

THE EFFECT OF METHYLATION UPON THE ANTIOXIDANT  
AND CHELATION CAPACITY OF QUERCETIN AND  
DIHYDROQUERCETIN IN A LARD SUBSTRATE

by

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# THE EFFECT OF METHYLATION UPON THE ANTIOXIDANT AND CHELATION CAPACITY OF QUERCETIN AND DIHYDROQUERCETIN IN A LARD SUBSTRATE

## INTRODUCTION

Lea (39, p. 200) defined rancidity in the broadest possible sense as any "off" odor or flavor which has developed in an oil or fat as a result of deterioration or storage. Rancidity in oils and fats can develop by (a) absorption of odors, (b) hydrolysis (enzymatic and nonenzymatic), (c) action of microorganisms, (d) autoxidation and (e) reversion.

The wide variety of changes which are grouped in the category as constituting "off" flavors or odors is indicated by the large number of terms which are used to describe various types of rancidity. Some of these are rancid, pungent, tallowy, soapy, oily, ester-like, metallic, musty, fishy, bitter, cardboard and burnt.

Oxidative rancidity is liable to occur in any material containing unsaturated lipid when it is exposed to the air. The mechanism of lipid oxidation is thought to be a free radical initiated chain reaction with the primary product being a hydroperoxide (27, p. 48-53; 40, p. 1303; 46, p. 126-131; 48, p. 61-66; 52, p. 239-241; 54, p. 78-85). The hydroperoxides formed are not the source of the typical, strong and disagreeable odor and flavor of oxidized lipids

(37, p. 48-53). It has been shown that the disagreeable odor and flavor are largely attributed to the complex mixture of degradation products resulting from oxidation and breakdown of hydroperoxides (7, p. 489; 34, p. 377; 35, p. 719; 39, p. 211; 59, p. 297-300).

There are various methods to combat oxidative rancidity. These methods include: hydrogenation, removal of susceptible minor constituents or precursors of off-flavor, limitation of oxygen, refrigeration, exclusion of light, minimization of metallic contamination, heat inactivation of heat labile catalysts, control of moisture content, addition of antioxidants, and preservation of natural antioxidants (42, p. 621).

The use of antioxidants in the non-food industry is widespread and of significant industrial importance. In comparison, the addition of antioxidants to food substances is not as extensive and it is in its infancy.

The addition of antioxidants to food products presents certain problems. Beside being adequately effective and economically feasible, an antioxidant must impart no undesirable color, flavor and odor to the product. Above all, the toxicity of the antioxidant added to the food must be very low. The rigid specifications regarding toxicity incorporated in the Food, Drug and Cosmetic Act emphasizes this importance. Also, the recent amendment to

the act prohibiting any additive which "induces cancer" in animal or man from food use indicates the stringent requirements to which food additives, which include antioxidants, must adhere.

At the present time there is no universally accepted mechanism of antioxidant action. However, it is generally thought that the antioxidants mode of action is one of providing a hydrogen atom to free radicals. This would terminate the free radical initiated chain mechanism of autoxidation (16, p. 5236; 37, p. 48-53; 52, p. 241).

Flavonoids, particularly quercetin and dihydroquercetin are distributed widely in nature (19, p. 451; 38, p. 433), in the free form or as the glucoside (3, p. 267; 19, p. 455; 28, p. 451-454). Quercetin and dihydroquercetin are found in commercial quantities (27, p. 433), are nontoxic (4, p. 183-184; 25, p. 96; 33, p. 430-433; 50, p. 272), and have certain biological significance (4, p. 183-184; 19, p. 451-453; 25, p. 3; 33, p. 430-433; 37, p. 118-120).

Pazina (5, p. 9-10) has listed the present and potential uses for flavonoids and in particular quercetin. They are: (a) pharmaceutical, (b) dye and dye intermediate, (c) complexing agent, (d) analytical reagent and indicator, (e) a phenol to be used in preparation of resins, (f) a dispersant and thinning agent and (g) as

an antioxidant for fats and oils.

Flavonoids such as quercetin and dihydroquercetin have been classified as multifunctional antioxidants. They can act as normal polyhydroxyphenolic, radical chain breaking antioxidants and as complexers or chelators of catalytic metal ions (26, p. 395; 42, p. 633). The antioxidant ability of quercetin and dihydroquercetin has been demonstrated in a wide variety of lipid substances (26, p. 398; 36, p. 402-407; 38, p. 234-235; 47, p. 2). There antioxidant ability compares favorably with other commercial and approved antioxidants (26, p. 394-398; 47, p. 2).

The characteristics of flavonoids which suggest their possible use as food antioxidants indicated the need for a detailed study of the molecular structural features which are the source of their antioxidant ability. Such a study may result in some contribution to the information known of the mechanism of antioxidant action and lipid autoxidation.

This investigation was designed to allow the study of the relationship of the hydroxyl group in various positions on the flavone and flavanone molecule to the effectiveness of the compounds as antioxidants. At the same time the relationship of the various complexing sites on the molecule to antioxidant capacity could be studied.

## LITERATURE REVIEW

Lipids in general have been considered to be those compounds, insoluble in water, but soluble in "fat solvents" (ether, chloroform, benzene, etc.) related to fatty acid esters and usable by living organisms (11, p. 2-3, 24, p. 38). Lipids are conveniently classified into groups, which include triglycerides, waxes, glycerophosphatides, sphingolipids, etc. (11, p. 3-6; 24, p. 38).

Mixed triglycerides are the major component of natural fats and oils (11, p. 179; 37, p. 48). They are esters which contain the glycerol molecule with its three hydroxyl groups esterified with three different fatty acid residues (11, p. 12-19; 37, p. 48). The fatty acid residues may be saturated or unsaturated and of various chain lengths (11, p. 12-19; 37, p. 48).

### Autoxidation

#### Primary oxidation

The mechanism, products formed and factors that influence autoxidation has been the subject of investigation for more than fifty years (52, p. 237). During this time sufficient evidence and experimental data have been accumulated to propose an acceptable mechanism for autoxidation.

Autoxidation results from the action of atmospheric oxygen with the triglyceride molecule (11, p. 281; 39, p. 237). Under ordinary conditions oxidation takes place only with the fatty acid portion. The unsaturation of the fatty acid moiety is necessary for oxidation to take place under ordinary conditions (37, p. 48-53). Any lipid substance containing unsaturation then is subject to oxidation under ordinary conditions (37, p. 48-53). Deterioration of this type is not limited to triglycerides and fat soluble pigments and vitamins, but is exhibited by many other substances of biological importance (42, p. 621).

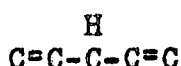
Double bonds are able to capture outside sources of energy, such as light and heat energy which raises their electrons to a higher energy level or excited state. When enough energy has been absorbed so that the electrons have reached a critical excitation level, the excess energy is dissipated by an electron breaking away from the molecule and taking a proton with it. This leaves the molecule with a carbon atom containing unpaired electrons, i.e., a free radical. The proton or hydrogen atom which escaped with the electron is also a free radical since it also has an unpaired electron (37, p. 49).

Farmer (14, p. 86) pointed out that his experimental results indicated that autoxidation could be interpreted

as a polar type reaction in which the participants are short-lived neutral entities displaying free radical characteristics. The formation of free radicals from fat molecules is dependent on hydrogen lability and this lability is determined by the pattern of unsaturation in the fatty acid residue (14, p. 88).

Unsaturation patterns of fatty acids have been grouped by Farmer (14, p. 88) in the following manner:

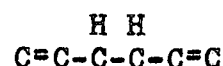
Group (1)



Group (3)



Group (2)



Group (4)



Group (1) has the highest degree of hydrogen lability due to the alpha methylene group flanked by two adjacent double bonds. Group (1) is a nonconjugated unsaturated system. The methylene carbon separates the two double bonds hindering the dissipation of excess energy through resonance. Group (2) shows less lability because the inference of the double bond is divided between the two methylene carbons. Group (3) has a still smaller hydrogen lability and Group (4), being a conjugated olefin, has lost most of its hydrogen lability. Conjugated unsaturated systems due to the close proximity of the double bonds allows electrons to dissipate their energy through

resonance (14, p. 86-88; 37, p. 48-53).

Direct experimental evidence is now available which demonstrates that hydroperoxides are the predominant, but not the exclusive, primary product of autoxidation (37, p. 48-53). Saunders, Ricciute, and Swern (54, p. 78-85) stated that as much as 95% of the peroxides formed in autoxidation of methyl oleate are alpha-methylenic hydroperoxides.

Privett and coworkers (48, p. 61-66) autoxidized methyl linoleate in the dark to a peroxide value of 671 me./kg. at approximately 0°C. They quantitatively separated an essentially pure concentrate of methyl octadecadienoate monohydroperoxide from the oxidized methyl linoleate. Infrared and ultraviolet spectrophotometric methods indicated that the concentrate contained at least 90% conjugated monohydroperoxides. They suggested that the remaining 10% were most likely nonconjugated diene hydroperoxides.

Lea (40, p. 1303) pointed out that monohydroperoxides, by what ever route they are produced, are very largely conjugated, and not likely to form dihydroperoxides. Some evidence is available which indicates that a cyclic peroxide may be formed by 1:4 addition to the monohydroperoxide of linoleic acid (40, p. 1303; 46, p. 127). After the cyclic peroxide is formed by 1:4 addition,

dimerization and polymerization can probably take place (40, p. 1303).

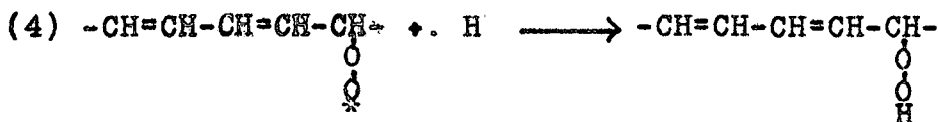
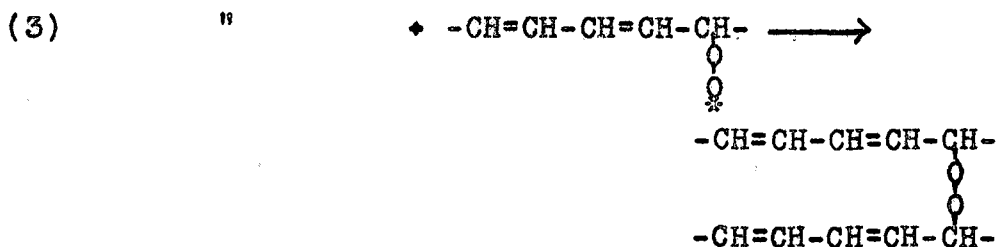
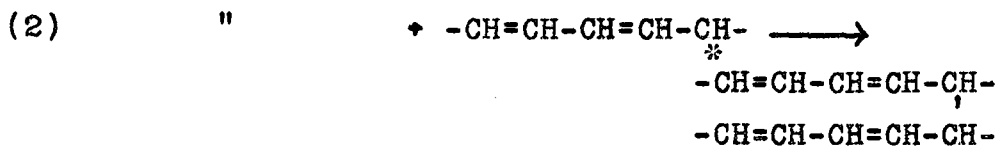
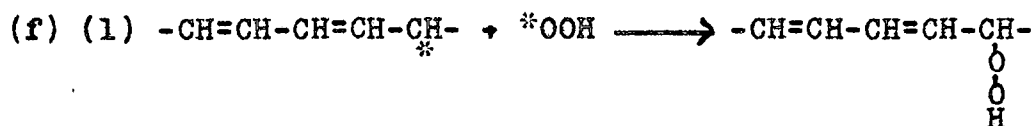
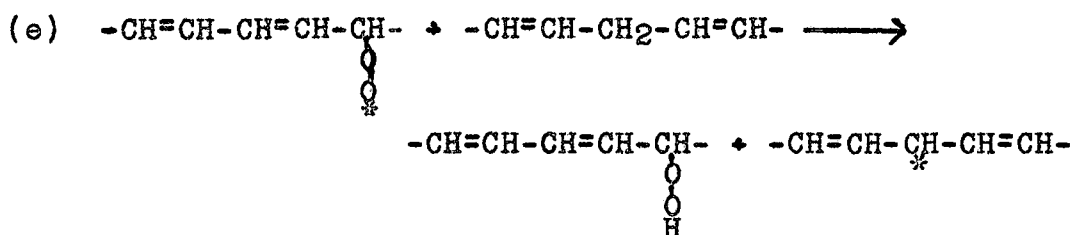
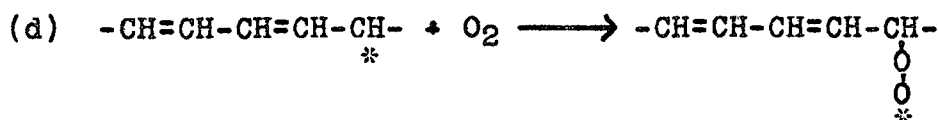
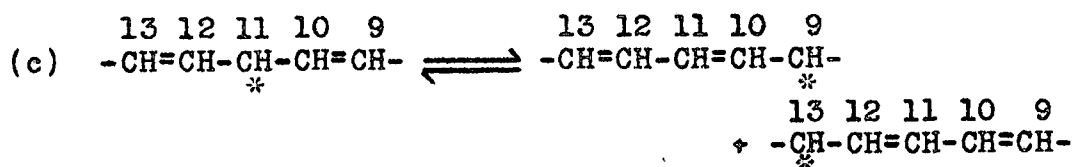
Morris (46, p. 127) in citing the work of Farmer, Bolland and Gee, and Gunstone and Hilditch stated that oxidation may take place initially at the double bond instead of at the alpha methylene group. They based their conclusion on thermodynamic data and the fact that additions of small amounts of methyl linoleate to methyl oleate greatly accelerated its oxidation. However, they agree that only a small amount of oxidation at the double bond is necessary to form enough free radicals for the usual alpha methylene chain reaction to proceed.

The reviews of Lea (40, p. 1303-1309), Riemenschneider (52, p. 239-241), Koch (37, p. 68-69) and Morris (46, p. 126-131) best covers the generally accepted mechanism of fat autoxidation. Linoleic acid is one of the principal unsaturated acids of our common edible fats (lard) and oils (soybean, cottonseed, peanut and corn) (52, p. 239). Linoleic acid contains the pentadiene unsaturated system upon which the mechanism of fat oxidation is based.

When sufficient energy is acquired, as thermal or light energy, to permit the removal of a labile hydrogen from the alpha methylene of the pentadiene system of linoleic acid as shown in (a) page 11, the reaction as shown in (b) page 11 takes place. Molecular oxygen which has

unpaired electrons reacts with the pentadiene system and an active hydrogen is removed from the alpha methylene carbon to form an intermediate unstable free radical (37, p. 68-69; 42, p. 1303; 46, p. 127; 52, p. 240). The unstable intermediate undergoes electronic stabilization to form conjugated resonance hybrids as shown in (c) page 12. The presence of free radicals on C<sub>9</sub>, C<sub>11</sub>, and C<sub>13</sub> is then possible (46, p. 127). Molecular oxygen may add to the free radicals on C<sub>9</sub> and C<sub>13</sub> as shown in (d) page 12 to form conjugated peroxides. It is also possible that molecular oxygen could add to C<sub>11</sub> to form a nonconjugated peroxide. Chain reaction propagation then takes place as shown in (e) page 12. The peroxide free radical removes an active hydrogen from the alpha methylene of another fatty acid residue producing a relatively stable hydroperoxide and a new free radical, which perpetuates the chain reaction (37, p. 48-53). Conjugated compounds with hydroperoxides on C<sub>9</sub> and C<sub>13</sub> and a nonconjugated compound with a hydroperoxide on C<sub>11</sub> could be formed. Morris (46, p. 127) cites the work of Lundberg who prepared hydroperoxides of methyl linoleate and concentrated them at low temperature. He obtained practically pure monhydroperoxide and found that the amount of conjugated hydroperoxides was considerably less than the total. He concluded then that there must be some nonconjugated hydroperoxides present,



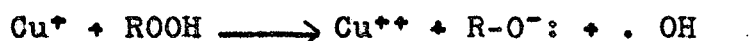


### Metal Catalysis of Primary Autoxidation

The autoxidation of fats and oils may be promoted by various agents. It is clear that the absorption of light

and heat energy will accelerate the process of autoxidation (11, p. 282; 52, p. 24). Light energy, particularly ultraviolet irradiation, is a powerful initiator and promoter of the free radical mechanism (52, p. 421). Certain organic peroxides (52, p. 240) also will promote the free radical mechanism. Metal salts and oxides, particularly those of the heavy metals such as copper and iron, are powerful catalysts for fat autoxidation (11, p. 282; 45, p. 105-107; 52, p. 240; 60, p. 31).

Uri (60, p. 32-35) stated that it is apparent that the elucidation of the intimate mechanism of metal catalysis is still in its infancy. Robertson and Waters (53, p. 206-209) grouped autoxidation catalysts into two classes: (a) "initial" catalysts, which attack the hydrocarbon, abstracting a hydrogen and producing an active hydrocarbon radical, and (2) secondary catalysts, such as salts of copper, cobalt, etc. which promote the decomposition of hydroperoxides thereby increasing the steady concentration of active hydroxyl radicals thus:



Organic peroxides can both take from and give electrons to many metallic cations.

It can be seen that the most active catalysis are those which can be oxidized by a one-electron transfer in

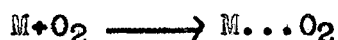
accordance with  $M^{n+} \longrightarrow M^{(n+1)+}$ . Very little activity in the catalysis of initial peroxide formation is obtained with metal ions which are reoxidized by transfer of two electron like  $Sn^{2+}$  or metals ions which are normally reduced by electron transfer such as  $Cu^{2+}$  ion. This appears to be contradictory to the general knowledge of the catalyzing effect of copper on the oxidation of fat (60, p. 31). Uri (60, p. 31) found that  $Cu^{2+}$  catalyst is active if either peroxides are present or if another reducing agent occurs in the system. Free radical intermediates are formed during the reduction of  $Cu^{2+}$  to  $Cu^+$ , while  $Cu^+$  is being continuously reoxidized by the oxygen in the air. Under these conditions  $Cu^{2+}$  becomes a powerful catalyst for fat oxidation.

Uri (60, p. 32-35) suggested that oxygen does not react with linoleic acid as proposed by the generally accepted chain mechanism for autoxidation, but that the reaction involves a trace metal catalyst. In principle the reaction is represented by  $M^{n+} + O_2 \longrightarrow M^{(n+1)+} + O_2^-$ . Kinetic evidence indicates that the heavy metal catalyst forms a complex with oxygen. Kinetic analysis of the initial stages of autoxidation of linoleic acid catalyzed by cobaltous stearate, indicate that this phase is marked by the presence of at least four separate reactions; one of which is inhibitory. They are as follows:



George and Robertson (20, p. 226) have indicated the free radical character of the heavy metal catalyzed oxidations. They suggested the primary reaction is the chain formation of the hydroperoxide in which the catalyst both starts and stops reaction chains, followed by the catalyzed unimolecular decomposition of hydroperoxides. They formulated a chain mechanism in which the heavy metal catalyst starts the reaction chains by the activation of oxygen. Their proposed mechanism is illustrated as follows:

Chain initiation:



Chain propagation:



Chain termination:



### Secondary Oxidation

Autoxidation of triglycerides results in a typical, strong and disagreeable odor and flavor (52, p. 237).

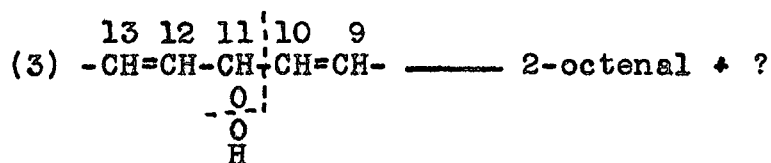
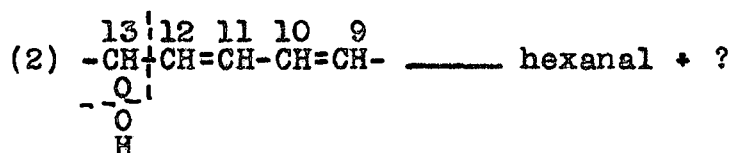
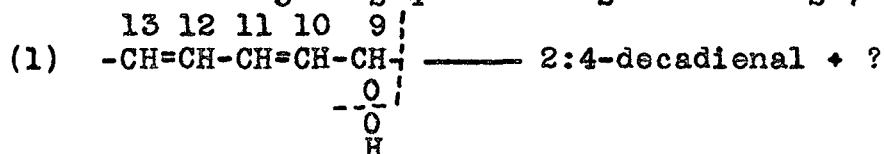
These disagreeable odors and flavors are attributed to the complex mixture of degradation products resulting from the further oxidation and breakdown of hydroperoxides (39, p. 211). The peroxides and hydroperoxides formed during primary oxidation are not responsible for the rancid odor and flavor of autoxidized triglycerides (37, p. 48-53). It is evident that there is a multiplicity of reactions which can occur simultaneously with the primary autoxidative mechanism (52, p. 240).

Keeney and Doan (35, p. 718) found that vacuum distillation of oxidized milk fat yielded materials with the characteristic flavor and odor of oxidized milk fat. They demonstrated that carbonyl compounds contributed to the characteristic odor of oxidized fat and that the contribution of volatile acids was insignificant. Kawahara and Dutton (34, p. 377) isolated a group of aldehydes from the volatile odor principle of reverted soybean oil. Buss and Mackinney (7, p. 489) have shown that large numbers of carbonyl compounds are present in autoxidized corn oil. They found fourteen petroleum ether soluble and sixteen insoluble 2,4-dinitrophenylhydrazine derivatives.

Swift and coworkers (59, p. 297-300) isolated 2:4 decadienal, 2-octenal and hexanal from oxidized cottonseed oil. They believe these aldehydes are the decomposition products of the three isomeric hydroperoxides of linoleic

acid, the principal fatty acid of cottonseed oil. Although the mechanism for their formation has not been elucidated they suggested the following decomposition:

Linoleate:  $\text{CH}_3-(\text{CH}_2)_4-\overset{13}{\text{CH}}=\overset{12}{\text{CH}}-\overset{11}{\text{CH}_2}-\overset{10}{\text{CH}}=\overset{9}{\text{CH}}-(\text{CH}_2)_7 \text{ COOR}$



### Antioxidants

Lea (42, p. 621) has classified antioxidants into two main types: (1) primary antioxidants and (2) synergists or metal deactivators. The line of demarcation between these two classes of antioxidants is not always clear-cut. Some substances may combine by their chemical nature, the ability to act as a primary antioxidant and as metal deactivators. Lea (42, p. 623) has designated these compounds as multifunctional antioxidants.

The classification of antioxidants made by Lea (42, p. 621) is based upon the mode of protective action.

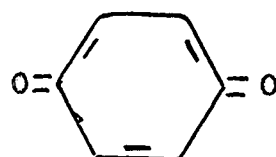
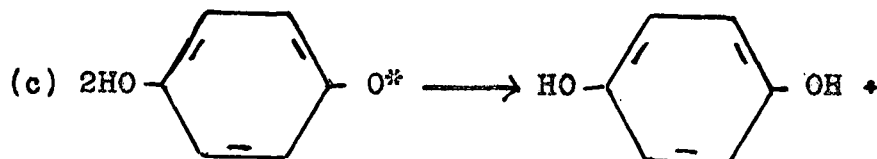
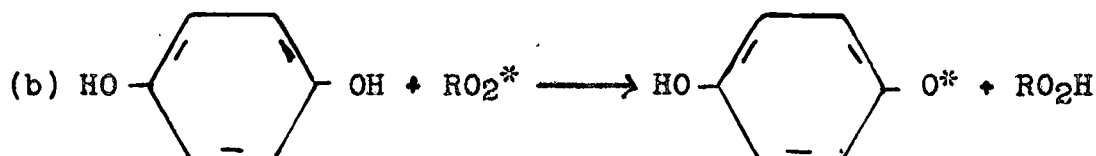
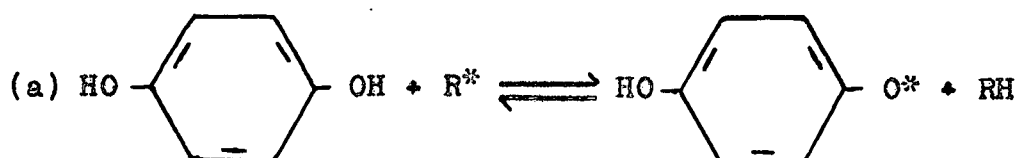
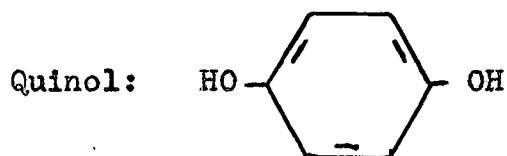
Primary antioxidants function by reacting with free radicals, particularly the peroxide radical which is the prime initiator of the chain autoxidation of fats. This essentially breaks the chain reaction. Synergists, on the other hand, function by inhibiting the proxidant activity of trace metals (42, p. 621-623).

Primary antioxidants are usually ortho or para substituted phenols or aromatic amines (42, p. 621). Thus far, only phenolic type antioxidants have been approved for food use by the Food and Drug Administration (37, p. 48-53; 42, p. 32-35). Aromatic amines are not used in foods but are of industrial importance as antioxidants for rubber and petroleum products (42, p. 623). Some of the aromatic amines and phenolic antioxidants not approved for food use are important as antioxidants for animal feeds (42, p. 623).

Riemenschneider (52, p. 241) stated that no definition of antioxidant based on mode of action in retarding the primary autoxidation by means of radical or chain termination has been universally accepted. This is probably due to the fact that little is known about the intimate mechanism of antioxidant action.

Riemenschneider (52, p. 241) and Koch (37, p. 48-53) in their reviews point to the work of such investigators as Golumbic, Conant, and Michaelis which suggested the

possible retarding mechanism of quinols, including tocopherol. The suggested mode of action is that the antioxidant furnishes a hydrogen atom to the lipid free radical which will terminate the propagating mechanism of autoxidation. The mechanism may be represented as follows where RH is from the linoleic pentadiene system with H as the labile hydrogen,  $R^*$  is the free radical,  $RO_2^*$  oxygenated free radical and  $RO_2H$  the primary product, a hydroperoxide.



The amount of energy required to remove a hydrogen from a hydrocarbon to form a free radical would make the

reaction quite sluggish (52, p. 241). Antioxidants have an affinity for free radicals, or to put it another way, they give up a hydrogen atom more readily to the free radical than does a fatty acid molecule (37, p. 48-53). When a free radical captures a hydrogen from an antioxidant its ability to propagate a chain reaction is terminated (37, p. 48-53). It is evident that small amounts of antioxidant present in a fat system would greatly retard the progress of autoxidation.

When an antioxidant gives up a hydrogen to stabilize a free radical of the fatty acid it becomes a free radical itself (37, p. 48-53; 52, p. 241). The free radical formed is called a semi-quinone. Its ability to be formed and the fact that it is stable enough to exist for an appreciable time has been shown (16, p. 5236; 52, p. 241). The stability of the free radical on the semi-quinone structure is attributed to resonance (37, p. 48-53). The electrons of the semi-quinone are able to move through the ring structure, thus stabilizing the molecule. The semi-quinone structure does not possess a strong enough attraction to remove a hydrogen from a fatty acid (37, p. 48-53). Hence, the free radical of the antioxidant cannot propagate the chain reaction of autoxidation.

The semiquinone free radical may undergo further change. Koch (37, p. 48-53) stated that if the semiquinone

structure encounters a free hydrogen atom they may react to form the original hydroquinone structure of the antioxidant. Thus, the antioxidant is regenerated with reference to its ability to furnish hydrogen. Another possibility, as shown by Riemenschneider (52, p. 241), is that two semiquinones may react to form a hydroquinone and a quinone. It is evident that semiquinones must undergo some further unknown reaction when used as antioxidants for a fat system, because they are eventually used up and the fat system is oxidized (37, p. 48-53). Riemenschneider (52, p. 241) stated that antioxidants are destroyed during the induction period of autoxidation. Filer, Mattill and Longenecker (17, p. 297) found that gallic acid gradually disappeared during the accelerated autoxidation of cottonseed oil. They showed that at the end of the induction period no gallic acid could be determined in the oxidized substrate.

The enhancing effect of certain compounds upon antioxidants was attributed in early work to synergistic action. Synergists are substances which are inactive or only slightly active in the retardation of primary oxidation (42, p. 623). Synergistic action has been shown by a large number of polyhydroxy or acidic compounds such as ascorbic, tartaric, galacturonic, and phosphoric acids (22, p. 1279-1280; 45, p. 105-107; 52, p. 242). In

addition, chelate compounds containing nitrogen as the coordinating atom have shown synergistic action (52, p. 268).

Koch (37, p. 48-53) stated in his review that it is thought that synergists may act in two ways: (1) by increasing the effectiveness of the antioxidant by supplying hydrogen atoms to the free radical and thereby regenerating the antioxidant and (2) by sequestering or by complexing of metal ions which are fat oxidizing catalysts. Recent work by Privett and Quackenbush (49, p. 323) disagree with the first proposed mode of synergistic action. They proposed that synergists function by inhibiting the antioxidant catalysis of peroxide decomposition.

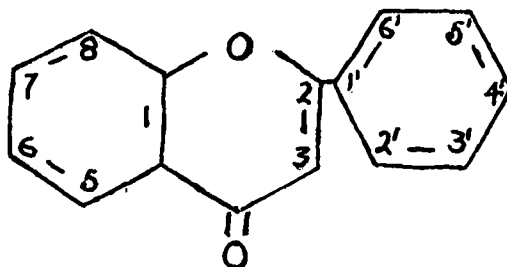
The possible relationship between oxidation potentials of antioxidants and synergists to their effectiveness has been suggested (32, p. 184-187). Golumbic (23, p. 186) showed that the most effective antioxidants fall in the potential region between 848 to 484 millivolts. If a synergist acts by regenerating the phenolic antioxidant, by furnishing hydrogen to the antioxidant free radical, then the oxidation potentials of the system would be in the order: fat peroxide > phenolic antioxidant > synergist (52, p. 242).

The term synergist continues to be used even though their main action is known to be as metal deactivators

(42, p. 623; 45, p. 107; 52, p. 264). It is thought that the effectiveness of a synergist is attributed to its ability to form a non-catalytic complex with metal ions (42, p. 623; 52, p. 241; 60, p. 32-35). Lea (42, p. 63) and Uri (60, p. 32-34) pointed out that metals already in foods may be bound in a stable complex, which may be much less or much more catalytic than the free metal ion. Synergists or metal deactivators have no effect upon such complex catalysts.

#### Flavonoids--Quercetin and Dihydroquercetin

Flavone Molecule



The distribution of flavone and flavanone compounds are wide and varied in nature. Enough is known about their distribution to support the statement that they occur throughout the higher plants; and probably in all parts of the plant: roots, bark, wood, stems, leaves, flowers, fruits and seeds (19, p. 451). The flavonoid quercetin and the flavonoid dihydroquercetin may exist in

the free form or as the glucoside (3, p. 267; 19, p. 455; 28, p. 451-454). Dihydroquercetin is found in the bark of the Douglas fir tree to the extent of 5% (38, p. 433). There is an annual supply of this bark in excess of two million tons (38, p. 433).

It has been suggested that flavonoid pigments may be involved directly in the physiological and reproductive processes of plants, but no proof or clear biochemical mechanism has been found. Rutin the glucoside of quercetin has had attributed to it the ability to inhibit the fertilization process in plants. Flavonoids have also been regarded as having a possible role in the oxidation-reduction process of plants (19, p. 451-453).

Clinical experience of the administration of rutin and quercetin to patients in doses up to 60 mg. for as long as five years have shown these flavonoids to be completely non-toxic to man (25, p. 96). Griffith and co-authors (25, p. 97) in citing the work of Ambrose, Robbins and DeEds stated that the flavonoids, rutin and quercetin, are nontoxic when given to rabbits in doses of 100-150 mg. per kilogram of body weight. Albino rats failed to produce evidence of abnormalities or injury on these flavonoids at a 1% or less diet level for 410 days. The appearance of abnormality was judged by growth, food consumption, organ weight, hemoglobin estimation, red and

white blood cell count and histopathological examination of tissue. The conclusion was that these compounds have a very low order of toxicity, if any.

Booth and DeEds (4, p. 183-184) found no significant toxic results when a dietary level of 1% dihydroquercetin was fed to albino rats for a long period of time. They showed that the metabolic fate of dihydroquercetin, quercetin and DOPA (3,4-dihydroxyphenylalanine) were the same. They speculated that the lack of toxicity of quercetin and dihydroquercetin is due to the fact that both compounds serve as substrate in the same metabolic pathway that accounts for the metabolism of DOPA.

Kallianas and co-workers (33, p. 430-433) have made a paper chromatographic study of the ether extracts of lyophilized rat stomach and its contents. The study was made following the feeding of randomly labeled quercetin with C-14. Radioactive phloroglucinol carboxylic acid, phloroglucinol, and protocathechuic acid were identified. A fourth radioactive compound still not identified plus a radioactive "quercetin-like" compound was also noted.

Ribelin, Masri, and DeEds (50, p. 272) fed rats a diet containing 1% quercetin and observed that the animals' bones were fluorescent when viewed under ultraviolet light. The fluorescence appeared after the third day of feeding and persisted at least 6 weeks after cessation of

feeding. The nature of the fluorescent substance and mode of its deposition are not clear.

The mechanism or modes of action of flavonoids as therapeutic agents and their role in animal physiology, are not fully understood (25, p. 4). Rutin and quercetin have the ability to restore to normal the capillary fragility and permeability in humans (25, p. 4). It has been demonstrated that flavonoids (rutin and quercetin) may work synergistically with epinephrine in effecting blood pressure (25, p. 99; 61, p. 399-405). Observations have been made of the antagonistic effects of rutin and quercetin on the anticoagulative properties of piperazine (25, p. 235). It is suggested that these flavonoids do not improve blood coagulation, but may have a favorable effect in preventing hemorrhage in certain conditions characterized by a fault in coagulation (25, p. 118).

Griffith and co-authors (25, p. 3, p. 119-120) cite the work of many investigators in their discussion of the vitamin "P" action of flavonoids. Vitamin "P" activity is best illustrated by the work of Ambrose and DeEds. They fed guinea pigs on an ascorbic acid free diet with sub-minimal doses of ascorbic acid. Part of the guinea pigs were given 100 mg. rutin or 50 mg. quercetin daily by injection. The pigs given a subminimal dose of ascorbic acid plus the rutin or quercetin lived longer. Pigs given

only quercetin and rutin with no dose of ascorbic acid died in a shorter time as did the pigs given the ascorbic acid free diet plus subminimal doses of ascorbic acid.

Quercetin and dihydroquercetin have been shown to be good antioxidants in a wide variety of substances. Dihydroquercetin has been shown to have good antioxidant ability in lard (38, p. 234; 47, p. 2), cottonseed oil (38, p. 235), butter oil (38, p. 235) and tallow (47, p. 2). Quercetin has shown superior antioxidant ability for lard (36, p. 402; 38, p. 235; 47, p. 2), butter fat (38, p. 406-407), tallow (47, p. 2) and for simple and multiple unsaturated fatty acid derivatives (26, p. 398).

Quercetin and dihydroquercetin are better antioxidants in lard than BHT and BHA at a level of 0.03%. Quercetin was shown to be superior to dihydroquercetin. Propyl gallate and NDGA showed better antioxidant properties than quercetin and dihydroquercetin in lard (47, p. 2).

Heimann and co-workers (26, p. 394-398) showed that quercetin in equal per cent additions (0.1%) produced practically the same strong antioxidant effect as the ethyl ester of protocatechuic acid, propyl gallate and ethyl gallate. In equal molar concentrations (0.0001) quercetin was superior to the gallates as an antioxidant. Alpha tocopherol was a better antioxidant than quercetin

in the initial stages of ethyl linoleate autoxidation. The action of alpha tocopherol was quickly exhausted and quercetin showed a higher inhibitory effect over a longer period of time.

Lea (42, p. 633) described flavonols as multifunctional antioxidants. Their chemical nature is such as to allow them to operate by both chain-breaking and metal-deactivating mechanism. Heimann and co-workers (26, p. 395) indicated that quercetin behaves as a natural radical chain breaking polyphenol. Quercetin and dihydroquercetin have shown concentration dependency (26, p. 396; 47, p. 2).

Lea (42, p. 623) pointed out that flavonols when suitably substituted are very powerful antioxidants with active groups in two or three parts of the molecule. Richardson, El Rafei and Long (51, p. 413) suggested the  $\text{-}\overset{\text{O}}{\underset{\text{O}}{\text{C}}}\text{-C=}\text{C-}$  group in the pyrone ring or in the open chalcone is responsible for the antioxidant ability of flavonoids.

Heimann and Reiff (27, p. 451-454) investigated the antioxidant activity of different flavone derivatives in a dioxane solution of ethyl linoleate. On the basis of their study they have pointed out the following molecular centers which are important in antioxidant effect:

(1) the double bond between  $\text{C}_2$  and  $\text{C}_3$  in combination with the keto group of the  $\alpha$ - $\beta$  unsaturated ketone structure in the pyrone ring or in the corresponding chalcones is

decidedly responsible for the antioxidant effect of the flavone derivatives, (2) the free (uncombined) hydroxyl group on C<sub>3</sub> in the chromone ring is of decisive importance, (3) the antioxidant effect of the chromone ring system is decreased by meta hydroxyls groups, and (4) the ortho hydroxyl group on the 2-phenyl ring increases the antioxidant effect of the flavones considerably.

Simpson and Uri (58, p. 956-957) confirmed the observations of Heimann and Rieff (27, p. 451-454) and add that a para-quinol structure in the 2-phenyl and benzenoid portions of the molecule appears to impart considerably higher activity than does the ortho-quinol structure.

Mehta and Seshadri (44, p. 24-28) have studied a large variety of flavonoids to ascertain the effect of position of the hydroxyl substituents to antioxidant ability. Their results indicated that meta hydroxyl groups on the chromone ring do not necessarily lower the antioxidant capacity of the molecule. The position of the two hydroxyl groups on the chromone ring is important as well as their orientation with each other. They suggested the tautomerism between the hydroxyl and the keto form on C<sub>3</sub> in conjunction with the carbonyl is of great importance in the antioxidant capacity of the molecule.

The metal chelating or complexing ability of polyhydroxy flavones has been studied and discussed by many

workers (5, p. 3; 9, p. 363; 36, p. 315; 43, p. 33). Dowd (12, p. 1184-1187) has used the color complex of quercetin with aluminum chloride as a quantitative determination of quercetin. Detty, Heston and Wender (10, p. 162-164) have studied the flavonoid metal combinations through amperometric titrations and have shown the importance of pH in metal complexing ability. Shifts in the ultraviolet absorption maxima in various chelating solvent systems has been used in structural determinations of flavones (30, p. 376; 31, p. 1618). The use of the complexing properties of flavones has also been utilized for the preparation of selectively alkylated flavones (29, p. 127-131; 32, p. 5531-5536).

Martell and Calvin (43, p. 28-34) indicated that the presence of chelates can be demonstrated by absorption spectra. Brode (6, p. 203-204) stated that rotational effects involving intramolecular binding are observed in the far infrared. Vibrational effects will occur in the near infrared and electronic bands will appear in the visible and ultraviolet portions of the spectrum. In the absorption spectra of many of the highly complicated molecules there is a transition from what one might call the atomic-electronic spectrum to true molecular spectrum involving a resonance or vibration of the molecule as a whole. It has been shown that with an increase in

resonance of ortho and para quinones there is a corresponding shift in absorption toward the visible from the ultraviolet region, an increase in intensity, together with a resolution effect (6, p. 228). Chalberek and Martell (8, p. 43) stated that chelation and the corresponding ultraviolet absorption maxima shifts results from electronic transitions in conjugated systems. In these systems metals may or may not take part. Metal ions alone do not result in absorption maxima shifts. Martell and Calvin (43, p. 32-34) stated that metal chelates of organic compounds, including o-hydroxyquinones, have an absorption spectra quite different from those of the organic compounds or the metal alone. For a given chelating agent the wave lengths of the absorption maxima were practically independent of the metal employed.

Geissman (19, p. 489) indicated that the formation of metal complexes is a characteristic property of the flavonoid compounds. He suggests the following complex or chelation systems for the flavonol molecule: (1) between the C<sub>5</sub> hydroxyl and the carbonyl, (2) between the C<sub>3</sub> hydroxyl and the carbonyl and (3) between the ortho hydroxyl group 1 on the 2-phenyl side ring.

Shaw, Simpson and Garden in a series of papers have studied hydrogen bonding in relation to chelation systems on the flavone molecule and its methoxyl substituted

derivatives. Simpson and Garden (57, p. 4638) by partition chromatograph have shown that the pyrone carbonyl group forms stronger bonds with the C<sub>5</sub> than with the C<sub>3</sub> hydroxyl. The ability of the carbonyl to form bonds simultaneously with both C<sub>5</sub> and C<sub>3</sub> hydroxyl groups also was demonstrated. Shaw and Simpson (55, p. 5027) added that the C<sub>4</sub>, hydroxyl, C<sub>7</sub> hydroxyl and C<sub>7</sub> methoxyl groups stabilize the carbonyl C<sub>3</sub> hydroxyl system more than the carbonyl C<sub>5</sub> hydroxyl system.

Shaw and Simpson (56, p. 656-658) confirmed earlier work with infrared spectra data which showed the C<sub>3</sub> and C<sub>5</sub> hydroxyl carbonyl chelation system exist. They also stated that an overall loss in hydrogen bonding by the introduction of C<sub>5</sub> hydroxyl onto a C<sub>3</sub> hydroxyl flavone indicates that the two chelation systems are opposed.

## EXPERIMENTAL METHODS

### Flavonoids and Derivatives

The flavonoids and derivatives used in this investigation were obtained from the Oregon Forest Research Center, Corvallis, Oregon. Paper chromatography was used as a criteria of purity. All flavonoids were dried over KOH in vacuum before use.

### Source and Characterization of Lard Substrate

Steam extracted lard, a sample from the middle portion of one large vat, was obtained from a local commercial source. The lard was packed at 70°C. in 307 X 409 "C" enamel cans and held at -28°C. until used. The lard had a free fatty acid content of 0.55% (oleic acid) (1, Ca 5a-40). No peroxides were found (1, Cd 8-53). The iodine number (Hanus method) of the lard was 57.53 (2, p. 464-465). After ignition, the residue from 25 g. of lard gave a negative copper ion spot test with phosphomolibdic acid (15, p. 84-85). The lard had an AOM stability of 8.8 hours (1, Cd 12-57). Lard containing the commercial antioxidant, propyl gallate at a concentration of  $5 \times 10^{-4}$  moles per kg., had an AOM stability of 97.0 hours (1, Cd 12-57).

### Antioxidant Activity of Flavonoids and Derivatives

Melted lard at 70°C. was added to known quantities of the flavonoids in a hot absolute ethanol (10 ml.) solution to adjust their molarity to  $5 \times 10^{-4}$  moles per kg. of lard. The ethanol was removed by holding the mixture at 70°C. under reduced pressure and flushing with nitrogen for 30 minutes. The stability of the treated lard was then determined by the Active Oxygen Method (1, Cd 12-57) using 125 milliequivalents of peroxide per kg. of lard as the end point of AOM stability.

### Antioxidant Activity of Copper-Quercetin Complexes

The copper-quercetin complex with the desired  $\text{Cu}^{++}$ /quercetin ratio was obtained by the addition of absolute ethanolic  $\text{CuCl}_2$  to a known quantity of quercetin in an absolute ethanol solution. The absolute ethanol solution containing the copper-quercetin complex was adjusted to 20 ml. The concentration of the copper-quercetin complex was adjusted with lard (70°C.) to  $5 \times 10^{-4}$  moles per kg. on the basis of quercetin present. The complex was then mixed into the lard by agitation for 5 minutes and its stability determined via the Active Oxygen Method (1, Cd 12-57) using 125 milliequivalents of peroxide per kg. lard as the AOM stability end point.

### Ultraviolet Absorption Spectra of Flavonoids and Copper-Flavonoid Complexes

Stock solutions of the flavonoids and  $\text{CuSO}_4$  were prepared according to the following procedure. Flavonoid stock solutions in a concentration range of  $2 \times 10^{-4}\text{M}$  were prepared with 95% redistilled ethanol. Aqueous  $\text{CuSO}_4$  stock solutions were prepared equal to five times the molarity of the flavonoid stock solutions. A 5.0 ml. aliquot of the flavonoid stock solution plus 6.0 ml. of distilled water was diluted to 50 ml. with redistilled 95% ethanol for absorption spectra determination. Copper-flavonoid complexes for absorption spectra determination were prepared by adding the  $\text{CuSO}_4$  stock solutions in quantities necessary to attain the desired  $\text{Cu}^{++}$ /flavonoid molar ratio with 5.0 ml. of the flavonoid stock solutions. The amount of water as added via the  $\text{CuSO}_4$  solutions was adjusted to 6.0 ml. with distilled water and the complex diluted to 50 ml. with redistilled 95% ethanol. The ultraviolet absorption spectra of flavonoids and copper-flavonoid complexes was determined from 220 to  $500 \text{ m}\mu$  on a DK-1 Beckman recording spectrophotometer.

## RESULTS

### Antioxidant Activity of Flavonoids in Lard

A list of the flavonoids used in this investigation with their effect upon the AOM stability of lard are shown in Table I. The AOM oxidation curves for the determination of the stability of lard containing the flavonoids listed in Table I are shown in Figures 2 and 3. All lard oxidation curves presented are from uninterrupted AOM analysis.

### Antioxidant Activity of the Copper-Quercetin Complexes

The  $\text{Cu}^{++}$ /quercetin molar ratios and the relationship of the complex constituents to the lard is illustrated in Table II. The AOM stability time is also given for the substrate and the substrate plus quercetin, copper ion, and copper-quercetin complexes. The end points for the substrate plus copper ion and copper-quercetin complexes are not exact AOM procedure end points. Only single determinations from one tube below and above 125 milli-equivalents were obtained. Peroxide formation, once initiated, was very rapid. This is illustrated by the complete oxidation curves of the substrate plus copper ion and copper-quercetin complexes in Figure 4. It should be noted that the AOM stability curve for lard as shown in Figure 4 is the lard described in Table II and contains  $5 \times 10^{-4}$  moles of  $\text{Cu}^{++}$  per kg. lard. The relationship

between the molar ratio of  $\text{Cu}^{++}$ /quercetin and AOM end point time as listed in Table II is graphically presented in Figure 5.

#### Ultraviolet Absorption Spectra of Flavonoids and Copper-Flavonoid Complexes

The ultraviolet absorption spectra of the flavonoids and their copper complexes are presented in Figures 6-15. The spectra of  $\text{Cu}^{++}$ /flavonoid molar complex ratios from 1.0 to 5.0 were determined. No absorption maxima shifts were observed beyond the shifts shown in the figures of absorption spectra and as listed in Table IV. Absorption maxima, minima and the  $\log \epsilon$  of the major absorption maxima of the flavonoid spectra are listed on Table III.

The absorption spectra of 4,7-dibenzylquercetin copper complexes was not demonstrated in an ethanol solvent system because the complexes were insoluble. However, by using an acetone system the ultraviolet absorption spectra of the complexes from 350-500  $\text{m}\mu$  was obtained.

Figure 1. Flavone Molecule

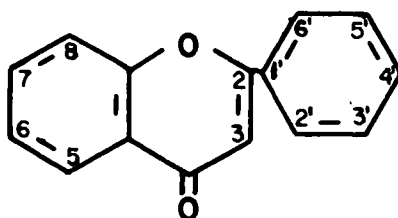


TABLE I

AOM STABILITY OF LARD CONTAINING FLAVONOIDS

<u>COMPOUND AT <math>5 \times 10^{-4}</math> M/kg. OF LARD</u>	<u>AOM STABILITY (HOURS)</u>
3,5,7,3',4'-PENTAHYDROXYFLAVONE (QUERCETIN)	66.1
3,5,7,3',4'-PENTAHYDROXYFLAVANONE (DIHYDROQUERCETIN)	47.0
3,5,7,2',4'-PENTAHYDROXYFLAVONE (MORIN)	27.0
3,5,3',4'-TETRAHYDROXY-7-METHOXYFLAVONE (RHAMNETIN)	48.6
3,7,3',4'-TETRAHYDROXY-5-METHOXYFLAVONE	59.7
3,3',4'-TRIHYDROXY-5,7-DIMETHOXYFLAVONE	37.7
5,3',4'-TRIHYDROXY-3,7-DIMETHOXYFLAVONE	45.8
5-HYDROXY-3,7,3',4'-TETRAMETHOXYFLAVONE	8.9
3,5,3',4'-TETRAHYDROXY-7-BENZYLOXYFLAVONE	71.0
3,5,3'-TRIHYDROXY-4',7-DIBENZYLOXYFLAVONE	10.8
LARD	8.8

Figure 2. AOM stability curves of lard containing flavanoids.

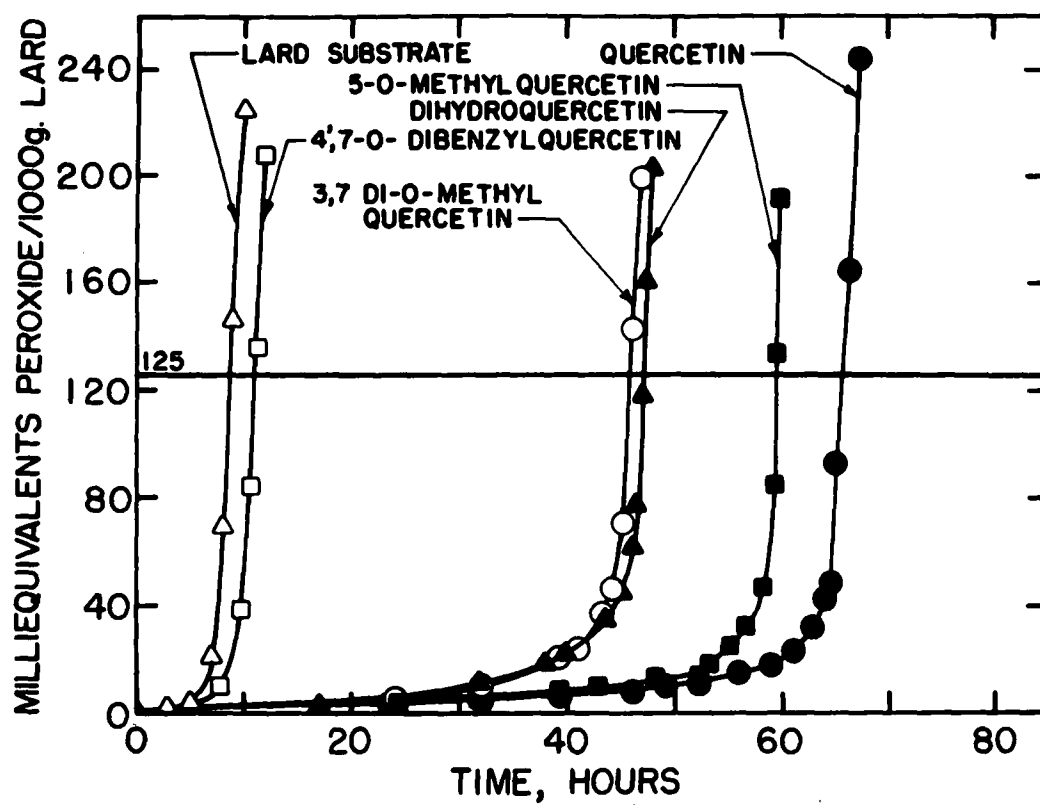


Figure 3. AOM stability curves of lard containing flavanoids.

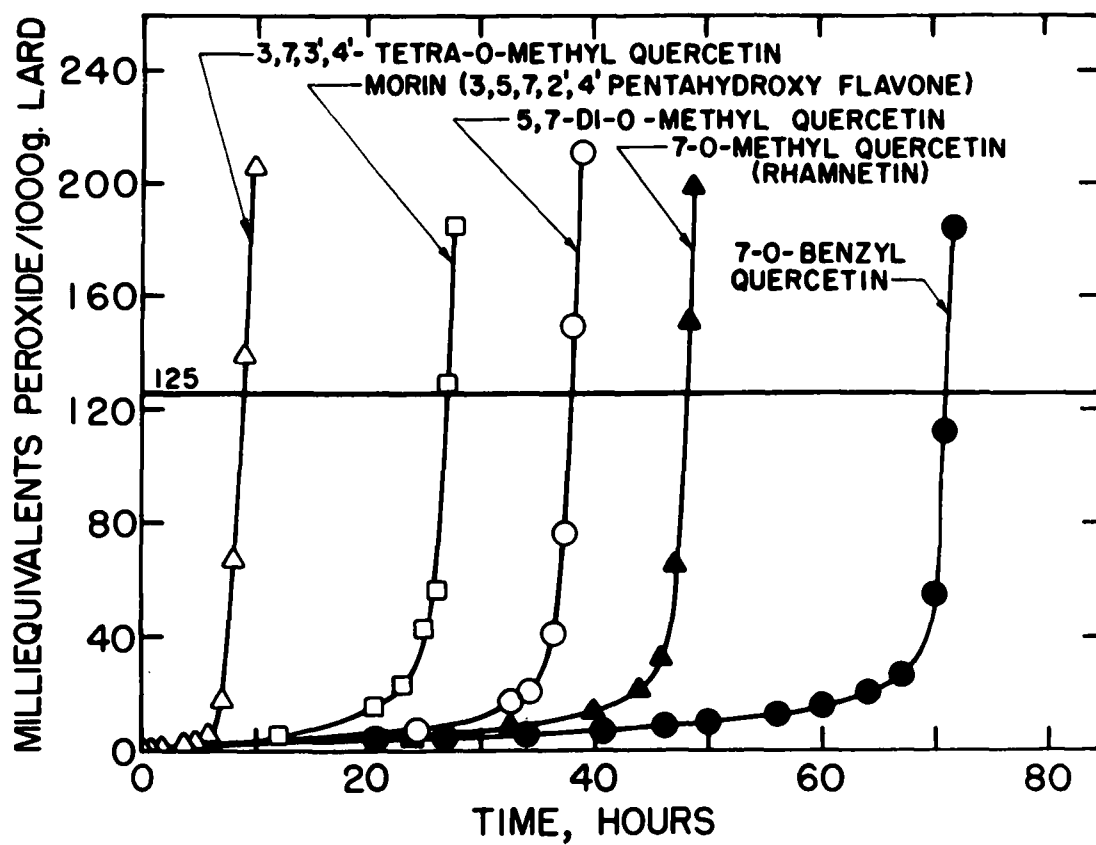


Figure 4. AOM stability curves of lard containing copper-quercetin complexes.

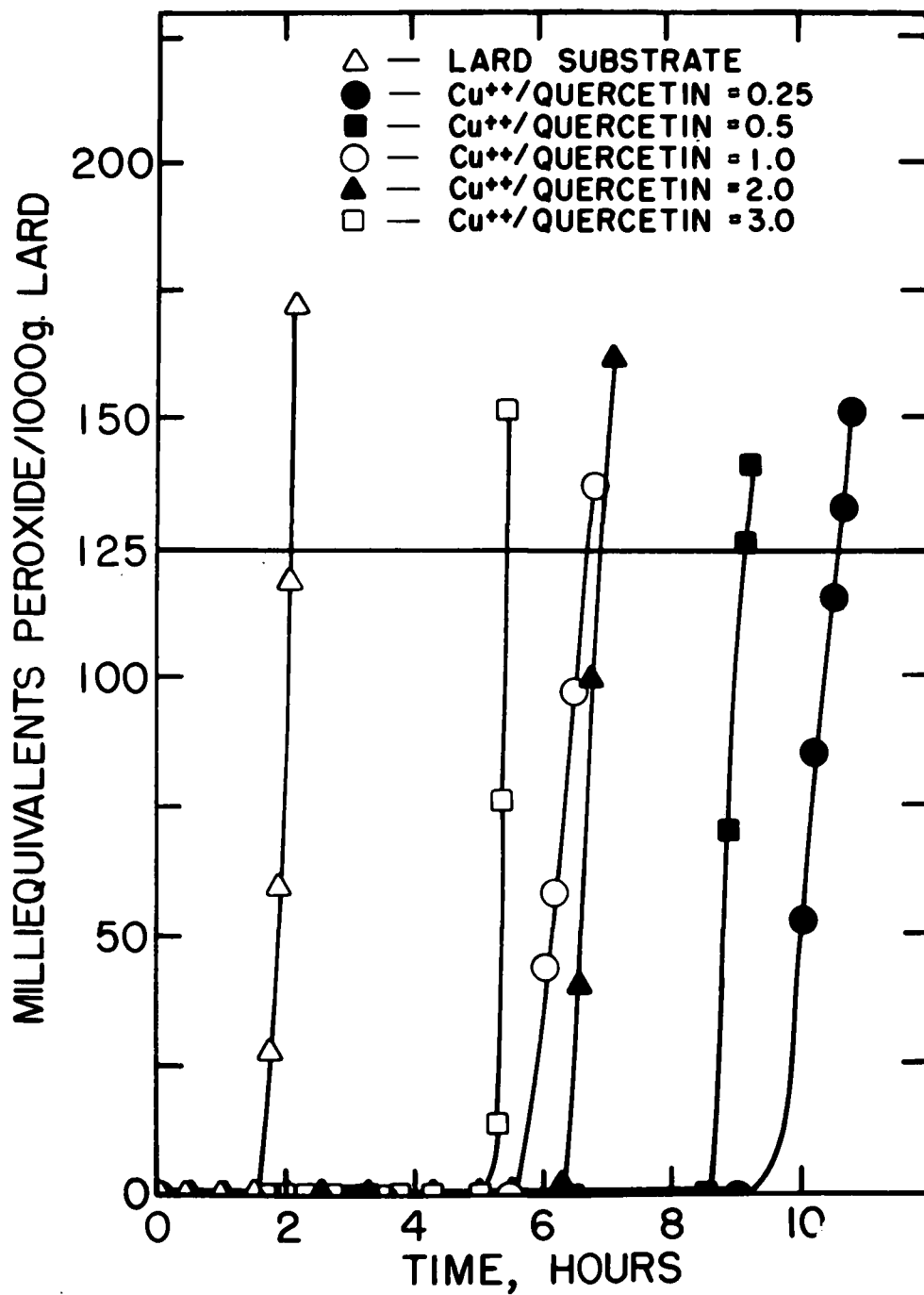


TABLE II  
RELATIONSHIP OF COPPER-QUERCETIN COMPLEX  
SUBSTITUENTS TO THE AOM STABILITY OF LARD

CONCENTRATION OF COMPLEXING SUBSTITUENT		Cu <sup>++</sup> /QUERCETIN	AOM STABILITY 125meq
MOLES QUERCETIN/Kg. LARD	MOLES Cu <sup>++</sup> /Kg. LARD		
			8.8
$5 \times 10^{-4}$			66.1
$5 \times 10^{-4}$	$1.25 \times 10^{-4}$	0.25	10.6
$5 \times 10^{-4}$	$2.5 \times 10^{-4}$	0.50	9.1
$5 \times 10^{-4}$	$5.0 \times 10^{-4}$	1.0	6.7
$5 \times 10^{-4}$	$10.0 \times 10^{-4}$	2.0	6.8
$5 \times 10^{-4}$	$15.0 \times 10^{-4}$	3.0	5.4
	$5.0 \times 10^{-4}$		2.1

Figure 5. Relationship of the complexed Cu<sup>++</sup>/quercetin molar ratios to AOM stability in lard.

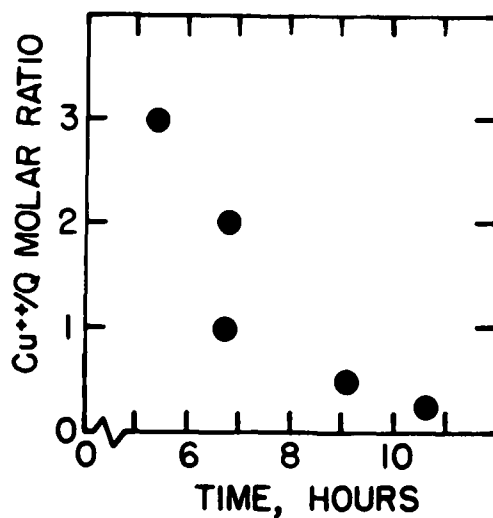
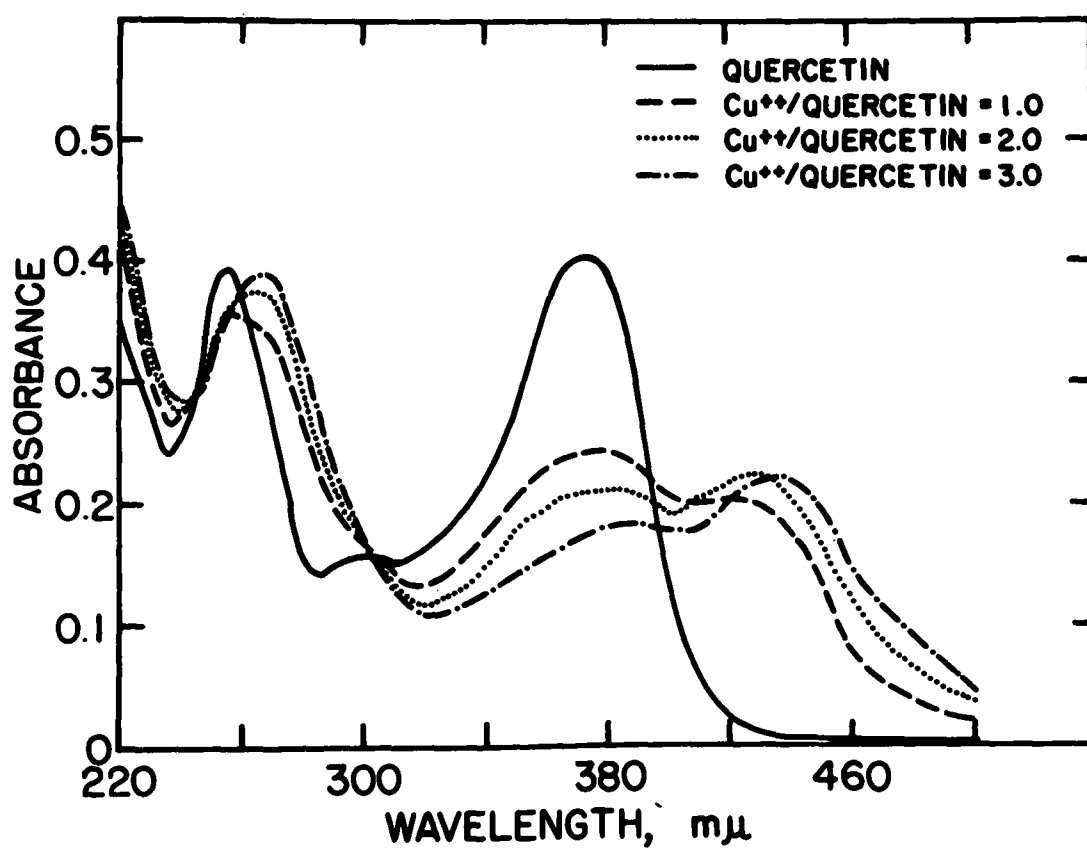


Figure 6. Ultraviolet absorption spectra of quercetin and copper-quercetin complexes.



**Figure 7. Ultraviolet absorption spectra of dihydroquercetin and copper-dihydroquercetin complexes.**

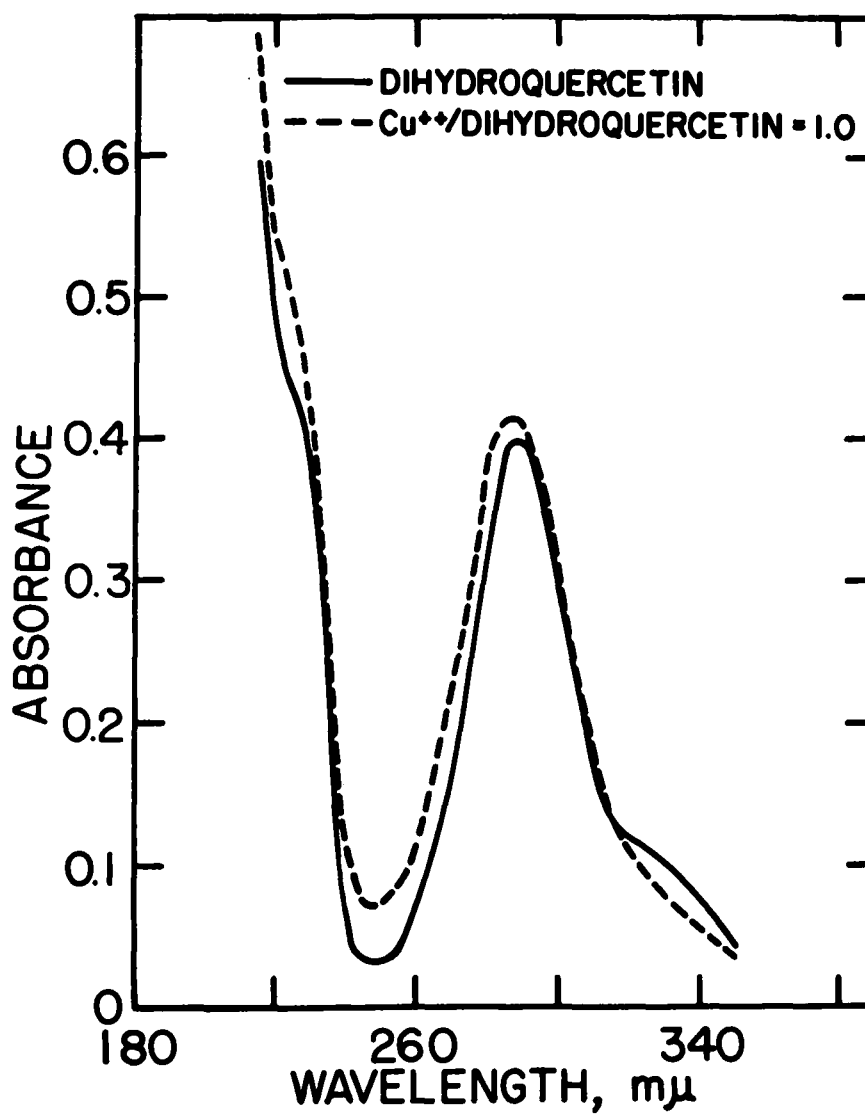


Figure 8. Ultraviolet absorption spectra of morin and copper-morin complexes.

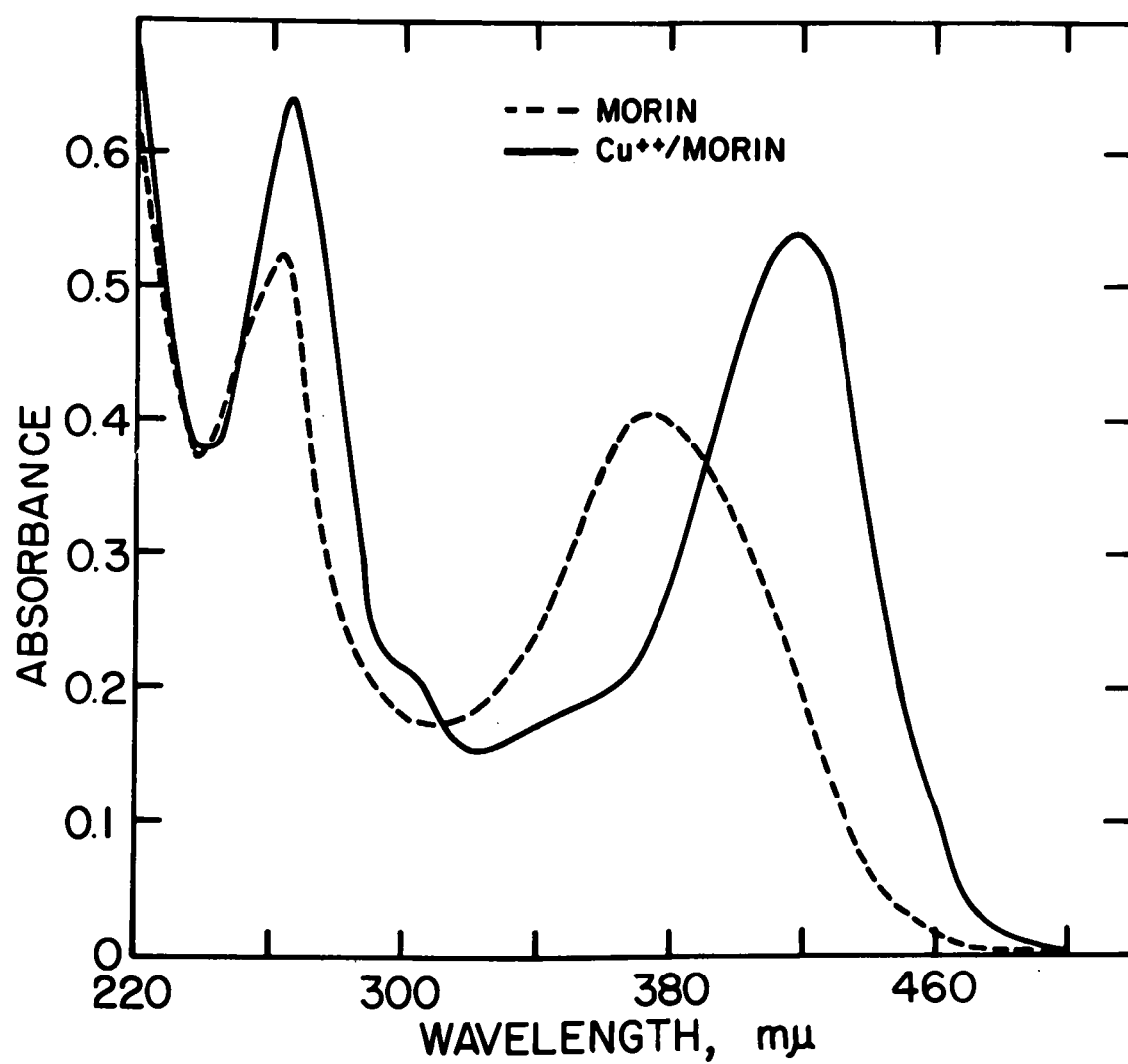


Figure 9. Ultraviolet absorption spectra of 7methylquercetin (rhamnetin) and copper-7methylquercetin complexes.

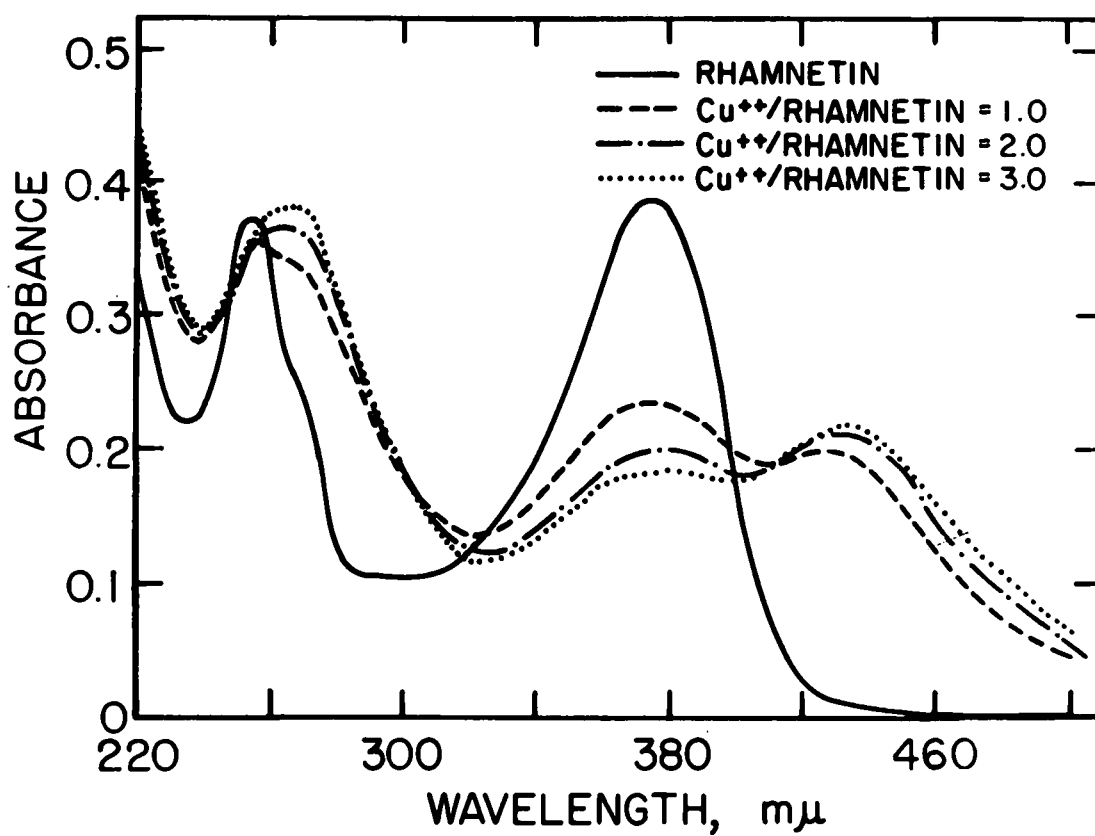


Figure 10. Ultraviolet absorption spectra of 5methylquercetin and copper-5methylquercetin complexes.

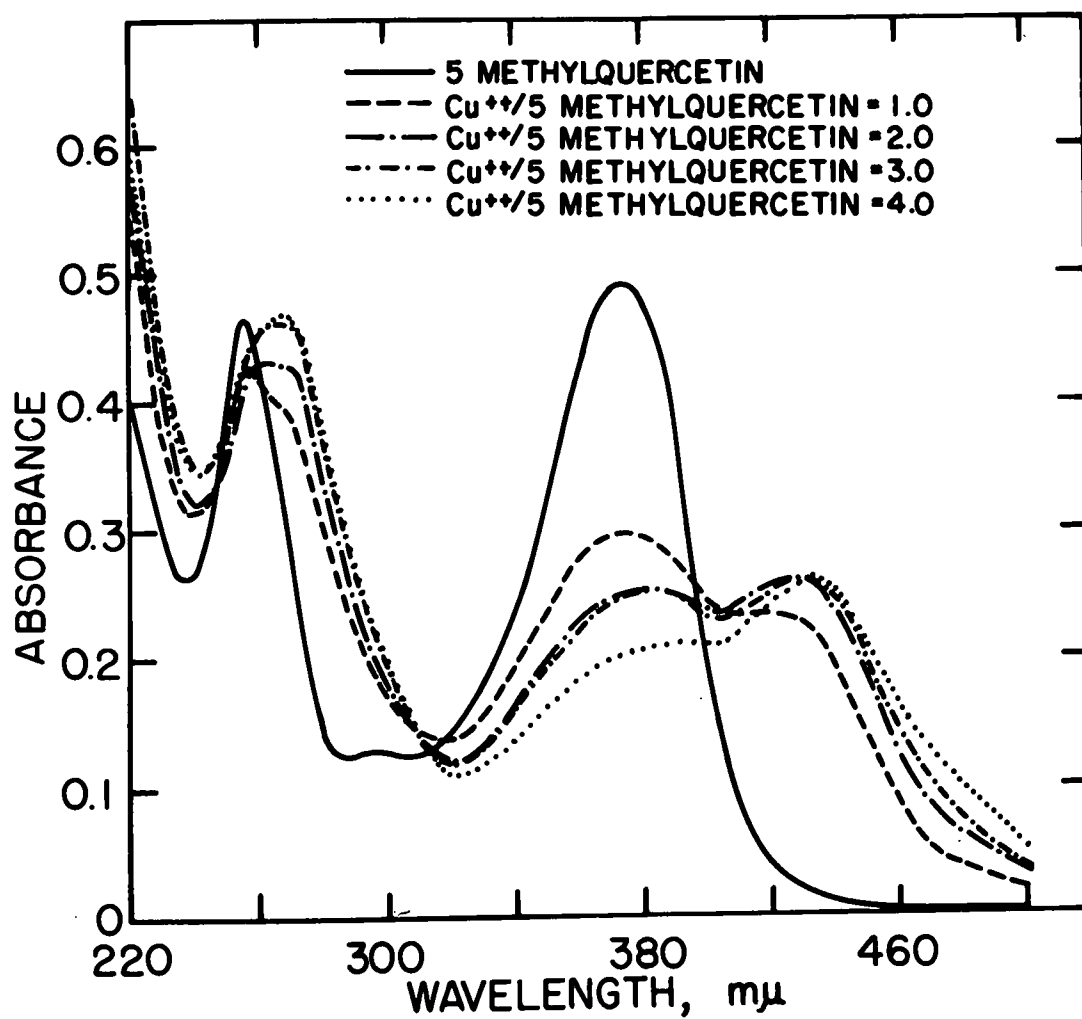


Figure 11. Ultraviolet absorption spectra of 3,7dimethylquercetin and copper-3,7dimethylquercetin complexes.

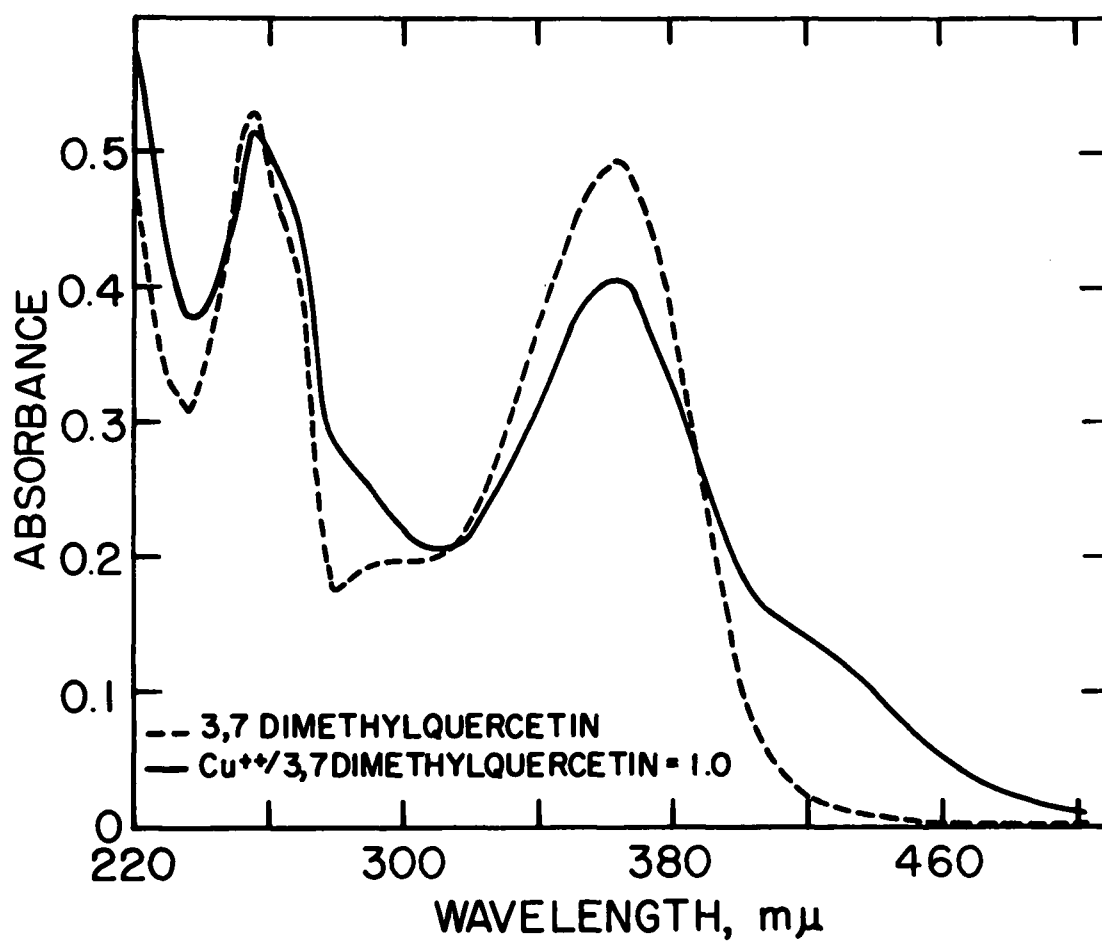


Figure 12. Ultraviolet absorption spectra of 5,7dimethylquercetin and copper-5,7dimethylquercetin complexes.

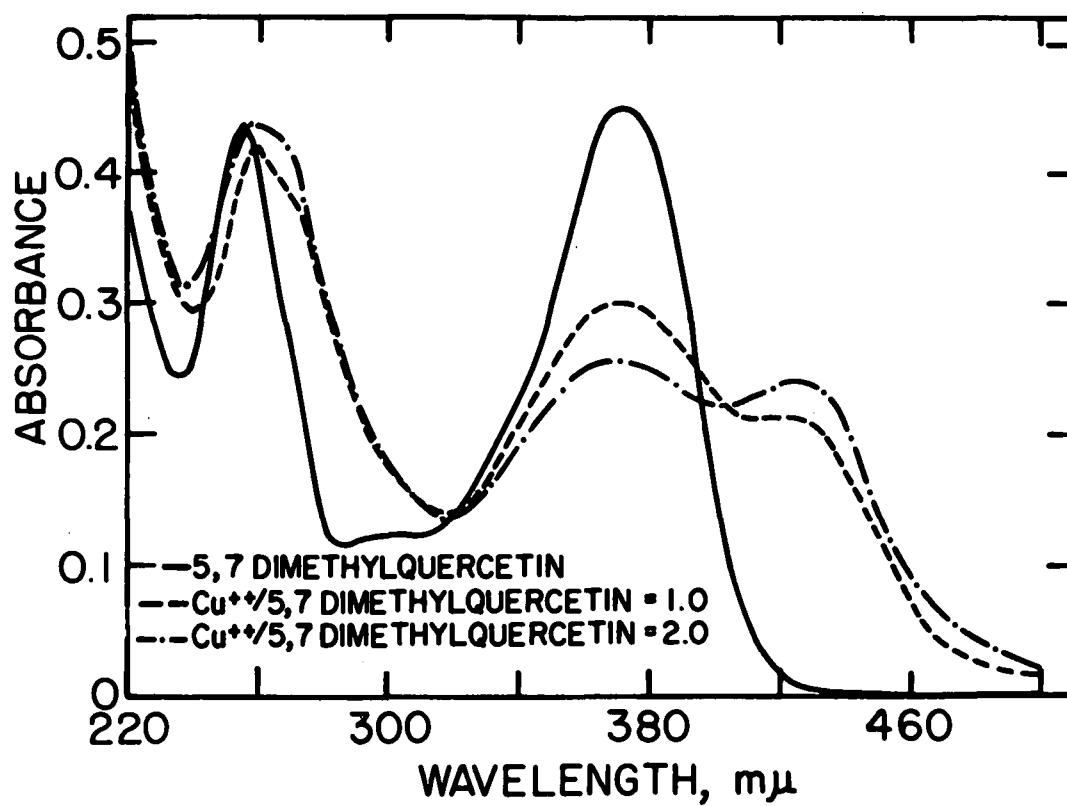


Figure 13. Ultraviolet absorption spectra of 3,7,3',4'tetramethylquercetin and copper-3,7,3',4'tetramethylquercetin complexes.

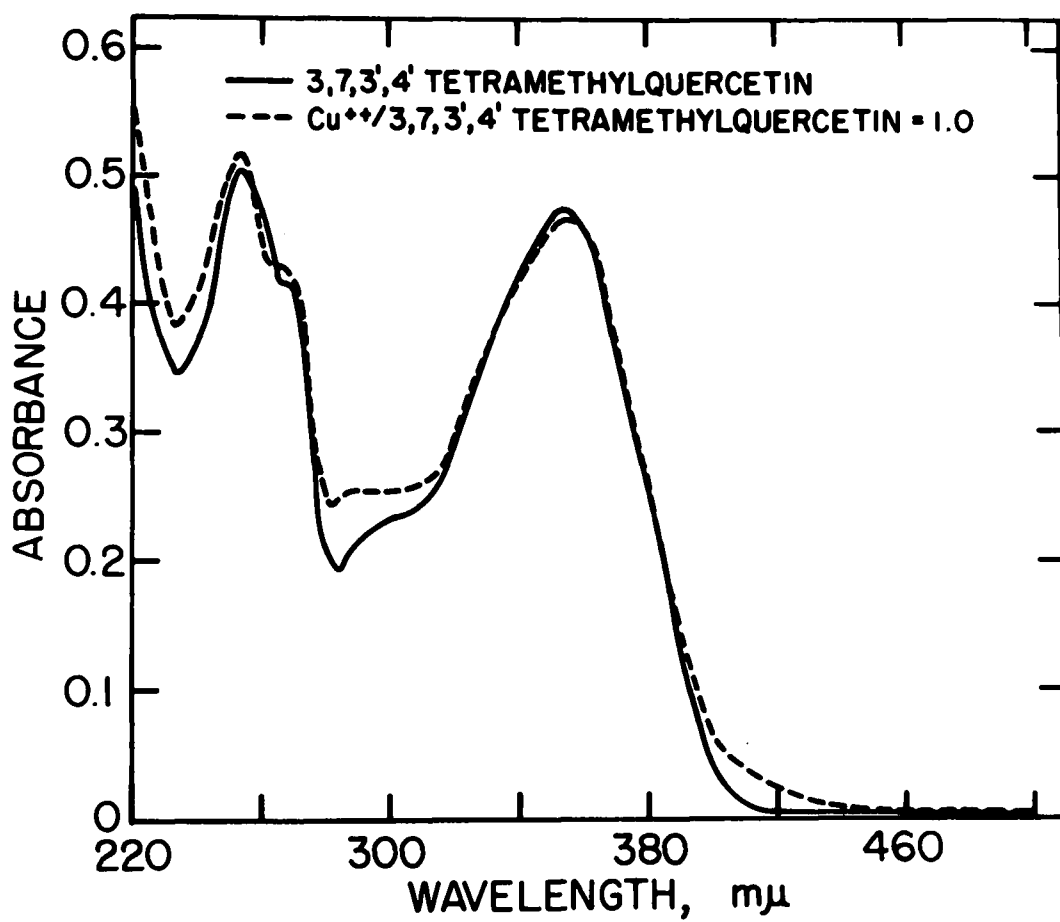


Figure 14. Ultraviolet absorption spectra of 7benzylquercetin and copper-7benzylquercetin complexes.

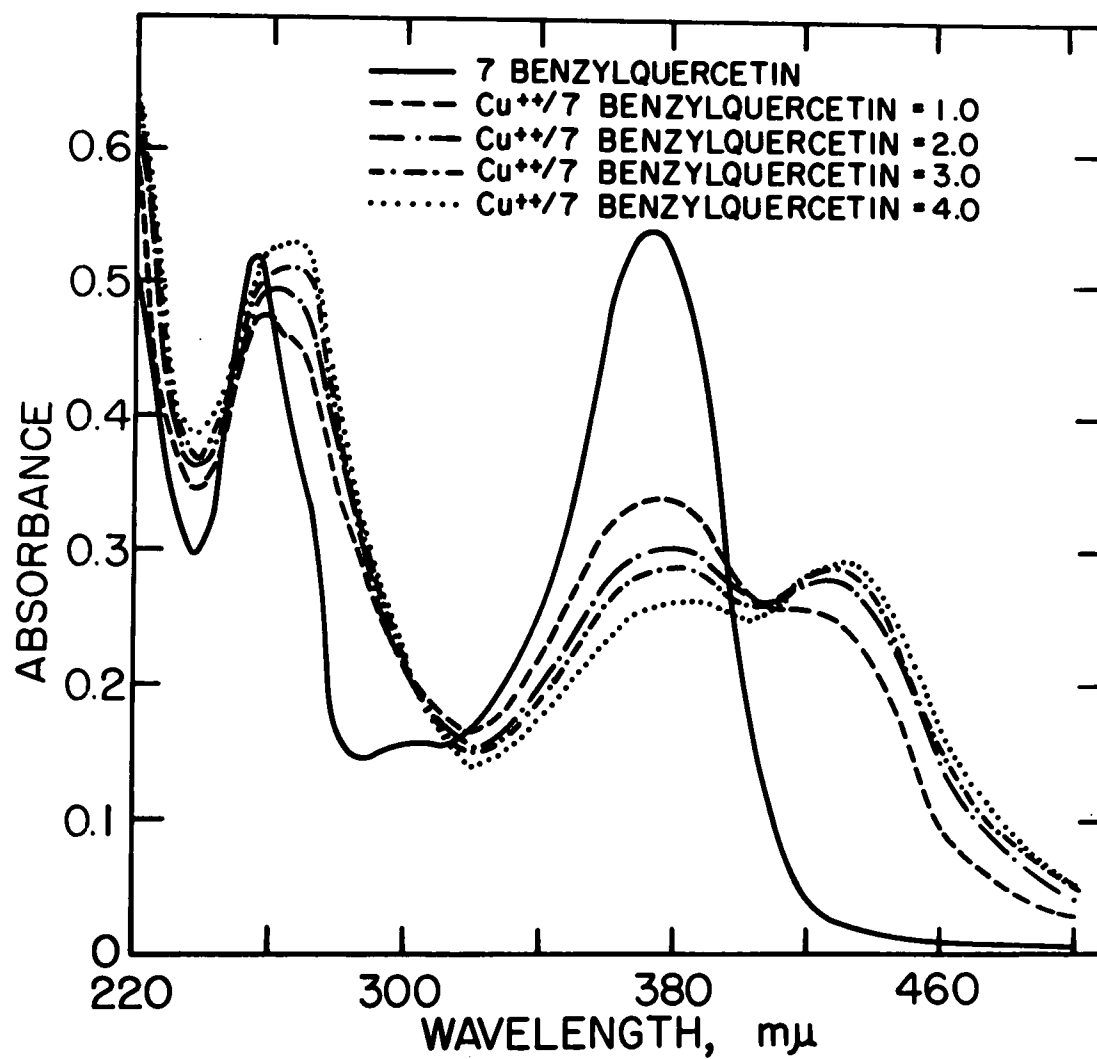


Figure 15. Ultraviolet absorption spectra of 4',7dibenzylquercetin and copper-4',7dibenzylquercetin complexes.

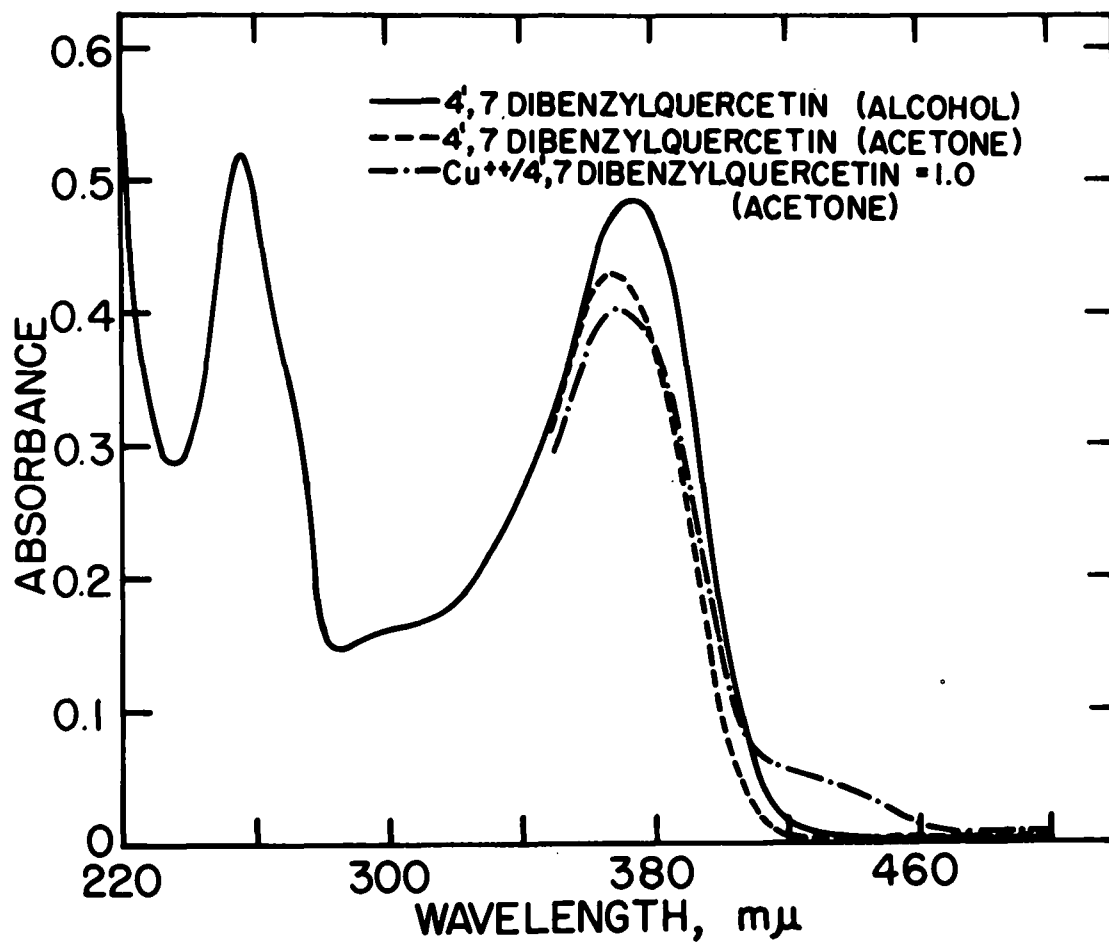


TABLE III  
Ultraviolet Absorption Spectra Data on Flavonoids

Flavonoid	Ultraviolet Absorption Maxima and Minima						Molar Extinction Coefficient		
	$\lambda_{\text{Max.}}$	$\lambda_{\text{Min.}}$	$\lambda_{\text{Max.}}$	$\lambda_{\text{Min.}}$	$\lambda_{\text{Max.}}$	$\lambda_{\text{Min.}}$	$\lambda_{\text{Max.}}$	$\epsilon$	Log $\epsilon$
3,5,7,3',4'-penta- hydroxyflavone	375	312	310 slight	285	256	236	375 256	22,470 22,134	4.35 4.34
3,5,3',4'-tetrahydroxy- 7-methoxyflavone	375	311-287 plat.			256	236	375 256	22,279 21,592	4.34 4.33
3,7,3',4'-tetrahydroxy- 5-methoxyflavone	375	309	295	288	256	238	375 256	23,108 21,929	4.36 4.34
3,3',4'-trihydroxy- 5,7-dimethoxyflavone	371	284			254	235	371 254	23,603 22,816	4.37 4.36
5,3',4'-trihydroxy- 3,7-dimethoxyflavone	365	310-291 plat.		290	256	239	365 256	21,005 22,581	4.32 4.35
5-hydroxy-3,7,3',4'- tetramethoxyflavone	355	284			254	235	355 254	21,025 22,353	4.34 4.35
3,5,7,3',4'-penta- hydroxyflavanone					288	248	288	20,772	4.32
3,5,7,2',4'-penta- hydroxyflavone	373			308	263	238	373 263	14,509 18,809	4.16 4.27
3,5,3',4'-tetrahydroxy- 7-benzyloxyflavone	375	311 slight	302 slight	286	256	238	375 256	28,226 27,076	4.45 4.43
3,5,3'-trihydroxy-4',7- dibenzyloxyflavone (ethanol)	375	284			256	236	375 256	25,653 27,506	4.41 4.44
3,5,3'-trihydroxy-4',7- dibenzyloxyflavone (acetone)	370						370	25,855	4.41

TABLE IV  
Ultraviolet Absorption Spectra Data on Copper-Flavonoid Complexes

Flavonoids	Maxima of Flavonoids and Complexes of Cu <sup>++</sup> /Flavonoid Molar Ratios					Cu <sup>++</sup> /Flavonoid Molar Ratio of Maximum $\lambda$ Shift	Maximum Shift in Maximum (m $\mu$ )
	0.0	1.0	2.0	3.0	4.0		
3,5,7,3',4'-pentahydroxy- flavone	375	421	428	436	436	3.0	61
	256	256	261-268	267	267	3.0	11
3,5,3',4'-tetrahydroxy-7- methoxyflavone	375	426	430	434	434	3.0	59
	256	256	265-270	267	267	3.0	11
3,7,3',4'-tetrahydroxy-5- methoxyflavone	375	421	426	427	434	4.0	59
	256	258	262	267	269	4.0	13
3,3',4'-trihydroxy-5,7- dimethoxyflavone	371	419	426	426	426	2.0	55
	254	258	258	258	258	1.0	4
5,3',4'-trihydroxy-3,7- dimethoxyflavone	365	365	365	365	365	**	
	256	256	256	256	256	**	
5-hydroxy-3,7,3',4'- tetramethoxyflavone	355	355	355	355	355	*	
	256	256	256	256	256	*	
3,5,7,3',4'-pentahydroxy- flavone	288	288	288	288	288	**	
3,5,7,2',4'-pentahydroxy- flavone	373	417	417	417	417	1.0	44
	263	266	266	266	266	1.0	3
3,5,3',4'-tetrahydroxy-7- benzyloxyflavone	375	419	428	428	434	4.0	59
	256	258	258	261-270	265	4.0	9
3,5,3'-trihydroxy-4',7- dibenzyloxyflavone (acetone)	370	370	370	370	370	**	

\* No apparent alteration in absorption spectrum.

\*\* No change in absorption maximum, but alteration of absorption curve at a Cu<sup>++</sup>/flavonoid molar ratio equal to 1.0.

## DISCUSSION OF RESULTS

Polyhydroxyflavones possess molecular structural features which enable them to function as free radical acceptors and as a metal complexing agents. It is difficult to evaluate the exact contribution of each mode of action to total antioxidant capacity. Uri (60, p. 32) has suggested that autoxidation of lipids is initiated by a trace metal catalyst involving an oxygen-metal complex rather than a direct reaction with oxygen. If his theory is correct then the metal complexing sites on the flavonoid molecule would be of primary importance in antioxidant action.

In this study the number of free hydroxyl groups on the flavone nucleus were altered by selective alkylation and arylation of the quercetin molecule. In addition a polyhydroxyflavone with free hydroxyls in different positions than quercetin was also studied. Changes in the number and positions of the hydroxyl groups on the flavone nucleus should influence both modes of action. It was anticipated that the differences in antioxidant capacity of these derivatives would indicate the importance of the various hydroxyl groups to antioxidant action. The role of the metal complexing sites to the antioxidant activity of the flavone molecule could also be indicated.

The results of this investigation show that both proposed mechanisms of antioxidant action could be operating. The exact contribution of each mechanism to total antioxidant ability was not readily apparent from the experimental results. There are theoretically three possible intramolecular chelation systems present on the quercetin molecule: (a) between the C<sub>5</sub> hydroxyl and the carbonyl, (b) between the C<sub>3</sub> hydroxyl and the carbonyl, and (c) between the ortho hydroxyl groups on the 2-phenyl ring. When the complexing sites on the quercetin molecule are blocked through methylation, benzylation or by positioning of hydroxyl groups the antioxidant activity is reduced. However, it is not clear whether this is due to the blocking of complexing ability or blocking of free radical capturing ability.

Certain results of this investigation suggest that free radical capture is the primary mechanism of antioxidant action of flavonoids. Metal complexing seems to be of secondary importance. Methylation of the C<sub>7</sub> hydroxyl group reduces the antioxidant capacity of quercetin more than C<sub>5</sub> hydroxyl methylation. The C<sub>5</sub> hydroxyl-carbonyl chelation system is blocked through methylation of the C<sub>5</sub> hydroxyl group. The C<sub>7</sub> hydroxyl can not participate in an intramolecular chelation ring. This result is unexpected in the light of the proposed importance of

metal deactivation to antioxidant action. Upon methylation of both C<sub>5</sub> and C<sub>7</sub> hydroxyl positions of quercetin a greater reduction in antioxidant ability occurs than when quercetin is methylated only in the C<sub>5</sub> hydroxyl position. Quercetin methylated in the C<sub>3</sub>, C<sub>7</sub>, C<sub>3</sub>, and C<sub>4</sub>, positions lacks antioxidant ability, even though the C<sub>5</sub> hydroxyl-carbonyl chelation ring is open. Morin (3,5,7,2',4'-pentahydroxyflavone), with meta hydroxyls on the 2-phenyl ring, and 4',7-dibenzylquercetin are very poor antioxidants even though they still retain a portion of their complexing ability. The blocking of the C<sub>3</sub> hydroxyl-carbonyl chelation ring as in 3,7-dimethylquercetin results in a reduction in antioxidant ability. This reduction is not of the magnitude that would be expected if chelation of catalytic metal ions was the primary mechanism of antioxidant action. Partial complexing of quercetin with copper ion as shown in Table II greatly reduces the antioxidant capacity of the molecule. There would probably be enough complexing ability left to inactivate any catalytic amounts of metal ion present. One may conclude that the molecule is more important in free radical capture and the metal complexing mechanism is of secondary importance.

There are certain conclusions that can be drawn regarding the relation of the hydroxyl groups to the

antioxidant capacity of quercetin. Methylation of the hydroxyl groups of quercetin results in a decrease in antioxidant capacity. This finding is in partial agreement with experimental results of Simpson and Uri (58, p. 956-957), Uri (60, p. 35) and Helmann and Reiff (27, p. 451-454).

Benzylation of the seven hydroxyl positions on the chromone ring of quercetin increases the antioxidant capacity of the molecule. The reason for this is not readily apparent. The resonance contribution of the benzyl substituent may be a possible explanation for the increased antioxidant effect. The greater degree of resonance of the benzyl substituted molecule is illustrated by the magnitude of its  $\log \epsilon$  as listed in Table III. The "bulkiness" of the substituted benzyl group also may be important. Gleim (21, p. 1-6) has stated that substitution of hydrocarbon groups particularly tertiary alkyl substituents increased the antioxidant ability of dihydroquercetin. Steric effects by substituted groups may also contribute to changes in antioxidant ability. This result indicates that further investigations of C<sub>7</sub> hydroxyl substitution is needed to extend and explain these observations.

The free ortho hydroxyl groups in the 3', 4' positions on the 2-phenyl ring of quercetin are essential to antioxidant capacity. The much greater antioxidant ability of

7-benzylquercetin over the 4', 7-dibenzylquercetin in lard is evidence for this observation. Morin (3, 5, 7, 2', 4'-pentahydroxyflavone) with free meta hydroxyl groups (2', 4') is a markedly poorer antioxidant than quercetin; this further verifies the importance of ortho hydroxyl groups on the quercetin molecule.

Methylation of the C<sub>3</sub> hydroxyl group of quercetin only slightly effects the antioxidant capacity of the molecule. Only a slight difference in stability of lard treated with 7-methylquercetin and 3, 7-dimethylquercetin was observed. No compound without the hydroxyl group in the C<sub>3</sub> position was tested, hence no conclusions can be drawn from this work as to the importance of its presence. However, in consideration of the importance attached to the C<sub>3</sub> hydroxyl group by other workers (27, p. 454; 44, p. 24-28; 58, p. 956-957) the result obtained was unexpected. Mehta and Seshadri (44, p. 24-28) presented conflicting results as to the importance of the C<sub>3</sub> hydroxyl group. Their data showed the quercetin molecule without the C<sub>3</sub> hydroxyl was a much poorer antioxidant. With the addition of a C<sub>3</sub> hydroxyl to the flavone nucleus with only hydroxyls in ortho or para positions on the 2-phenyl ring an increase in activity was observed. However, upon addition of a C<sub>3</sub> hydroxyl to the flavone nucleus with only hydroxyls on the chromone ring in the C<sub>5</sub> and C<sub>7</sub> positions

no effect was noted. This same effect was observed in the results presented by Simpson and Uri (58, p. 956-957). In an effort to explain the results of this investigation as to the antioxidant ability afforded by the C<sub>3</sub> hydroxyl certain possible conclusions can be made. It seems probable that its main function may be to promote formation of resonance forms which contribute to the antioxidant action of the rest of the molecule. Methylation would not necessarily block this mechanism. Methylation would, however, block the antioxidant contribution of the C<sub>3</sub> hydroxyl as it participates in the ortho semiquinone structure of the pyrone ring.

The C<sub>5</sub> hydroxyl group is of minor importance to the antioxidant capacity of quercetin. This is demonstrated by the absence of antioxidant protection for lard afforded by 3, 7, 3', 4'-tetramethylquercetin. Methylation of only the C<sub>5</sub> hydroxyl group slightly decreases the antioxidant capacity of quercetin.

Considerable decrease in antioxidant action is observed when the C<sub>7</sub> hydroxyl group is methylated. When both the C<sub>5</sub> and C<sub>7</sub> hydroxyl groups are methylated, an even further decrease results. This decrease is greater than that obtained by methylation of the C<sub>3</sub> and C<sub>7</sub> hydroxyl groups. Therefore, the C<sub>5</sub> and C<sub>7</sub> hydroxyl groups together must influence the antioxidant capacity

of the molecule. This conclusion is in conflict with those of Simpson and Uri (58, p. 956-957) and Heimann and Reiff (27, p. 451-454). They concluded that meta hydroxyls in the C<sub>5</sub> and C<sub>7</sub> position do not contribute to antioxidant ability and may even exert a pro-oxidative effect. They base their conclusion on the fact that quercetin methylated in the C<sub>7</sub> position was a better antioxidant. This result was not obtained in this study. Simpson and Uri (58, p. 956-957) presented results which showed no increase in antioxidant ability of certain flavones by methylation of C<sub>7</sub> hydroxyl or by methylation of both C<sub>5</sub> and C<sub>7</sub> hydroxyl groups. Also in most cases the addition of C<sub>5</sub> and C<sub>7</sub> hydroxyls to the molecule did not decrease antioxidant ability. Mehta and Seshadri (44, p. 24-28) concluded that the meta hydroxyls on the chromone ring do not lower activity. Not only was the position of the hydroxyl groups important, but also their orientation to each other. Their results also indicated that the addition of C<sub>7</sub> hydroxyl to the flavone nucleus increases antioxidant activity, particularly in the presence of a C<sub>3</sub> hydroxyl group. It is apparent from the experimental results that the C<sub>7</sub> hydroxyl group must play an important role in the antioxidant effect of the quercetin molecule and that the C<sub>5</sub> hydroxyl enhances this effect in some way.

The reduction of the double bond between C<sub>2</sub> and C<sub>3</sub>

of the chromone ring of quercetin results in an appreciable loss in antioxidant capacity. This is illustrated by the greater stability exhibited by lard with additions of quercetin than with dihydroquercetin. This result is in agreement with other workers (27, p. 451-454; 38, p. 433-436; 47, p. 1-3).

Morris et al. (45, p. 106) noted in their study that the presence of metals in a fat system markedly decreased the protective effect of antioxidants. In many instances there was still an appreciable antioxidant effect, since the stability of the metal containing fat system plus antioxidant was greater than the control fat system. The question that naturally arose was whether the antioxidant deactivated the metal, leaving some unreacted antioxidant, or whether no metal deactivation took place and antioxidant action was via the free radical capture mechanism. In this investigation a different approach was used. The metal ion ( $\text{Cu}^{++}$ ) was complexed with quercetin in an absolute ethanol system and then fat was added to it. The complexing of quercetin with copper ion in an ethanol system takes place as indicated by the shifts in ultraviolet absorption spectra observed in Figure 6. The objective of this method was to determine the maximum chelating ability of the quercetin molecule and at the same time ascertain the effect of the chelation on anti-

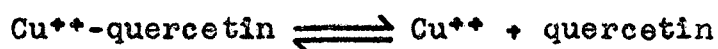
oxidant ability.

The lard containing the copper-quercetin complexes was more resistant to oxidation than the lard containing copper ion alone as illustrated in Table II. This indicates that some antioxidant ability was retained by the complex. However, the stability of lard treated with  $\text{Cu}^{++}$ /quercetin molar ratios of 1.0 or greater was less than the lard itself. The degree of protection afforded the lard by the complexes was much less than that given by uncomplexed quercetin. It should be noted that once a peroxide value was obtained (Figure 4), the reaction proceeded at almost an instantaneous rate. This is in contrast to the much slower accumulation of peroxides observed with uncomplexed quercetin. It is evident also from the results presented in Table II and Figure 5 that the oxidation rate is dependent on the copper concentration in the complex. This dependency exists up to a molar ratio of  $\text{Cu}^{++}$ /quercetin of one and beyond the ratio of two.

These results suggest certain conclusions. Copper complexed flavonoids cannot act in their full capacity as free radical acceptors, and the metal complexing sites are of great importance to the antioxidant ability of the molecule. This is shown by the AOM stability of lard treated with complexed and uncomplexed quercetin. Since

molar ratios of  $\text{Cu}^{++}$ /quercetin of one and two exhibit the same protection for lard, it is possible that an equilibrium may exist between the complex and the uncomplexed substituent. The rapid change in the oxidation pattern of the lard treated with the complexes may be interpreted as additional evidence of this equilibrium. A  $\text{Cu}^{++}$ /quercetin molar ratio complex of 1.0 gave an AOM stability of less than that of the lard itself. Ultraviolet absorption shifts indicate that quercetin will complex at least two moles of copper per mole. This may be explained by the existence of an equilibrium. An interesting visual observation was noted that also suggests an equilibrium. Copper-quercetin complexes imparted a yellow color to the fat. This yellow color changed to a greenish cast upon the outset of rapid peroxide formation. This may indicate the presence of free  $\text{Cu}^{++}$  ion in the fat.

The proposed equilibrium may be represented as follows:



The uncomplexed  $\text{Cu}^{++}$  ion could catalyze the free radical formation reaction of autoxidation. The free radicals produced could be captured by the free quercetin. The free quercetin could react according to this mechanism and be removed. The equilibrium reaction then would proceed to the right producing more free  $\text{Cu}^{++}$  ion and quercetin.

The quercetin could be thus used up in the capture of the free radicals produced by the copper catalyzed autoxidation and peroxide formation would begin very rapidly.

It is apparent that this suggested equilibrium is not a simple one. Since there is more than one complexing system present on the quercetin molecule, there could be more than one equilibrium system. Further investigation is necessary for the conformation of this proposed equilibrium. Such an investigation would be of great value in understanding the relationship of metal deactivation and metal catalyzed autoxidation.

Resonance phenomena have long been recognized in phenolic compounds, and its importance in their action as antioxidants has been postulated. With an increase in resonance there is a corresponding shift in absorption spectrum from the ultraviolet to the visible and an increase in intensity. The absorption maxima and  $\log \epsilon$  of the flavonoids used in this investigation may suggest certain conclusions as to the effect of substitution on resonance and its corresponding effect on the antioxidant ability of the molecule.

As stated earlier, methylation of quercetin results in a decrease in antioxidant ability. Methylation generally shifts the absorption maximum toward the ultraviolet, indicating a decrease in resonance. The shift seems to

depend upon the extent and position of methylation. Methylation in either the C<sub>5</sub> or the C<sub>7</sub> position results in no shift of absorption spectrum, however, methylation in both the C<sub>5</sub> and C<sub>7</sub> position did result in a shift. This decrease in resonance of quercetin methylated in the C<sub>5</sub> and C<sub>7</sub> position corresponds to a large decrease in antioxidant capacity. Quercetin methylated in the C<sub>3</sub>, C<sub>7</sub>, C<sub>3'</sub>, and C<sub>4'</sub> positions exhibited a 20 m $\mu$  shift toward the ultraviolet, indicating a large decrease in resonance. This parallels the lack of antioxidant ability of 3, 7, 3', 4'-tetramethylquercetin. A shift toward the ultraviolet with a corresponding decrease in antioxidant ability is also shown by 3, 7-dimethylquercetin.

No shift in absorption maximum toward the visible was observed with an increase in antioxidant capacity when quercetin was benzylated in the C<sub>7</sub> position. However, a significant increase in log  $\epsilon$  was observed.

It should be noted that an increase or decrease in resonance as measured by a shift in absorption maximum or change in intensity of absorption does not necessarily indicate the corresponding change in antioxidant ability. The position of substitution may be at an active site participating in the free radical capture mechanism of antioxidant action, thus blocking this action. This is illustrated by the poor antioxidant ability of quercetin

benzylated in the C<sub>4</sub>, and C<sub>7</sub> positions with a corresponding significant increase in intensity of absorption. Benzylation would block the possible ortho quinol structure contributing to antioxidant capacity, but still may increase the resonance of the molecule.

Ultraviolet absorption maxima shifts to flavonoids when molar ratios of copper ion are added, demonstrate that complexes of the flavonoids with copper ion are being formed. The cessation of absorption maximum shifts with additions of copper ion should indicate maximum complexing capacity of a flavonoid molecule. The absorption spectra (Figures 6-15) and the corresponding absorption maxima shifts listed on Table IV show that the C<sub>3</sub> hydroxyl-carbonyl and the ortho hydroxyl complexing systems must be open to observe a shift in absorption maximum. This is shown by the absorption spectra of 3, 7-dimethylquercetin, 4', 7-dibenzylquercetin and 3,7,3',4'-tetramethylquercetin with additions of copper ion in molar ratios.

No significant change in the absorption spectra was noted when only the C<sub>5</sub> hydroxyl-carbonyl complexing system was open. The stability of the absorption spectrum of 3,7,3',4'-tetramethylquercetin (Figure 13) with molar additions of copper ion is evidence for this. It should be noted that not only was the other intramolecular complexing sites blocked but also all probable chances for

intermolecular complexing. This may indicate that the C<sub>5</sub> hydroxyl-carbonyl complexing system does not function.

Morin (3,5,7,2',4' pentahydroxyflavone) with meta hydroxyl groups on the 2-phenyl ring has only two possible intramolecular complexing systems available; the C<sub>3</sub> hydroxyl-carbonyl and the C<sub>5</sub> hydroxyl-carbonyl systems. Simpson and Garden (57, p. 4641) suggest that these two systems may exist simultaneously. However, they point out that the chelation of one system may involve the deformation of the electronic cloud of the carbonyl-oxygen atom, and render its electrons less available for chelation with the second hydroxyl group. The greatest absorption maximum shift was observed with a Cu<sup>++</sup>/Morin molar complex ratio of 1.0. This indicates that only one of the systems was operating. Since 3,7,3',4'-tetramethylquercetin apparently did not exhibit chelation, then probably only the C<sub>3</sub> hydroxyl-carbonyl system was operative and there is no simultaneous chelation by the two systems.

When quercetin is methylated in the C<sub>5</sub> and C<sub>7</sub> positions only the C<sub>3</sub> hydroxyl-carbonyl and the ortho hydroxyl systems are open for complexing. The absorption maximum shifts as shown in Figure 12 ceased at a Cu<sup>++</sup>/5,7-dimethylquercetin molar ratio of 2.0. This indicates that these two systems do exist. Since only those hydroxyls necessary for intramolecular chelation were available, no

intermolecular chelation was probable. Further evidence that these two systems function was shown by the significant alteration of the absorption spectra of 3,7-dimethylquercetin and 4',7-dibenzylquercetin (Figures 11 and 15) upon molar additions of copper ion. Maximum alteration was observed at a  $\text{Cu}^{++}$ /flavonoid complex molar ratio of 1.0.

It seems probable that due to molecular proximity, chelation first occurs intramolecularly. Intermolecular chelation may also occur. This makes possible a multitude of chelate structures for the unsubstituted quercetin molecule to participate in. Quercetin and 7-methylquercetin exhibited a cessation of absorption shift at a  $\text{Cu}^{++}$ -flavonoid molar ratio of 3.0. In the light of the preceding discussion this may be best explained by the presence of intermolecular complexing.

Ultraviolet absorption spectra of 5-methylquercetin and 7-benzylquercetin show a cessation of absorption shift upon the addition of copper ion at a  $\text{Cu}^{++}$ /flavonoid molar ratio 4.0. This observation is significant in that this occurred only with these two derivatives of quercetin. Perhaps these two substitutions in their respective positions promote a different and more complicated complexing system.

## SUMMARY AND CONCLUSIONS

This investigation was designed to allow the study of the relationship of the hydroxyl group in various positions on the flavone and flavanone molecule to the effectiveness of the compounds as antioxidants. At the same time the relationship of the various complexing sites on the molecule to antioxidant capacity could be studied.

In this study the number of free hydroxyl groups on the quercetin molecule was altered. This was accomplished through selective alkylation and arylation. In addition a flavone with different positioning of hydroxyl groups was used. The flavanone molecule in the form of dihydroquercetin was also used.

To accomplish the objectives of this investigation using the compounds available the following sources of experimental data were used: (1) AOM oxidation patterns of lard containing the compounds. (2) AOM oxidation patterns of lard containing copper-quercetin complexes in various molar ratios. (3) Ultraviolet absorption spectra of the compounds. (4) Ultraviolet absorption spectra of the compounds complexed with copper ion in various molar ratios.

The following conclusions were made in the light of the experimental data obtained from the above listed

## sources:

- (1) The antioxidant action of the polyhydroxyflavone molecule seems to result from a combination of free radical accepting and metal complexing. It is difficult to evaluate the contribution of each mechanism to total antioxidant ability, but experimental evidence points to free radical acceptance as the most important mechanism.
- (2) Methylation of the hydroxyl groups of the quercetin molecule results in a decrease in antioxidant capacity.
- (3) The following molecular structural features were found to be of great importance to the antioxidant ability of the quercetin molecule: (a) ortho hydroxyl groups on the 2-phenyl ring, (b) the double bond between C<sub>2</sub> and C<sub>3</sub>, (c) the C<sub>7</sub> hydroxyl group, and (d) the combination of the C<sub>5</sub> and C<sub>7</sub> hydroxyl groups.
- (4) The C<sub>7</sub> hydroxyl group itself does not contribute significantly to the antioxidant ability of the quercetin molecule.
- (5) The C<sub>3</sub> hydroxyl as it participates in the antioxidant action of the ortho semiquinone structure in the pyrone ring does not contribute significantly to antioxidant ability.
- (6) Metal complexing is involved in the antioxidant ability of quercetin. The copper-quercetin complex retains some antioxidant ability although it is greatly reduced from that of the uncomplexed quercetin. The metal complexing sites are then of great importance to the free radical capture mechanism of antioxidant action.
- (7) Experimental evidence from the AOM analysis of lard containing copper-quercetin complexes in molar ratios suggests a relationship between the complexed metal ion and the autoxidation of lard. An equilibrium between the complexed and the uncomplexed substituents is postulated.
- (8) Ultraviolet absorption spectra of the flavonoids and the copper-flavonoid complexes in various molar ratios suggest the following conclusions:

- (a) Methylation results in a decrease in resonance as measured by maxima shifts toward the ultraviolet and changes in intensity of absorption. This decrease in resonance corresponds to a decrease in antioxidant ability.
- (b) Intramolecular chelation between the carbonyl and the C<sub>3</sub> hydroxyl and between the ortho hydroxyl groups on the 2-phenyl ring was demonstrated.
- (c) Intramolecular chelation between the C<sub>5</sub> hydroxyl and the carbonyl was not demonstrated.
- (d) Intermolecular complexing and the presence of more complicated and significant chelation systems is indicated.

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