides.

Other factors believed to be involved in the development of oxidized flavors are the carotene content, the oxidation-reduction potential, the dissolved oxygen content, the microflora and the ascorbic acid content of the milk (30, p. 1-13). Brown et al (2, p. 925-935) decreased the carotene content of milk by feeding bleached alfalfa. They found that this did not increase the susceptibility
toward oxidation. From this they concluded that some substance, or substances, associated with the carotene probably had a greater effect than did the carotene itself. Postulation as to what these compounds might be was not made.

Webb and Hileman (41, p. 47-57) found no correlation between the development of oxidized flavor and the oxidation-reduction potentials of milks from individual cows. Milk samples from individual cows with a low potential developed oxidized flavor, while summer milks resisted the flavor development although the potential was high. Thurston (39, p. 112-118) raised the potential of non-susceptible milk by passing oxygen through it, but an oxidized flavor did not develop. Greenbank (16, p. 373-384) found that milks, poorly poised as to oxidation-reduction potential toward addition of copper, were susceptible to oxidized flavor development and that those well-poised did not develop the flavor. He postulated that this poising action of milk was a possible means of detecting susceptible milks.

Dissolved oxygen in milk increased the intensity of oxidized flavor and de-aeration and replacement of oxygen with nitrogen prevented the development of an oxidized flavor in susceptible milks (36, p. 31 and 17, p. 359-369). Microorganisms appear to exert their protective effect by utilizing the dissolved oxygen and thus lowering the potential (32, p. 140).

Chilson and co-workers (4, p. 925-928) found that the addition of propyl gallate to milk retarded oxidized flavor development but did not prevent ascorbic acid oxidation. From this, Riel and Sommer
postulated that ascorbic acid oxidation and oxidized flavor development do not necessarily stem from the same causes (30, p. 7). Krukovsky and Guthrie (20, p. 565-579 and 21, p. 293-306) reported that the presence of ascorbic acid was necessary for the development of a tallowy flavor in milks stored at low temperatures. Partial oxidation of the ascorbic acid was found by them to accelerate the development of the flavor while complete and rapid oxidation by either light of chemicals prior to pasteurization and storage was found to prevent the development of the tallowy flavor. Subsequent addition of ascorbic acid to the latter milk was found to produce a tallowy flavor.

Constituents of Milk Responsible for the Oxidized Flavor

Milk fat itself does not appear to be the constituent responsible for oxidized flavor. In 1937, Brown, Dustman and Thurston (3, p. 599-604) reported that there was no apparent difference between the iodine value of the fat from oxidized milk and that from non-oxidized milk. From this they concluded that the milk fat itself is not a source of the oxidized flavor. Swanson and Sommer (37, p. 201-208) found that the iodine value of the phospholipid fraction in oxidized milk was much lower than that in the original milk. From this they concluded that the probable source of oxidized flavor in milk was the phospholipid fraction.

According to Lea (25, p. 1-13) autoxidation of phospholipids stems from the action of atmospheric oxygen on the unsaturated fat acid components. He states that the major factor in this phospholipid
oxidation is undoubtedly the tendency for poly-unsaturated fat acids to be included in the phospholipid molecules. He further states that phospholipids isolated from fats often are appreciably more unsaturated than the fat triglycerides and that these highly unsaturated fat acids usually are present in very small quantities. These acids in the phospholipid fraction not only increase the susceptibility of the total lipid toward oxidation but also are the probable source of the strongly-flavored decomposition products encountered in the early stages of autoxidation.

Forss, Pont, and Stark, (13, p. 91-102) using chromatographic techniques, isolated and identified the following compounds from the steam distillate of oxidized skim milk: acetone, acetaldehyde, n-hexanal, croton aldehyde, and C₅ to C₉ unsaturated aldehydes. Presumptive evidence for the presence of several 2,4 di-unsaturated aldehydes of medium chain length was also obtained. They considered the 2-enals, particularly the C₈ and C₉ to be the principal constituents of the typical oxidized flavor. Acetone and acetaldehyde also were isolated from normal skim milk and consequently were considered not likely to be among the oxidized flavor constituents. Addition of the C₈ and C₉ enals in dilutions of 1 part in 10⁷ to 10⁹ to normal skim milk imparted a typical cardboard flavor to the milk. From this the authors deduced that the oxidized or cardboard flavor in milk is caused by preferential oxidation of di- and poly-unsaturated fat acids.
In further work, Forss et al (12, p. 345-348) found that the most abundant individual compounds in the steam distillates from oxidized skim milk were 2-octenal, 2-nonenal, 2,4-heptadienal, and 2,4-nonadienal. These compounds were added to normal skim milk in dilutions of 1 part in $10^7$ to $10^9$ and it was again found that typical cardboard flavor was obtained. They concluded that these compounds originate from the more highly unsaturated fatty acids in the milk lipids and are the compounds responsible for oxidized flavor.

**Theories of Lipid Autoxidation**

In 1956, Koch (19, p. 43-53,68,71) published a compilation of what is presently thought to be the mechanism of fat oxidation. The author recognized that the mechanism of fat oxidation is not completely understood and presented what appear to be the most logical pathways consistent with past investigations and present theories. These pathways and theories are presented below.

Oxidation can occur only in the fat acid portion of a lipid because the presence of double bonds is essential for oxidation. Fat oxidation can occur in two ways, either by autoxidation or enzymatic oxidation. In autoxidation, a hydrogen atom escapes from the $\alpha$-methylene carbon atom leaving an unstable free radical. An oxygen atom then replaces the hydrogen atom producing an unstable peroxyde free radical. The latter subsequently adds a hydrogen atom to give a fairly stable hydroperoxide. Under normal conditions, the following is the over-all mechanism for oxidation of mono-unsaturated and unconjugated poly-unsaturated fat acid esters:
Fat acid esters with conjugated double bonds will show more resistance to autoxidation because, instead of utilizing excess energy to break away from the molecule, the hydrogen atom expends the excess energy through resonance.

The hydrogen atom breaks away by being put into a higher energy state by some outside source of energy such as heat or light. When sufficient energy has been absorbed, the electrons reach a critical level and break away. When breaking away, the electron takes a
proton with it, making the net effect one of losing a hydrogen atom. This leaves the molecules as extremely reactive free radicals.

Since free radicals are very unstable, they will seek another electron. This electron can come from either molecular oxygen or from another \( \alpha \)-methylene hydrogen atom. If the hydrogen is from the latter, a chain reaction is started which continues until the free radical reacts with another free radical or an antioxidant. The law of mass action precludes many reactions with free radicals because usually there is a much greater quantity of the reactive fat acids than free radicals; so the chain reaction is perpetuated.

The oxidation of the non-conjugated polyethanoic structure is thought to be at the methyl group midway between the two double bonds. As this methyl group is alpha to either double bond it seems logical that this would be the most reactive point.

The fat acid ester in a free radical is very unstable. It is thought that in the free radical state the double bonds instantaneously rearrange to the more stable conjugated system. Spectrophotometric studies in the ultra violet spectra have confirmed this view.

Enzymatic oxidation is thought not to occur in milk, thus making the chemical autoxidation the most probable mechanism in the development of oxidized flavor in milk. (30, p. 16) Reimenschneider (32, p. 50-63) theorized that antioxidants functioned by interrupting the chain reaction of free radicals. He gave the following scheme as a possible mode of action, where \( F = \text{fat}, \ F_2\text{O}_2 = \text{peroxide}, \) and \( \ast = \) activated molecule:
\[ F + \text{energy} = F^*; \quad O_2 + \text{energy} \rightarrow O^* \]
\[ F^* + O_2 = F_2O^*; \quad F + O_2 \rightarrow F_2O^* \]
\[ F_2O^* + F = F_2O_2 + F^* \]
\[ F^* + O_2 \rightarrow F_2O_2^*, \text{ etc.} \]

When an antioxidant (A) is present, it presumably interferes with the reaction chains by one or more of the following reactions:

\[ A + F^* \rightarrow A^* + F; \quad A^* + O_2 \rightarrow AO_2 \]
\[ A + O_2^* \rightarrow AO_2 \]
\[ A + F_2O_2^* \rightarrow A^* + F_2O_2 \]
\[ A^* + F_2O_2^* \rightarrow AO^* + F_2O^* \rightarrow A + F + O_2 \]
\[ AO^* + F \rightarrow A + FO^* \]

Koch (19, p. 48-53, 68, 71) stated that the phenolic type of antioxidant functions by giving up hydrogen and forming a free radical which is more stable than the fat acid ester free radical. Thus the antioxidant prevents autoxidation by interfering with the chain reaction. Synergistic action of other compounds used with phenolic-type antioxidants has been attributed to chelation of traces of metals and/or furnishing of hydrogen atoms to antioxidant free radicals thus providing regeneration. Koch considers the former action to be the most likely. The mechanism for formation of carbonyl compounds from the hydroperoxides is not known. It is known that if autoxidative production of hydroperoxides is prevented there will be no carbonyl production (17, p. 48-53, 68, 71).
Methods of Detecting Oxidation in Milk

Riel and Sommer (30, p. 17) list numerous methods for the detection of fat oxidation but state that most do not have enough sensitivity to detect oxidation in milk. The two methods which are the most useful for detecting oxidation milk are organoleptic evaluation and the 2-thiobarbituric acid test. Patton and Kurtz (27, p. 669-674) investigated the latter test with milk fat and Dunkley and Jennings (8, p. 1064-1069) developed a method for detecting oxidized flavor in fluid milk using this reagent. Dunkley found that the TBA test (9, p. 342-346) could be correlated with organoleptic evaluations.

Flavonoids as Antioxidants in Dairy Products

The first workers to study the antioxidant properties of flavone and flavone derivatives were Richardson, El-Rafey and Long (29, p. 397-413). They found the flavones, quercetin, quercitrin, and rutin, to be effective antioxidants for milk fat and lard. The flavanone glycoside, hesperidin, was found to have little if any antioxidant properties but its chalcone was found to be active. They postulated that it was the lability of the pyrone ring which was responsible for the antioxidant properties of the flavones. Preliminary work on the flavones as antioxidants for fluid milk showed prevention of a typical oxidized flavor in winter milk when added after pasteurization.
In 1951, Kurth and Chan (23, p. 433-436) reported on dihydroquercetin as an antioxidant. They found dihydroquercetin to be an effective antioxidant for lard, cottonseed oil, and butter oil. The dihydroquercetin imparted no color and was both odorless and tasteless. Quercetin was found to impart a yellow color to the lard, but was slightly more effective than the same concentration of dihydroquercetin (0.03%) as an inhibitor of rancidity in lard.

To date, no other work with flavones on dairy products has been reported.
Preparation and Method of Handling Milk Samples

Before starting experimental work it was necessary to assure a known source of milk. Six cows, three Holsteins and three Jerseys, from the college herd were found to give milk susceptible to oxidation when 2 ppm of copper were added after pasteurization. All of these cows were in the first or second month of lactation. For convenience of handling, two cows, a Jersey (#331) and a Holstein (#643), were arbitrarily chosen. They were tested for mastitis and found to be mastitis-free. Their ration consisted of alfalfa hay, grass silage, and grain mix. The milks obtained at the time of regular milking were combined, brought to the laboratory in a newly-timed container and immediately pasteurized at 62° C. for 30 minutes. Three hundred ml. portions were placed in clear glass pint bottles after pasteurization and the copper was added in ppm quantities (weight/volume) in the form of a copper sulfate solution. The samples were then stored in the dark at 1-2° C. until removed for analysis. The milks were usually placed in the cooler in triplicate, thus providing a sample for each examination interval.

The antioxidants were added in the dry state either before pasteurization or after, depending on the study being conducted. If added after pasteurization, they were added at the same time as the copper.
Organoleptic Examinations

Organoleptic evaluations by at least three trained judges familiar with oxidized flavors were made at definite intervals during a particular test period. The milks were warmed to 20 - 25°C and scored as unknowns. A special evaluation sheet was developed and used; a facsimile of the evaluation sheet is shown in Figure 1.

It was not possible to have the same number of judges or even the same judges at all times; however, there were always at least three competent judges and the data obtained were evaluated by noting the comments and using the responses of the majority. The milks were judged semi-quantitatively for oxidized flavor by the use of the following notations:

0 - no oxidized flavor
? - questionable oxidized flavor
+ - slight oxidized flavor
++ - definite oxidized flavor
+++ - strong oxidized flavor

2-Thiobarbituric Acid Test (TBA test):

The TBA test procedure used was that of Dunkley and Jennings (3, p. 1064-1069) with some modifications. The procedure as outlined by these workers is as follows:

Reagents:

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

Extraction mixture: 2:1 mixture of iso-amyl alcohol and pyridine.
<table>
<thead>
<tr>
<th>SAMPLE NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown</td>
</tr>
<tr>
<td>Sample No.</td>
</tr>
<tr>
<td>No Comment</td>
</tr>
<tr>
<td>Questionable</td>
</tr>
<tr>
<td>Oxid. Flavor</td>
</tr>
<tr>
<td>Slight Oxid.</td>
</tr>
<tr>
<td>Flavor</td>
</tr>
<tr>
<td>Oxidized</td>
</tr>
<tr>
<td>Flavor</td>
</tr>
<tr>
<td>Definite</td>
</tr>
<tr>
<td>Oxid. Flavor</td>
</tr>
<tr>
<td>Other Flavor</td>
</tr>
<tr>
<td>Other Comments</td>
</tr>
</tbody>
</table>

Figure 1. Facsimile of evaluation sheet used in Organoleptic examinations.
Method:

Ten ml. of milk are pipetted into a centrifuge tube and mixed thoroughly with 5 ml. of the TBA reagent. The tube is then placed in a boiling water bath for exactly ten minutes and subsequently cooled in a cold water bath.

Fifteen ml. of the extraction mixture are then added to the tube and the tube shaken vigorously for 30 seconds and centrifuged at 3000 rpm for 5 minutes in a centrifuge with 16 inch peripheral diameter. Part of the solvent layer is then removed and read at 535 mu in a Beckman Model DU spectrophotometer.

The modification found to be necessary in this test was the insertion of a filtration step when the solvent layer is removed for spectrophotometric examination. Even at speeds in excess of 3000 rpm it was not possible to obtain an absolutely clear extract by centrifugation alone. This made it impossible to obtain duplicate results in optical density determinations. The filtration modification was found satisfactory and was used in the experimental studies.

A Coleman Junior Spectrophotometer, Model A, was used in this study because a Beckman Model DU Spectrophotometer was not immediately available. This instrument proved to be suitable.

Oxidation-Reduction Potential Determinations

Oxidation-reduction potentials were determined, using platinum electrodes patterned after those developed by Webb and Hileman, (41, p. 47-57) and a battery-operated Hellige potentiometer. A saturated calomel half-cell was used as a reference. Webb and Hileman
(41, p. 47-57) used 2 electrodes for each sample and averaged the results, but in this study it was found that replicate results were obtainable with one electrode. The electrodes were prepared for use by treatment in boiling detergent solution for at least 1 hour followed by immersion in hot (100-110° C.) dichromate-sulfuric acid cleaning solution (23, p. 1680) for at least two hours. The electrodes were then rinsed with distilled water and stored in distilled water for at least 24 hours before use. The agar-KCl bridges were prepared by adding 3 grams of agar to 100 ml. of saturated KCl solution. This was then introduced into glass tubing. These salt bridges were freshly prepared for each experiment.

The milk samples were transferred to 1/2 pint bottles; the agar-KCl salt bridge and platinum electrodes were introduced through a close-fitting rubber stopper. The milk, thus sealed, was placed in the dark at 1-2° C. The salt bridges and platinum electrodes were not removed from the milk until the end of the experiment thus allowing the milk to remain quiescent. Replicate samples for the TBA test were obtained from aliquots of the same milks in a series of individual tubes. At the time of each oxidation-reduction reading a tube corresponding to the sample being read was taken for the TBA test. In this manner, the TBA test at each time interval represented milk in the same quiescent state as that used for oxidation-reduction potential determinations.
Feeding of Quercetin

Quercetin was fed to the two experimental cows at a suggested rate of 0.25 grams of quercetin per 100 pounds of body weight (25). The quercetin was pre-mixed with cottonseed meal and fed daily at the PM feeding. Cottonseed meal was utilized because the cows had been receiving it previously in the grain mix. For ease of calculation and weighing, 2.5 grams of quercetin were mixed with 47.5 grams of the cottonseed meal making the ratio 50 grams of pre-mix per 1000 pounds of body weight.

Milk samples were taken initially and every two days thereafter. After pasteurization and the addition of 0.25 ppm copper, they were stored in the dark at 1-2° C. They were examined organoleptically at 24-hour intervals.

Standard Plate Count

For the first three trials, standard plate counts were made to assure that bacterial action was not exerting a protective effect. The plates were poured on the first and last day of the experimental periods and counted after 48 hours storage at 1-2° C. (9, p. 84-95).
RESULTS

Trial I was a preliminary trial to determine the susceptibility of the milks from six cows to copper-induced oxidation and to determine if added dihydroquercetin would impart a flavor to milk. All six milks showed susceptibility and none appeared to be spontaneous. It was also found that the presence in milk of dihydroquercetin added in increments of 5, 10, 20, 30, and 50 mg. % was not detected by the judges.

In Trial II, the milks from the two cows chosen for this study were re-examined and found to remain susceptible. The results of this trial are shown in Table I. Organoleptic examination at the 24-hour interval indicates the possibility of spontaneous oxidation in the milk from cow number 643. The TBA values at the 42-and 66-hour intervals, however, were no higher than the initial value indicating no oxidation. It should be pointed out that when this experiment was conducted, the results from the TBA test were still erratic because of the turbidity in the solvent layer which increased the optical density. The increase in optical density stemming from turbidity of the solvent layer is demonstrated by the high values obtained at the 42-and 66-hour interval in those milks to which copper had been added.

The effects of increasing levels of dihydroquercetin added to the mixed milk of cows 643 and 331 after pasteurization (Trial III) are shown in Table II and Figure 2. The added dihydroquercetin at
TABLE I

Susceptibility to copper-induced oxidation of the milk from cows 331 and 643. Trial II.

<table>
<thead>
<tr>
<th>Cow Number</th>
<th>Breed</th>
<th>Milk Treatment</th>
<th>18 hours TBA</th>
<th>18 hours Organ.</th>
<th>42 hours TBA</th>
<th>42 hours Organ.</th>
<th>66 hours TBA</th>
<th>66 hours Organ.</th>
</tr>
</thead>
<tbody>
<tr>
<td>331</td>
<td>Jersey</td>
<td>Control</td>
<td>0.042</td>
<td>0</td>
<td>0.042</td>
<td>0</td>
<td>0.032</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.25 ppm Cu added</td>
<td>0.046</td>
<td>0</td>
<td>0.085</td>
<td>++</td>
<td>0.082</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.50 ppm Cu added</td>
<td>0.060</td>
<td>+</td>
<td>0.077</td>
<td>++</td>
<td>0.077</td>
<td>++</td>
</tr>
<tr>
<td>643</td>
<td>Holstein</td>
<td>Control</td>
<td>0.043</td>
<td>0</td>
<td>0.036</td>
<td>?</td>
<td>0.027</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.25 ppm Cu added</td>
<td>0.046</td>
<td>+++</td>
<td>0.342</td>
<td>+++</td>
<td>0.301</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.50 ppm Cu added</td>
<td>0.053</td>
<td>+++</td>
<td>--</td>
<td>+++</td>
<td>0.347</td>
<td>+++</td>
</tr>
</tbody>
</table>
TABLE II

The effect of adding dihydroquercetin after pasteurization to milks containing added copper as reflected by organoleptic examinations and the TBA test. Trial III.

<table>
<thead>
<tr>
<th>Milk Treatment</th>
<th>Initial</th>
<th>18 hours</th>
<th>42 hours</th>
<th>66 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TBA</td>
<td>TBA</td>
<td>Organ.</td>
<td>TBA</td>
</tr>
<tr>
<td>Control</td>
<td>0.029</td>
<td>-</td>
<td>0</td>
<td>0.037</td>
</tr>
<tr>
<td>0.25 ppm Cu added</td>
<td>0.044</td>
<td>0.094</td>
<td>+++</td>
<td>0.124</td>
</tr>
<tr>
<td>0.25 ppm Cu plus 5 mg. % DHQ</td>
<td>0.036</td>
<td>0.041</td>
<td>0</td>
<td>0.034</td>
</tr>
<tr>
<td>0.25 ppm Cu plus 10 mg. % DHQ</td>
<td>0.046</td>
<td>0.041</td>
<td>0</td>
<td>0.040</td>
</tr>
<tr>
<td>0.25 ppm Cu plus 20 mg. % DHQ</td>
<td>0.041</td>
<td>0.035</td>
<td>0</td>
<td>0.040</td>
</tr>
<tr>
<td>0.25 ppm Cu plus 30 mg. % DHQ</td>
<td>0.035</td>
<td>-</td>
<td>0</td>
<td>0.037</td>
</tr>
</tbody>
</table>
Figure 2. The progressive changes of Eh values of pasteurized milks when Cu and DHQ are added after pasteurization. Trial III.
all levels prevented the development of oxidized flavor as reflected by both the organoleptic examinations and the TBA test. In this trial the filtration step, previously mentioned, was used in the TBA test and clear solvent extracts were obtained in every case. At the 18-hour interval, two of the TBA values were lost due to breakage in the centrifuge. Both TBA values and organoleptic examination indicate the development of an oxidized flavor in the control sample. This may have resulted from exposure to the light left burning in the cold room, the samples being contained in clear glass bottles. As shown in Figure 2, Eh values of the milks containing dihydroquercetin progressively increased at the 18-and 32-hour intervals faster than that of the milk containing only copper. The reverse was found at the 66-hour interval. Standard bacterial plate counts initially and at the end of the trial were less than 1000 ml. in both cases, indicating that bacterial activity was negligible. Because of the possibility of light-catalyzed oxidation in Trial III, Trial IV entailed a partial repetition of that trial. Dark storage was assured by removal of the light bulbs in the cooler.

Table III shows the results of this trial. The TBA values of the samples containing added copper and dihydroquercetin remained practically identical to the control. The subjective organoleptic tests were somewhat irregular. This disagreement between TBA and organoleptic results may be attributed to the presence of a fairly strong feed flavor in the milk which made it difficult to identify
TABLE III

TBA values and organoleptic evaluations of milks stored at 1-2° C. containing 0.25 ppm plus dihydroquercetin added after pasteurization. Trial IV.

<table>
<thead>
<tr>
<th>Milk Treatment</th>
<th>Initial TBA</th>
<th>24 hours TBA</th>
<th>24 hours Organ.</th>
<th>48 hours TBA</th>
<th>48 hours Organ.</th>
<th>72 hours TBA</th>
<th>72 hours Organ.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control - no added Cu</td>
<td>0.022</td>
<td>0.022</td>
<td>0</td>
<td>0.036</td>
<td>0</td>
<td>0.036</td>
<td>0</td>
</tr>
<tr>
<td>0.25 ppm copper</td>
<td>0.022</td>
<td>0.108</td>
<td>++</td>
<td>0.115</td>
<td>+++</td>
<td>0.125</td>
<td>++</td>
</tr>
<tr>
<td>0.25 ppm copper plus 5 mg. % DHQ</td>
<td>0.022</td>
<td>0.022</td>
<td>+</td>
<td>0.040</td>
<td>0</td>
<td>0.036</td>
<td>0</td>
</tr>
<tr>
<td>0.25 ppm copper plus 10 mg. % DHQ</td>
<td>0.022</td>
<td>0.022</td>
<td>0</td>
<td>0.037</td>
<td>0</td>
<td>0.039</td>
<td>?</td>
</tr>
<tr>
<td>0.25 ppm copper plus 15 mg. % DHQ</td>
<td>0.022</td>
<td>0.022</td>
<td>+</td>
<td>0.039</td>
<td>0</td>
<td>0.036</td>
<td>0</td>
</tr>
</tbody>
</table>

* Optical density (2-log %T) at 535 mu, 19 x 105 mm. cell.
or recognize other flavors in the milk. The standard bacterial plate counts were again less than 1000/ml. at both the beginning and end of this trial.

To assure the absence of feed flavor, the milk for Trial V was obtained from the morning milking. The cows had had no feed except grain for the 2 to 3 hours prior to milking. In this trial, quercetin and dihydroquercetin were added both before and after pasteurization to copper-containing milk. As indicated in Table IV and Figures 3 and 4, the addition of the two compounds was equally effective when added either before or after pasteurization. The TBA tests and organoleptic examinations were in agreement in this trial. Standard bacterial plate counts indicated no increase in bacteria during the course of the trial being less than 1000/ml. at both the beginning and end. No bacterial counts were made subsequent to this trial because the results thus far indicated no bacterial increase under the experimental conditions. In Trial V it was found that the quercetin had not wholly dissolved, the excess forming a sediment in the sample bottles. This indicated that this antioxidant might be effective in concentrations less than 10 mg. per cent. In Trial VI, the quercetin and dihydroquercetin were added at the 1 mg. per cent level and the copper concentration was increased stepwise to 1 ppm. The antioxidants were added before pasteurization. As shown in Table V, the TBA values do not indicate oxidation in those milks with added antioxidants; the organoleptic examinations indicated the presence of a questionable oxidized flavor in three cases.
TABLE IV

TBA values and organoleptic evaluation of milks stored at 1-2\(^\circ\) C. containing 0.025 ppm Cu plus added dihydroquercetin (DHQ) or Quercetin (Q) added both before (BP) and after (AP) pasteurization. Trial V.

<table>
<thead>
<tr>
<th>Milk Treatment</th>
<th>Initial TBA*</th>
<th>24 hours TBA</th>
<th>24 hours Organ.</th>
<th>48 hours TBA</th>
<th>48 hours Organ.</th>
<th>72 hours TBA</th>
<th>72 hours Organ.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control - no added Cu</td>
<td>0.031</td>
<td>0.029</td>
<td>0</td>
<td>0.032</td>
<td>0</td>
<td>0.032</td>
<td>0</td>
</tr>
<tr>
<td>0.25 ppm Cu</td>
<td>0.031</td>
<td>0.065</td>
<td>+++</td>
<td>0.102</td>
<td>+++</td>
<td>0.137</td>
<td>+++</td>
</tr>
<tr>
<td>0.25 ppm Cu plus 10 mg. % DHQ BP</td>
<td>0.033</td>
<td>0.032</td>
<td>0</td>
<td>0.036</td>
<td>0</td>
<td>0.036</td>
<td>0</td>
</tr>
<tr>
<td>0.25 ppm Cu plus 10 mg. % DHQ AP</td>
<td>0.032</td>
<td>0.032</td>
<td>0</td>
<td>0.034</td>
<td>0</td>
<td>0.032</td>
<td>0</td>
</tr>
<tr>
<td>0.25 ppm Cu plus 10 mg. % Q BP</td>
<td>0.036</td>
<td>0.032</td>
<td>0</td>
<td>0.039</td>
<td>0</td>
<td>0.036</td>
<td>0</td>
</tr>
<tr>
<td>0.25 ppm Cu plus 10 mg. % Q AP</td>
<td>0.029</td>
<td>0.029</td>
<td>0</td>
<td>0.039</td>
<td>0</td>
<td>0.037</td>
<td>0</td>
</tr>
</tbody>
</table>

* Optical density (2-log %T) at 535 m\(\mu\), 19 x 105 mm. cell.
Figure 3. Effect of addition of 10 mg/70 Q before and after pasteurization on the development of oxidation of milk containing 0.25 ppm Cu and stored at 1-2°C as reflected by the TBA test.
**Figure 4. Results of adding 10 mg% dihydroquercetin to milk with 0.25 ppm Cu added, as reflected by the 2-thiobarbituric acid test. Trial V**
TABLE V

TBA values and organoleptic evaluations of milk containing varied amounts of copper plus dihydroquercetin and quercetin added before pasteurization at the 1 mg. % level. Trial VI.

<table>
<thead>
<tr>
<th>Milk Treatment</th>
<th>Initial TBA*</th>
<th>24 hours TBA</th>
<th>24 hours Organ.</th>
<th>48 hours TBA</th>
<th>48 hours Organ.</th>
<th>72 hours TBA</th>
<th>72 hours Organ.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control - no added Cu</td>
<td>0.031</td>
<td>0.029</td>
<td>0.029</td>
<td>0.029</td>
<td>0.029</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.25 ppm added Cu</td>
<td>0.036</td>
<td>0.122</td>
<td>+++</td>
<td>0.122</td>
<td>+++</td>
<td>0.122</td>
<td>-</td>
</tr>
<tr>
<td>1 mg. % DHQ plus 0.25 ppm Cu</td>
<td>0.039</td>
<td>0.036</td>
<td>0.034</td>
<td>?</td>
<td>0.036</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1 mg. % DHQ plus 0.50 ppm Cu</td>
<td>0.041</td>
<td>0.036</td>
<td>0.036</td>
<td>0</td>
<td>0.036</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1 mg. % DHQ plus 1.00 ppm Cu</td>
<td>0.039</td>
<td>0.034</td>
<td>0.039</td>
<td>0</td>
<td>0.039</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1 mg. % Q plus 0.25 ppm Cu</td>
<td>0.036</td>
<td>0.032</td>
<td>0.032</td>
<td>?</td>
<td>0.032</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1 mg. % Q plus 0.50 ppm Cu</td>
<td>0.039</td>
<td>0.036</td>
<td>0.036</td>
<td>0</td>
<td>0.036</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1 mg. % Q plus 1.00 ppm Cu</td>
<td>0.031</td>
<td>0.043</td>
<td>0.046</td>
<td>?</td>
<td>0.043</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Optical Density (2-log %T) at 535 mu.
Trial VII involved, primarily, the study of Eh changes in the milks up to the end of the induction period. This was supplemented by the TBA determinations and organoleptic examinations at the 48 and 72 hour intervals. The progressive Eh changes are shown in Figure 5, and the TBA values and organoleptic results are shown in Table VI. As in Trial III, the milk with both dihydroquercetin and copper showed an increase in Eh value comparable to the increase in the milk containing only copper. As shown in Table VI, the copper-containing milk with no dihydroquercetin developed an oxidized flavor along with the increase in Eh, but that containing dihydroquercetin did not develop the flavor even though the Eh increased.

The progressive Eh changes in copper-containing milks were again studied in Trial VIII. The Eh readings are shown in Figure 7, the corresponding TBA values in Figure 6. As found previously in Trials III and VII, the copper-containing milks with added quercetin and dihydroquercetin increased in Eh with no oxidized flavor development, while in the copper-containing milk with no quercetin or dihydroquercetin the increase in Eh was accompanied by the development of an oxidized flavor. The corresponding TBA values show an increase only in the milk with added copper; those milks with the copper and antioxidant show no increase and, consequently, no oxidized flavor development.
Figure 5. Progressive changes in oxidation-reduction potentials of milks stored at 1-2°C with added Cu and/or dihydroquercetin. Trial VII
TABLE VI

TBA values and organoleptic evaluations of milk containing varied amounts of copper plus dihydroquercetin and quercetin added before pasteurization at the 1 mg. % level. Trial VII.

<table>
<thead>
<tr>
<th>Milk Treatment</th>
<th>Initial TBA</th>
<th>48 hours TBA</th>
<th>72 hours TBA</th>
<th>48 hours Organ.</th>
<th>72 hours Organ.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control - no added Cu</td>
<td>0.032</td>
<td>0.027</td>
<td>0.027</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.25 ppm Cu added</td>
<td>0.035</td>
<td>0.097</td>
<td>0.107</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>1 mg. % DHQ plus 0.25 ppm Cu</td>
<td>0.035</td>
<td>0.036</td>
<td>0.037</td>
<td>0</td>
<td>?</td>
</tr>
<tr>
<td>1 mg. % DHQ plus 1.00 ppm Cu</td>
<td>0.035</td>
<td>0.043</td>
<td>0.043</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 mg. % DHQ plus 2.00 ppm Cu</td>
<td>0.045</td>
<td>0.047</td>
<td>0.050</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 mg. % Q plus 1.00 ppm Cu</td>
<td>0.036</td>
<td>0.041</td>
<td>0.043</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 mg. % Q plus 2.00 ppm Cu</td>
<td>0.042</td>
<td>0.048</td>
<td>0.051</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 6. Progressive changes of TBA values of milk stored at 1-2°C with added Cu, quercetin, and dihydroquercetin as indicated. Trial VIII.
Figure 7. Progressive changes of Eh values of milks stored at 1-2°C with added Cu, Quercetin, and Dihydroquercetin added before pasteurization as indicated. Trial VIII.
The feeding experiment in which quercetin is being added to the grain mixture is still in progress. Data obtained from a ten-day observation indicate no apparent antioxidant effect in the milk resulting from the additive. This experiment will continue for at least 4 weeks.
DISCUSSION

Milk is a complex, biological fluid which contains many components with varied properties. This complexity precludes any precise description of the actual mechanism of protection of added antioxidants. Modern theories of antioxidant action postulate chelation or complexing of trace metals or a system of hydrogen or electron transfer (19).

Chelation of trace metals could be influenced by pH or by the presence of other cations such as calcium present in milk. The hydrogen transfer theory is obviously complicated by the differing oxidation-reduction potentials of the various milk components, such as; riboflavin, ascorbic acid, phospholipids, tocopherol, glutathione, etc. Each of these components exists in a separate equilibrium with respect to its oxidized and reduced states and any shift of equilibrium of one of these components probably will affect the equilibrium states of the others. The actions of the enzymes involved cannot be completely ignored, although pasteurization inactivates the enzymes, partially or completely.

In previous work by Richardson and associates (29, p. 397-413) the antioxidant activity of some flavonoids was considered to be centered in the labile pyrone ring. This was deduced from the fact that the flavonoid compounds containing the unsaturated pyrone nucleus exhibited antioxidant activity while those compounds, such as hesperidin, lacking this grouping showed little or no antioxidant
activity. It was thought that the saturated pyrone ring of hesperidin was the reason for its inactivity. Both quercetin and dihydroquercetin, which were used in this study, contain this grouping as is shown in Figure 8. Kelley and Watts (18, p. 308-315) postulate that the antioxidant effect of the flavonoids, e.g. quercetin, is exerted through the chelation of trace metals, and suggest that the metal is chelated through \(-C=O\)-metal bridges from carbons 3 and 4, and 3' and 4'. This would appear to be in agreement with the findings of Richardson and associates (29).

If the antioxidant properties of quercetin and dihydroquercetin in milks containing copper are a result of chelation of the copper, it would seem logical to expect that the copper present in milk would be chemically inactivated. It would be expected, therefore, that the oxidation-reduction potential of the milk would not be affected. In Trials III, VII, and VIII, however, in these studies, oxidation appeared to take place. The oxidation-reduction potential of the milks containing both copper and quercetin or dihydroquercetin increased as much or more than that of the milks containing copper only. The milks with copper only developed an oxidized flavor while that with both copper and antioxidant did not. The control samples in all the trials showed little or no increase in oxidation-reduction potential but all samples with copper present showed increases, thus indicating that the copper was not being inactivated.
A possible explanation of the rise in Eh without oxidized flavor development might be due to the oxidation of ascorbic acid. Chilson, Martin and Whitnah (4, p. 925-928) found that propyl gallate, a phenolic-type antioxidant, prevented the development of an oxidized flavor in milk, but did not prevent the oxidation of ascorbic acid. Although quercetin and dihydroquercetin have not been classed as phenolic-type antioxidants, they do show similarity to these phenolic-types in that the two aromatic rings have the necessary hydroxyls (Figure 8). Reimenschneider (32, p. 50-63) reported that the most effective phenolic-type antioxidants have two hydroxyl groups in ortho or para relation to each other. Two hydroxyls in ortho relationship are found in quercetin and dihydroquercetin at the 3' and 4' positions as shown in Figure 8. These two compounds, therefore, may act in the same manner as the phenolic-type antioxidants. This concept would be in agreement with the inactivity of hesperidin as found by Richardson et al (29) because in hesperidin, the hydroxyl group in the 4' position is methylated. It might be that this methylation is the cause of inactivity rather than the saturated pyrone ring.

If the two flavonols studied in this work act in the same manner as phenolic-type antioxidants, the ascorbic acid present could act synergistically. Koch (19) believed that the synergistic action of ascorbic acid may be due to either a furnishing of electrons for regeneration of the antioxidant or to chelation of trace metals. From the results obtained in this work it would appear that the former mode of action is the most likely function because of the rise
Figure 8. Structural Formulas of Quercetin and Dihydroquercetin
in $E_h$ found in the presence of both copper and antioxidant. It might then be theorized that the flavonols, quercetin and dihydroquercetin, prevent oxidation by providing hydrogen atoms to the free radicals formed from unsaturated fatty acids through the catalytic action of the copper, thus preventing the initiation of chain reactions. The antioxidant could then be regenerated by receiving an electron from the ascorbic acid which would go to dehydroascorbic acid. The free radical would then be in the more stable hydroperoxide form and would not form the objectionable and typical carbonyl products associated with the oxidized flavor. The rise in $E_h$ could then be attributed to the oxidation of the ascorbic acid. The oxidation of ascorbic acid was not followed in these studies thus making the foregoing suggestions purely speculative. It is also possible that some of the other components existing in an oxidation-reduction equilibria could contribute to the rise in $E_h$.

The suggested oxidation of ascorbic acid would also be in agreement with the theory of Krukovsky and Guthrie (21, p. 293-306) that a certain equilibrium between ascorbic acid and dehydroascorbic acid is necessary for the development of an oxidized flavor and if ascorbic acid is completely oxidized to dehydroascorbic the flavor will not develop.

Although the mode of action of quercetin and dihydroquercetin remains obscure, it was found that these two compounds were effective antioxidants at low concentrations in pasteurized fluid milk. Incidental observations made during this study indicate that of the
two compounds used, dihydroquercetin would probably be the most suitable because it is colorless, more water soluble and tasteless in the concentration used. The solubility of quercetin is 0.008% at 20° C. (1, p. 2) while that of dihydroquercetin is 0.51% in water at 15° C. (23, p. 433-436)

Thus far, ingested quercetin does not appear to be secreted in the milk. The studies are being continued. Dihydroquercetin might prove to be more suitable for feeding than quercetin because of the difference in solubility. The lack of a specific method of assaying for these compounds in milk precludes any conclusive results at present.

The results of this study suggest that dihydroquercetin and quercetin may be effective antioxidants for other dairy products and other foods. The apparent stability of these compounds to pasteurization temperatures indicates the distinct possibility that they might be effective antioxidants for dry whole milk and dry buttermilk.
SUMMARY AND CONCLUSIONS

The mixed milks from two cows whose milks were proved to be susceptible to copper-induced oxidation were subjected to a series of tests to determine the antioxidant efficiency of two flavone-type compounds, quercetin and dihydroquercetin. These tests included the addition of the two compounds, before or after pasteurization and at different concentrations, to milks containing varying levels of copper added after pasteurization.

Experimental results were obtained by organoleptic examinations, the 2-thiobarbituric acid test, and oxidation-reduction potential determinations. The TBA test was modified by including a filtration step. It was found to correlate with the organoleptic examinations.

Dihydroquercetin and quercetin prevented oxidized flavor development when added either before or after pasteurization, at the 1 mg. per cent level, in the test milks containing up to 1 ppm added copper.

The milks containing both copper and dihydroquercetin, or quercetin, showed initial increases in Eh equal to or greater than that of the milks containing copper only. The latter milks, however, developed an oxidized flavor while the former did not. Since neither the organoleptic examinations nor the TBA test indicated oxidation of a lipid fraction, it is suggested that the increase in Eh may be due to the oxidation of ascorbic acid.
The initial studies in the addition of quercetin to the ration of the test cows have shown no lessening of the susceptibility of the milk to oxidation. They are being continued.

Some possible application of dihydroquercetin and quercetin as antioxidants in other dairy products, such as dry whole milk and dry buttermilk, are suggested.
BIBLIOGRAPHY


