DETERMINATION OF ANTIOXIDANTS IN FATS AND THEIR BEHAVIOR IN FOODS

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THE DETERMINATION OF CERTAIN ANTIOXIDANTS IN FATS AND THEIR BEHAVIOR IN FOOD PRODUCTS

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INTRODUCTION

A number of the constituents of foods, including unsaturated lipids, lipid- and water-soluble vitamins, pigments and flavouring substances, are capable of reacting with atmospheric oxygen, during the interval between the preparation and consumption of food. These oxidative processes do not proceed in the living tissues in spite of the presence of complicated oxidation - reduction systems in the cell; this fact is ordinarily ascribed to the maintenance of a highly specific state of organization of these systems in the living cell. Once the cell is dead, as in most food materials, the destruction of organization at the level designated as "living", leads to degradative oxidative processes. These processes may actually be hastened by fragments of the disorganized cellular biochemical mechanisms. The effect of haematin pigments and certain oxidases are cases in point. On the other hand, the disorganized material contains other substances which act as inhibitors of such oxidative changes. The resistance to oxidation of extracted triglyceride oils and fats, especially in the purified state, is largely dependent on the presence of substances of this class.

Lea (79) has listed several types of rancidity in fats,

including the action of enzymes present in the tissues, action of micro-organisms, and atmospheric oxidation. The term "oxidative rancidity" is somewhat unprecise, although it is employed to denote the deterioration in flavour and odour that occurs when atmospheric oxygen reacts with the triglycerides or other components of natural fats. There are at least four types of oxidative rancidity in edible fats. Namely, "oxidative rancidity", "oxidized flavour" in milk products, "flavour reversion" and lipoxidative rancidity.

For lack of a more specific term, "common oxidative rancidity" is used in this thesis to denote that type of fat deterioration which occurs typically in lard and other rendered animal fats and in vegetable fats. Such rancidity develops sooner or later in all substances that contain triglycerides composed of long-chain fatty acids. At a very early stage, this type of rancidity is characterized by a sweetish but undesirable odour and flavour, which becomes more pungent and penetrating as the deterioration progresses. Powick (115) attributed the flavour to the formation of heptylic aldehyde, although other substances are undoubtedly involved.

Economically, one of the most important types of flavour deterioration associated with fats is the development of "oxidized flavour" in milk and milk products. Many workers

have believed that oxidized flavour results from the oxidative breakdown of lecithin. Salt in butter is said to hasten the breakdown of lecithin, resulting in a fishy or oxidized flavour (52). In the later stages, when the deterioration is advanced, more profound breakdown products of the triglycerides contribute to the off-flavours.

Bailey (5) defined "flavour reversion" in fats as the appearance of objectionable flavours from a lesser degree of oxidation than is required to produce true rancidity. The term is a misnomer and is misleading since the flavour, variously described as "painty", "fishy" and so on, is generally not related to the original flavours present in the fresh fats. Flavour reversion occurs principally in fish and vegetable oils that contain linolenic acid. According to Bailey (5), an extremely small amount of oxidation, involving less than one-fiftieth as much oxygen absorption as is required to produce true rancidity, will in some cases produce flavour deterioration. At the present time the problem of reversion is of greatest economic importance in the production of soybean oil.

Lipoxidative rancidity results from the presence of lipoxidase enzymes which are known to be present in pork (78), fish (6), soybeans (2) and many other vegetable tissues. These enzymes are responsible for one type of

oxidative deterioration of lipids in food products. This form of rancidification is of particular importance to the frozen food industry.

While the ease and rapidity with which an oil oxidizes depends primarily upon its content of reactive double bonds, it is also influenced considerably by the presence of certain accessory substances, which may occur in the oil either naturally or through addition. Those substances which favour the oxidative process are termed pro-oxidants, while those inhibiting oxidation are known as antioxidants.

All naturally occurring fats and oils contain characteristic antioxidants. Vegetable oils generally possess a larger antioxidant content than do animal fats, and consequently are usually more stable than animal fats of an equivalent degree of unsaturation. A remarkable characteristic of the antioxidants is their great effectiveness at low concentrations in the fat or oil. The natural antioxidants of an oil rarely exceeds one-tenth of 1 percent.

Food antioxidants are conveniently divided into two chemical categories. In one category are found antioxidants such as the polyphenols and substituted polyphenols. These represent the primary type of antioxidants, that is, they markedly inhibit the oxidation of fats when present at low concentrations. A classic example of a primary antioxidant is hydroquinone. The second category of antioxidants include

the "synergists" which play a secondary role, in that they have little or no effect by themselves upon the resistance of fats to oxidation. However, when such an antioxidant is added to a fat together with a phenolic antioxidant, a "synergistic" effect is observed, namely, the effect on the induction period is greater than the sum of the effects exerted by each antioxidant when added to the fat alone. Citric acid is a widely used "synergistic" antioxidant.

In commercial fat products, the pro-oxidants most likely to be encountered are metallic soaps, usually formed by the action of free fatty acids upon the metallic equipment employed in processing and storage of the oils. King et al. (70) tested the effect of different metallic soaps on the stability of lard held at 208°F. These workers found that the following amounts of metal, in p.p.m., were required to reduce the keeping time of the fat to one-half: copper, 0.05; manganese, 0.6; chromium, 1.2; nickel, 2.2; vanadium, 3.0; zinc, 19.6; and aluminium approximately 50. These findings illustrate the fact that copper, in particular, is a very strong pro-oxidant. Consequently, copper and copper-bearing alloys are avoided in the fabrication of equipment for handling edible fats and oils. In this connection Hills and Conochie (59) have reported that the pro-oxidant effect of common salt in dry fats is due to the presence of magnesium chloride in the salt.

The mechanism by which metals and other pro-oxidants accelerate the oxidation of fats is still obscure. However, the theory has been advanced (79) that they function principally as catalysts for the destruction of natural or added antioxidants. Some of the synergists for edible fats, such as commercial lecithin, citric acid and phosphoric acid, have the property of inactivating heavy metal pro-oxidants through the formation of metal complexes. It has been suggested by Dutton et al. (32) that metal deactivation rather than a synergistic effect is actually responsible for the effectiveness of acidic "synergists".

The phenols constitute the largest and most important class of primary antioxidants. Practically any phenolic substance with two or more hydroxyl groups in the ortho or para positions to one another, at least one of which must be free, or any naphthol, is likely to exhibit some antioxidant properties, although the activity varies widely with the nature and position of the substituent groups. Rosenwald and Chenicek (125) illustrated the effect of structure, on the antioxidant activity of more than forty anisoles and related phenols when added to lard. Bickoff (10) also investigated 108 phenolic antioxidants for their effectiveness in preventing the destruction of carotene in mineral oil.

The generally accepted explanation of the powerful inhibitory effects of mere traces of antioxidant on the

oxidation of fats is essentially that advanced by Christiansen (21). According to his so-called chain reaction theory, the fat is pictured as uniting with oxygen to form a peroxide only after acquiring the necessary energy of activation. After the peroxide is formed, the activating energy may be made available for the activation of a new molecule and so on, leading to a chain reaction. Thus, unless the chain is broken by the absorption of the activating energy which is then lost in an extraneous reaction, an almost unlimited number of peroxide molecules may be formed as a result of the initial activation of a single molecule.

The antioxidants are presumed to be substances which, because of their specific structure are able to absorb the activating energy, thus preventing the propagation of the chain reaction. If the average chain of reactions is very long, then the presence of even trace amounts of an antioxidant may be sufficient materially to reduce its length, leading to a reduction in the rate of oxidation which in turn results in a prolonged induction period. According to this theory the antioxidant should be oxidized during its role of breaking the reaction chains. Generally speaking, this appears to be so. For, when fatty materials have been analyzed for antioxidant content during the course of oxidation experiments, the complete disappearance of the antioxidant has generally coincided with the end of the

induction period (42,50,87,90,141). From such observations it may be assumed that any fat exposed to oxygen will resist rancidification for a period of time related to the rate at which its antioxidants are destroyed. It is probable, however, in view of the many anomalies encountered, that the role of the antioxidant is more complex than the foregoing statements might be taken to imply.

The simplest interpretations of the chain reaction theory leads to the expectation that the protective action of any antioxidant will be in direct proportion to its concentration in the fat or fatty substance. This expectation is seldom fulfilled in practice, except perhaps within narrow limits of concentration. In general, the addition of an antioxidant to a fat yields progressively diminishing returns, in terms of stability, as the amount of antioxidant is increased (50,87,90,102,111). It is generally assumed that at high concentrations an appreciable portion of the antioxidant present is wasted in side reactions, and thus fails to act as a breaker of the peroxide reaction chain. It is also possible that these side reactions, if they occur, lead to the formation of pro-oxidant compounds (111).

Of particular importance from the viewpoint of the food chemist are those inhibitors which are naturally associated with fats and oils in the tissues, and which remain in solution in the crude oil when the latter is expressed or

extracted, but which may subsequently be removed in greater or lesser degree during the refining processes. A large number of such substances must exist. Comparatively few have been isolated and identified. Of these, the tocopherols (Vitamins E) are the best known and are almost universally distributed in vegetable oil-bearing tissues and also, though to a smaller extent, in animal tissues (75). The tocopherols were discovered and named by Evans et al. (39) in the course of their nutritional studies on Vitamin E. Olcott and Emerson (110) subsequently demonstrated that the tocopherols were effective antioxidants. The tocopherols were also shown to be the active substances in the "inhibitols" previously isolated from a variety of vegetable oils by Olcott and Mattill (108). Structurally, the tocopherols can be regarded as mono-ethers of hydroquinone with a long lipophilic side chain which renders them much more fat-soluble than the simple phenols. At the present time four tocopherols have been isolated (72,136) and designated as α-, β-, γ- and δ-tocopherol. δ-tocopherol is the most potent antioxidant (136), while γ-tocopherol is more effective than β-tocopherol (110) which in turn is more effective than α-tocopherol (121). Therefore, the order of antioxidant activity appears to be the reverse of their biological activity as measured by anti-sterility test on rats (62). In crude vegetable oils the tocopherols are often present in amounts

approaching the optimum for prevention of oxidative change and little or no additional stabilization can be secured by further additions (50). Above the optimal concentration, tocopherol actually functions as a pro-oxidant (111). However, animal fats are generally deficient in tocopherols and are greatly stabilized by the addition of tocopherols (110). The loss of tocopherols in the commercial processing of edible vegetable oil products is relatively small. A maximum of about 6 percent of the total has been reported lost in bleaching or continuous alkali refining (121) and no more than a few percent during deodorizing (121), whereas hydrogenation occasions no loss whatsoever (121).

Sesamol, another naturally occurring phenolic antioxidant (13,109) was isolated from sesame oil and seems to be mainly, though not entirely, responsible for the exceptional stability of sesame seed and hydrogenated sesame seed oils (14). The antioxidant is present in the crude sesame oil in an inactive bound form as sesamolin: it is partially liberated during bleaching and almost completely liberated during hydrogenation (13). Sesamol is a methylene ether of hydroxy-hydroquinone (13).

Gossypol is a complex phenolic substance which is present in crude cottonseed oil. It is an effective antioxidant (61,91,126), but it is quite toxic (23). Fortunately, gossypol combines with caustic soda, and is, therefore,

removed more or less completely by the alkali refining process.

Several other naturally occurring substances that are not present in fats and oils have been employed commercially as antioxidants in fats and oils. One such substance is gum guaiac (53) which is a secretion from the tropical tree Guaiacum officinalis. For several years gum guaiac was employed to stabilize lard (53). The gum was dissolved in acetic acid (29) and this solution was added to lard before deodorization (114). As a result an almost colourless lard with good flavour and stability was obtained. Gum guaiac is not as powerful as some of the more recently developed synthetic antioxidants, but it has the advantages of continuing to protect the fat in baked goods (57) and being non-toxic (63). Nevertheless, gum guaiac has fallen into disuse since the introduction of the newer, more potent synthetic antioxidants.

Another important antioxidant development in the food field was the introduction of nordihydroguaiaretic acid (NDGA) for use in lard by Lundberg et al. (84). NDGA is extracted from an American desert plant Larrea divaricata, or creosote bush (128). The chief disadvantages of NDGA have been its relative high cost and the fact that its antioxidant activity is not carried over into baked goods (129). Consequently, NDGA has not been used as widely as the synthetic

antioxidants.

Norconidendrin is an antioxidant derived indirectly from a natural source, namely, from conidendrin, a substance found in the sulphite waste of Western hemlock (43). Norconidendrin has been shown to exhibit antioxidant properties when added to hydrogenated or unhydrogenated cottonseed oil, peanut oil and lard (44); acidic synergists enhance its effectiveness (44). The toxicological characteristics of norconidendrin have not been adequately investigated, consequently, this antioxidant has not been used commercially.

Dihydroquercetin is a pentahydroxyflavanone found in large quantities in the bark of Douglas fir and Jeffrey pine and isolated by Kurth and Chan (76). Dihydroquercetin is an effective antioxidant for lard, cottonseed oil and butter (76) but has not been used commercially.

Recently Clopton (25) isolated two substances from the osage orange (Bois D'Arc) namely, osajin and pomiferin. These are isoflavone pigments and have been shown to possess antioxidant activity when added to lard (25).

At the present time synthetic antioxidants are of great importance as food antioxidants. Ten years ago Boehm and Williams (11) proposed the use of propyl gallate as an antioxidant. Lea (80) has shown that other esters of gallic acid derived from lower aliphatic alcohols are also effective antioxidants. Propyl gallate exhibits a low level of

toxicity (112) and is an extremely effective antioxidant especially in lard (57,73,90), but is ineffective in baked goods (129).

Five years ago butylated hydroxyanisole (BHA) (74) was introduced as an antioxidant for food fats. Commercially prepared BHA is primarily composed of the isomers 3-tertiary-butyl-4-hydroxyanisole and 2-tertiary-butyl-4-hydroxyanisole (31) which will be referred to hereafter as 3-BHA and 2-BHA respectively. BHA is highly soluble in fats (73) and virtually insoluble in water (73). When used alone BHA is not unusually effective in increasing the stability of lard (73, 90) but, has the property of carrying-through into baked goods made from lard (30,73,129). Therefore, BHA has found wide usage in the stabilization of animal fats, particularly lard which is to be employed in baking.

The first large commercial use of these newer antioxidants was in their application to lard. NDGA, which is relatively insoluble in lard, was dissolved in an alcoholic or acetic acid solution in combination with citric acid and added to the lard, which was then heated to remove the solvent (86). Propyl gallate was incorporated in an oil-soluble combination (54,55), which included propyl gallate, citric acid, lecithin and corn oil, and was sold under the trade name of "G-4" which is readily soluble in oils and melted fats. BHA is now widely used in combination with

propyl gallate and citric acid in a propylene glycol solution. This antioxidant combination was originally designated "AMIF-72" (74) by the American Meat Institute foundation and is known commercially under the names such as "G-4(BHA)", "Tenox II", and "Sustane". BHA is also used in combination with NDGA and citric acid in a propylene glycol solution under the name of "Tenox N". The advantage of "Tenox N" over "Tenox II" is that "Tenox N" produces no colour when exposed to iron salts, which may be encountered in foods such as enriched flours. The propyl gallate in "Tenox II" produces a bluish colouration in the presence of iron salts.

The determination of specific antioxidants in edible oils and fats presents an analytical problem of considerable importance. Mattil and Filer (93) had developed a method for determining gallic acid in fats, while Lundberg and Halvorson (85) had developed methods for the determination of a number of antioxidants if present singly in fats. These methods were valuable in determining the solubilities of such antioxidants in fats, studying the kinetics of the destruction of single antioxidants in oxidizing fats and in making control determinations. However, no methods for the determination of antioxidant mixtures in edible oils and fats were available when the researches now described were begun in 1949.

The methods for single antioxidants had found application in kinetic studies of oxidative deterioration. Thus Filer et al. (41) reported that the rate of disappearance of gallic acid in oxidizing fat was virtually constant with respect to time and almost independant of the initial concentration. Golumbic (50), on the other hand, found that the rate of disappearance of α-tocopherol was dependant on the initial concentration, although the time for disappearance of a given fraction of the α-tocopherol was independant of the initial concentration. Golumbic (50) observed that when fats containing ascorbic acid were oxidized, some of the ascorbic acid remained after the end of the induction period. On the other hand, with gallic acid the end of the induction period coincided approximately with the disappearance of the last traces of gallic acid (50).

A number of studies have been made on the kinetics of antioxidation in fats containing α-tocopherol (111,141). Golumbic (50), in the work already cited, found that there was an optimal concentration for α-tocopherol. Rancidity developed more rapidly if the concentration was above or below this optimum. This positive catalytic effect at higher concentrations has been observed also with other phenolic antioxidants. Lundberg et al. (87) reported a positive catalytic effect upon the formation of peroxides during the initial stages, when 0.5 percent of hydroquinone,

catechol, NDGA or gallic acid were added to lard stored at 100°C with a stream of oxygen passing through the sample. Lower initial peroxide values were obtained when 0.02 percent of these antioxidants was employed (87). In view of these findings, the concentration of phenolic antioxidants added to lard seldom exceeds 0.02 percent. Above 0.01 percent the effectiveness of phenolic antioxidants, such as propyl gallate and BHA, obeys a law of diminishing returns (90).

In order to evaluate the stability of fats or fatty foods within a reasonable period, it is necessary, in the laboratory, to adopt some means of accelerating their normal rate of oxidation. It has been suggested that the oxidative process should be hastened by the use of strong light or by adding metallic pro-oxidants (7,126). However, both these methods are difficult to control, and the use of heat has been more widely accepted in practice because of its greater ease of control.

For routine testing of edible fats, a method widely acclaimed is that originally suggested by Wheeler (146) and later modified and standardized by King et al. (70). This method is referred to as the "Active Oxygen Method" or the "Swift Stability Test" and involves the continuous aeration of the fat sample at 97.8°C. Sub-samples are withdrawn at intervals for analysis in order to determine the time

required to attain a given peroxide value. Mehlenbacher (97) devised a modification of the foregoing procedure by aerating the sample at 110°C. The stability times thus obtained are about 40 percent of those obtained by the Active Oxygen Method. The "Schaal test" or "Oven Method" (64) is also widely used. It consists of storing the fat or fatty food in an oven at 60° to 62.5°C, until a specified peroxide value is attained or the sample exhibits organoleptic rancidity. Several other methods and modifications have been suggested from time to time.

If the object of the tests is a comparison of the practical utility of antioxidants or synergists in fats and oils, then serious errors can arise from the use of tests wherein oxidation is accelerated by the use of elevated temperatures. Nagy et al. (106) report a classic example of such error. A lard sample was tested by the Active Oxygen Method at 97.8°C. The addition of 0.1 percent of D-isoascorbyl palmitate increased the keeping time from eight hours to forty hours. However, when compared with an oxygen absorption method at 70°C, the addition of the palmitate acted as a pro-oxidant and decreased the keeping time from thirty-five hours to six hours. Storage experiments conducted at approximately 20°C gave results in accordance with this latter finding.

In view of the widespread use of mixtures of antioxidants, such as propyl gallate plus BHA or NDGA plus BHA in edible fats, quantitative analytical methods applicable to the determination of these antioxidant mixtures were required. The present thesis deals in part with the development of new analytical procedures for a number of antioxidants when present in fats, oils or pie crust. A study has also been made of the characteristics of a number of these agents as antioxidants when incorporated in lard and pie crust.

DEVELOPMENT OF ANALYTICAL METHODS FOR ANTIOXIDANTS IN FATS

A. Propyl Gallate

1. INTRODUCTION

It is generally accepted that polyphenolic compounds carrying hydroxyl groups in the ortho or para relation to each other are most effective as antioxidants (82). A powerful antioxidant of this type is gallic acid. One disadvantage of this antioxidant is its low solubility in fats and oils, which gives rise to technical difficulties in the process of incorporating the antioxidant into fat.

Golumbic and Mattil (51) studied the antioxidant properties of gallic acid. They reported that it was unusual in that, although a powerful phenolic antioxidant in itself, it was also capable of exerting a powerful synergistic effect on other antioxidants. Other workers have corroborated the results of Golumbic and Mattil and have further studied the antioxidant properties of this substance (41,92).

Although gallic acid is inexpensive and effective as an antioxidant, its use in edible fats has never been approved, presumably on the ground that it has not been shown to be nontoxic, but perhaps also in part because of its low solubility in fats. However, its solubility has been shown to

be higher than the concentration needed to stabilize fats (85). Lea (82) has pointed out that gallic acid is widely distributed in nature in combined form as the gallotannins, and it is said to occur as the free acid in the pomegranate (107) and in tea (124). A practical method of improving the fat solubility of the hydroxybenzoic acids, for example, gallic acid, is to esterify the carboxyl group with fatty alcohols. A United States patent was issued to Sabalitschka and Bohm (128) in 1941 for the use of methyl-, ethyl-, propyl- and butyl gallates as antioxidants for fats and oils. These gallates have been classified among the best antioxidants for animal fats and are generally considered nontoxic in the small amounts required for use as fat antioxidants.

The lower esters of gallic acid are rather sparingly soluble both in water and in fat; the higher esters are only slightly soluble in water but are readily soluble in fats (102). Lea (80) has shown that a number of esters of gallic acid derived from lower aliphatic alcohols are also effective antioxidants. Ethyl gallate has been found to be a useful antioxidant in a number of fats and foods (82), and propyl gallate has also proved to be an effective phenolic antioxidant (11,12,57). Propyl gallate has been widely sponsored commercially both alone and, more frequently, under trade names in combination with such substances as

butylated hydroxyanisole and citric acid in a solution of propylene glycol (74), or with citric acid in a solution of corn oil and lecithin.

Gallic acid and its lower esters have a bitter astringent flavour which increases with increasing molecular weight from ethyl- to n-amyl- (142), but which weakens again as solubility in water decreases with increasing molecular weight (142). However, in the low concentrations required, these gallates do not impart any flavour to the products to which they are added.

At the present time propyl gallate is the only gallate ester permitted to be added to foods in Canada (17) or the United States (122). It has been widely used, but there is one respect in which propyl gallate is not fully satisfactory: its antioxidant effectiveness is not carried-over into baked foods, such as crackers (73) or pie crust (102).

2. TOXICITY OF PROPYL GALLATE

Extensive toxicological studies using various laboratory animals (112) indicate that no detectable toxic effects follow the prolonged ingestion of propyl gallate in amounts at least 100 times those that would be consumed by human subjects if all the dietary fat contained the recommended amount of propyl gallate. The LD-50 for propyl gallate administered orally in aqueous suspension to rats, was found to be 3800 mg. per kg. of body weight (117) and

380 mg. per kg. when administered intraperitoneally (117). Previous work (13) had indicated an LD-50 for propyl gallate of 2000 mg. per kg., when dissolved in propylene glycol and given orally to mice. These results suggest that there is a large margin of safety for propyl gallate, considering the small amounts employed in foods. Many manufacturers employ only 0.006 or 0.003 percent propyl gallate in lard or shortening, which further increases this safety factor.

3. DEVELOPMENT OF AN ANALYTICAL METHOD FOR PROPYL GALLATE

a. Extraction of Propyl Gallate from Fats

Gallic acid is virtually insoluble in edible fats having a solubility of only 0.016 percent in lard at 45°C and 0.016 percent in cottonseed oil at 30°C (85). On the other hand, gallic acid is highly soluble in hot water, 1.16 percent at 25°C and 33 percent at 100°C (19). Mattil and Filer (93) took advantage of these solubility characteristics for the quantitative extraction of gallic acid from fats by means of a single extraction with boiling water. Propyl gallate, on the other hand, is less soluble in water, 0.35 percent at 25°C (56) and more soluble in fats, 1.14 percent in lard at 45°C and 1.23 percent in cottonseed oil at 30°C (85). Preliminary studies were directed to the possibility of applying the procedure of Mattil and Filer

(93) to the extraction of propyl gallate from fats. It was found impossible to extract propyl gallate quantitatively from fats by a single extracting with boiling water as is possible with gallic acid. Even several successive extractions of lard with boiling water were found to recover only about 80 percent of the propyl gallate present. This failure is presumably related to the greater solubility of propyl gallate in fats. Further experimentation showed that propyl gallate is more soluble in dilute aqueous ammonium acetate solution than in distilled water. Approximate solubilities of propyl gallate were found to be 0.32 percent in water at 22°C as compared to 0.37 percent in 1.67 percent aqueous ammonium acetate at 22°C. Therefore, dilute ammonium acetate solution was employed for the extraction of propyl gallate. The ammonium acetate extracting solution was chosen because Glasstone (48) determined gallic acid colorimetrically with ferrous tartrate in the presence of an ammonium acetate buffer. Ammonium acetate serves two purposes; it increases the solubility of propyl gallate in water so as to facilitate extraction and it acts as the buffer in the subsequent colorimetric determination.

Of the common organic solvents, light petroleum (boiling range 30-60°C) has the least solvent power for propyl gallate. The solubility of propyl gallate in various solvents is given in Table I.

Table I

Solubility of Propyl Gallate in Various Organic Solvents (56)

Solvent	Solubility g. per 100 g. solvent
Acetone	121.
Ethyl ether	83.
Propylene glycol	67.
Benzene	0.16
Carbon tetrachloride	0.01
Light petroleum (30 - 60°C)	0.001

Accordingly, 30 - 60°C light petroleum was used as the fat solvent and the resulting solution was extracted repeatedly with aliquots of cold (20 - 25°C) aqueous ammonium acetate. This procedure recovered up to 98 percent of the propyl gallate and was adopted as the basis of the method for the extraction of propyl gallate from lard and shortening. However, the fat solvent employed had to be modified. Subsequent work showed that if 60 - 100°C light petroleum was employed as the solvent, the extraction of BHA was satisfactory but that there was a greater tendency for emulsion formation during the aqueous extraction of propyl gallate. This tendency to form emulsions was particularly noticable in the case of fat samples undergoing rancidification. On the other hand, the use of 30 - 60°C light petroleum as fat solvent overcame the emulsion formation but resulted in

slightly reduced recovery of BHA. After investigating various mixtures of these two light petroleum fractions, a mixture containing three volumes of 60 - 100°C and one volume of 30 - 60°C boiling range light petroleum (hereafter referred to as 3+ 1 light petroleum) was found satisfactory, permitting good recoveries of both propyl gallate and BHA.

The following experiment was carried out in order to determine the efficiency of this extraction procedure. Ten g. of fat containing 0.01 percent of propyl gallate was dissolved in 50 ml. of 3+ 1 light petroleum and extracted with successive aliquots of 1.67 percent aqueous ammonium acetate solution. Each extract was analysed for propyl gallate with the results given in Table II.

Table II

Proportion of Propyl Gallate Extracted from a Fat Solution in Light Petroleum by Successive Extractions with 1.67 Percent Aqueous Ammonium Acetate

Volume 1.67% Ammonium Acetate ml.	Duration of Extraction Minutes	Propyl Gallate Removed per Extraction %	Total Propyl Gallate Extracted %
20	2.5	72.8	72.8
20	2.5	14.1	86.9
20	2.5	7.2	94.1
Water			
15	1.0	4.1	98.2

The results presented in Table II show that this extraction procedure recovered 98 percent of the propyl gallate added. If more than 0.012 percent propyl gallate is present in the fat, an additional extraction with 20 ml. of the ammonium acetate solution is necessary to attain an equally high recovery. However, since more than 0.006 percent propyl gallate is seldom used, the following extraction procedure was adopted:- Dissolve 10 g. of lard or shortening in 50 ml. of 3+1 light petroleum. Extract this solution with three 20 ml. aliquots of 1.67 percent ammonium acetate, each extraction being accompanied by 2.5 minutes shaking. Next extract the fat solution for one minute with 15 ml. of water. These four extracts are combined, filtered and analysed for propyl gallate.

b. Choice of Reagents for Determination of Propyl Gallate

Mitchell (101) reported that gallic acid and other trihydroxyphenols react with ferrous tartrate to produce a purple colour. He believed this reaction to be specific for the pyrogallic grouping. Glasstone (48) subsequently investigated the influence of hydrogen-ion concentration on this reaction and recommended the use of an ammonium acetate buffer of pH 7.0. Although this reaction takes place equally well in the absence of tartrate, the latter is said to be necessary to stabilize the reaction (101). Lundberg and Halvorson (85) have stated that ferrous tartrate gives a

coloured reaction product with most, if not all polyphenols, but that the products are insoluble in many cases and, therefore, cannot be estimated colorimetrically. Mattil and Filer (93), using Glasstone's (48) modification of the ferrous tartrate reagent, found that the soluble ferrous tartrate-gallic acid complex displayed an absorption maximum at 540 mμ and obeyed Beer's Law over the range of 0.2 to 1.0 mg. per 100 ml. of solution.

i. Specificity of the Ferrous Tartrate Reagent

The ferrous tartrate reagent in the presence of one percent ammonium acetate buffer (pH 7.0) was found to be specific for propyl gallate among the common antioxidants permitted for addition to foods. A number of antioxidants were investigated as follows:- Five mg. was dissolved in 1 ml. aliquots of 72 percent ethyl alcohol and this solution diluted to 200 ml. with water. Twenty ml. portions of this solution, containing 0.5 mg. of antioxidant was placed into Evelyn colorimeter tubes. To each tube was added 2.5 ml. of 10 percent ammonium acetate solution, 1.5 ml. of water and 1 ml. of ferrous tartrate reagent (0.1 percent $FeSO_4 \cdot 7H_2O$ plus 0.5 percent Rochelle Salt). The resulting absorbancy was measured in an Evelyn photoelectric colorimeter fitted with a No. 540 filter, the instrument having been set at 100 percent transmission with a "reagent blank" containing 21.5 ml. of water, 2.5 ml. of 10 percent ammonium acetate

solution and 1 ml. of the ferrous tartrate reagent. The results of these experiments are reported in Table III.

Table III

Relative Intensity of Colour Formed with the Ferrous Tartrate Reagent

Antioxidant	Absorbancy at 540 mμ/500 microg. antioxidant/25 ml.	Absorbancy Relative to Propyl Gallate
Gallic acid	0.643	111.8
Propyl gallate	0.574	100.0
Lauryl gallate *	0.003	0.5
NDGA	0.346	60.0
NDGA (after centrifuging)	0.009	1.8
Dithiopropionic acid	0.000	0.0
Dilaurylthiopropionate **	0.003	0.5
2-BHA	0.002	0.3
3-BHA	0.002	0.3
Hydroquinone	0.002	0.3
Gum Guaiac	0.001	0.2
α-Tocopherol	0.000	0.0

* Since lauryl gallate is insoluble in water, it was precipitated from the alcoholic solution upon the addition of water. It was, therefore, necessary to filter the lauryl gallate solution before measuring the absorbancy.

** Dilaurylthiopropionate was precipitated upon dilution of the original alcoholic solution with water and this precipitate was filtered off before measuring the absorbancy.

Ferrous tartrate produces a blue-purple colour in the presence of high concentrations of NDGA. However, this colour is due to the formation of a very fine blue-purple precipitate. This precipitate can be removed easily by

centrifuging and it has been shown that formation of this blue-purple precipitate does not introduce any appreciable error in the determination of propyl gallate (see Table III).

In the United States NDGA can be added to edible fats. The Canadian Food and Drug regulations have recently been amended to permit the use of NDGA (17). However, combinations of propyl gallate and NDGA are not permitted either in Canada (17) or the United States (122). Therefore, fat samples ought not to contain both propyl gallate and NDGA. Only gallic acid remains as a possible seriously interfering substance when the ferrous tartrate reagent is used to determine propyl gallate. However, gallic acid is not permitted to be added to foods. Consequently, ferrous tartrate is specific for propyl gallate among the antioxidants naturally present or permitted to be added to edible fats.

ii. Relative Sensitivity of the Ferric Chloride - α,α'-Bipyridine and Ferrous Tartrate Reagents

Hill (60) employed α,α'-bipyridine plus sodium bisulphite for the estimation of iron in biological material. Later Emmerie and Engel (35) used ferric chloride plus α,α'-bipyridine for the colorimetric determination of tocopherol. The literature contains numerous applications of this reaction to the determination of reducing substances, including propyl gallate (85), NDGA (85) and BHA (73). Although ferric chloride plus α,α'-bipyridine is nine times more sensitive for the determination of propyl gallate than

ferrous tartrate (employing equal volumes of final solution), the ferric chloride - α,α'-bipyridine reagent is non-specific. Accordingly, the ferrous tartrate reagent was adopted for the determination of propyl gallate.

c. Characteristics of the Ferrous Tartrate - Propyl Gallate Colour Reaction

i. Absorption Curve

An absorption curve was prepared for the ferrous tartrate - propyl gallate reaction product as follows:-

Twenty ml. of 1.25 percent aqueous ammonium acetate solution containing propyl gallate was placed in a test tube. To this was added 4 ml. of water and 1 ml. of ferrous tartrate reagent. After five minutes the absorption curve of the resulting coloured solution was measured relative to a reagent "blank", using a Beckman Model B spectrophotometer. The resulting data are given in Figure 1.

The results presented in Figure 1 show that the ferrous tartrate - propyl gallate colour exhibits a single broad absorption peak between 400 and 700 mμ with a maximum at 530 mμ. A similar absorption curve was prepared using a Coleman Universal Spectrophotometer, and again the maximum was found to be at 530 mμ. Because of the broad zone of near-maximum absorption, it was found that the absorbancy for the propyl gallate - ferrous tartrate colour using a No. 515 filter was 0.8 percent greater than that obtained

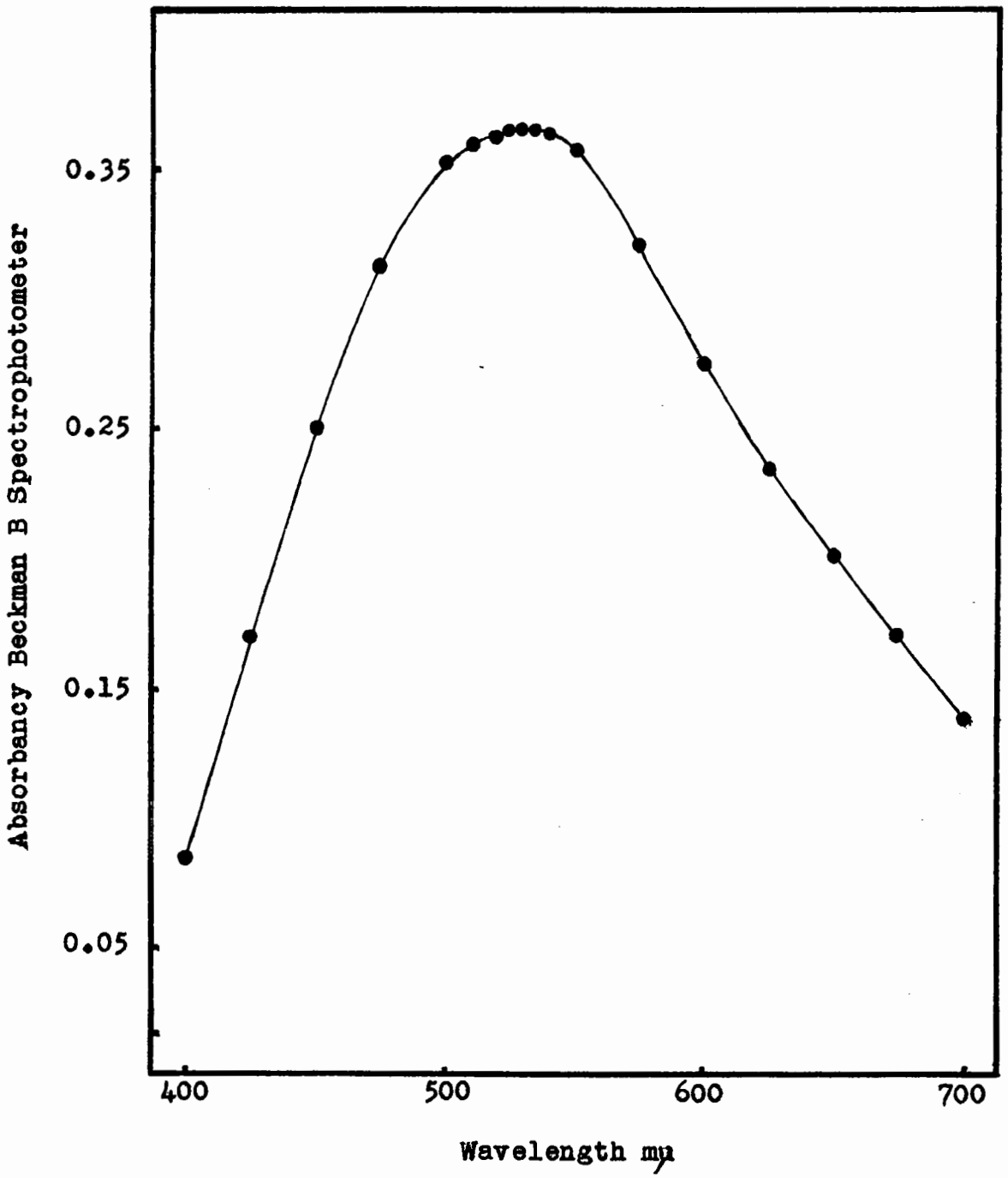


Figure 1 Propyl Gallate - Ferrous Tartrate Absorption Curve

employing a No. 540 filter in an Evelyn colorimeter. However, the ferrous tartrate "blank" gave an absorbancy of 0.006 with a No. 515 filter relative to water as compared to 0.001 when a No. 540 filter was employed. For this reason an Evelyn colorimeter fitted with a No. 540 filter was employed to measure the absorbancy of the ferrous tartrate - propyl gallate colour. When a spectrophotometer was used, absorbancies were measured at 530 mμ.

ii. Colour Stability

The ferrous tartrate - propyl gallate colour was developed in an Evelyn colorimeter tube as previously described. The resulting absorbancy was measured in an Evelyn Photoelectric colorimeter fitted with a No. 540 filter, relative to a "reagent blank" tube containing 20 ml. of 1.25 percent aqueous ammonium acetate, 4 ml. of water and 1 ml. of ferrous tartrate reagent. The tubes were stoppered and the absorbancy measured at intervals for 125 hours with the results given in Table IV.

Table IV shows that the ferrous tartrate reagent in the presence of 1 percent ammonium acetate buffer produced maximum colour intensity with propyl gallate within one minute. This maximum absorbancy remained constant for about fifteen minutes and then faded very slowly, at the rate of approximately 0.8 percent per twenty-four hours at room temperature. In the case of propyl gallate extracted

Table IV

Effect of Time Upon the Ferrous Tartrate - Propyl Gallate Colour

Time in Minutes	Absorbancy in Evelyn Colorimeter with 540 mμ Filter	
0.25	0.400	
0.5	0.418	
1	0.425	
2	0.425	
4	0.425	Maximum
8	0.425	
16	0.424	
32	0.423	
64	0.423	
128	0.422	
256	0.422	
1024	0.416	
2048	0.414	
4096	0.409	
8192	0.407	

from fat samples undergoing rancidification the rate of fading is more rapid and measurements should, therefore, always be made within fifteen minutes. The rapidity of colour formation and stability of the colour were additional factors in favour of the choice of the ferrous tartrate reagent for the analysis of propyl gallate

If the ferrous tartrate reagent was kept as much as forty-eight hours away from direct sunlight, the same maximum absorbancy was obtained. However, after five minutes in direct sunlight, the reagent turns colourless but the same maximum absorbancy was obtained. Next day this

reagent was brownish and absorbancies were 2.5 percent lower.

iii. Calibration Curve

Varying amounts of propyl gallate in 1.25 percent ammonium acetate solution were placed into Evelyn colorimeter tubes. The contents of each tube was diluted to 20 ml. with 1.25 percent ammonium acetate solution, 4 ml. of water and 1 ml. of ferrous tartrate reagent added. After ten minutes the absorbancy was measured in an Evelyn colorimeter fitted with a No. 540 filter relative to a reagent "blank" containing 20 ml. of 1.25 percent ammonium acetate solution, 4 ml. of water and 1 ml. of ferrous tartrate reagent. The resulting measurements are recorded in Table V.

Table V

Ferrous Tartrate - Propyl Gallate Calibration Curve

Propyl Gallate Micrograms	Absorbancy with a No. 540 Filter in Evelyn Colorimeter	Absorbancy/microgram of Propyl Gallate in 25 ml. "K-Value"
50	0.056	0.00112
100	0.112	0.00112
200	0.217	0.00109
300	0.334	0.00111
400	0.447	0.00112
500	0.564	0.00113
600	0.674	0.00112
700	0.796	0.00114
800	0.890	0.00111
900	0.963	0.00107
1000	1.037	0.00104

The results presented in Table V show that the propyl gallate - ferrous tartrate colour reaction obeys Beer's law over the range of 50 to 800 micrograms of propyl gallate per 25 ml. of solution, employing an Evelyn colorimeter with a No. 540 filter. The "K-value" was found to be 0.00112 per microgram of propyl gallate in 25 ml. using an Evelyn colorimeter. Employing a Coleman Universal Spectrophotometer with 40 m.m. absorption cells and a wavelength of 530 mμ it was found that Beer's law applied over the range of 20 to 360 micrograms of propyl gallate in 25 ml. of solution and that the "K-value" was 0.00223 per microgram.

4. ANALYTICAL METHODS

a. Reagents

Light Petroleum (3+1) - Mix 3 volumes of 60° - 100°C boiling range light petroleum (Skellysolve H) with 1 volume of 30° - 60°C light petroleum and shake this mixture with one-tenth its volume of concentrated sulphuric acid for five minutes. Run off the yellowish acid layer and wash the light petroleum repeatedly with water and then with 1 percent potassium hydroxide solution until free of acid. Distill the light petroleum, using an all-glass apparatus.

Ferrous Tartrate - 0.1 percent ferrous sulphate ($FeSO_4.7H_2O$) and 0.5 percent of Rochelle salt in distilled

water, freshly prepared.

Ammonium Acetate - 1.10, 1.25, 1.30, 1.67 and 10.0 percent aqueous solutions. Solutions containing 1.30 and 1.67 percent ammonium acetate in 5 percent ethyl alcohol solution may also be required.

Ammonium Hydroxide - concentrated ammonium hydroxide (28 - 29 percent NH_3) is required in the qualitative test for propyl gallate.

b. Qualitative Procedure

In order to avoid the need for conducting long extraction and analytical procedures only to find that the fat sample contained no propyl gallate, it was necessary to have a rapid, specific and sensitive qualitative test for propyl gallate. Experimentation provided a simple reagent that fulfilled these requirements. Propyl gallate in aqueous or alcoholic solution (not exceeding 80 percent ethyl alcohol by volume) was found to produce an intense transitory rose colour upon the addition of a small amount of ammonium hydroxide. Under these conditions gallic acid produces an intense orange colour. Thus, this simple test will differentiate propyl gallate from gallic acid. NDGA and gum guaiac give a pale yellow colour, while BHA, tocopherol, thiodipropionic acid and dilaurylthiopropionate produced no colour. Lauryl gallate (not permitted to be added to foods)

also gives an intense rose colour identical with that obtained with propyl gallate. However, since lauryl gallate is insoluble in water an alcoholic solution of lauryl gallate must be employed.

The following test was conducted to determine whether a fat contains propyl gallate:- Place 10 g. of the fat in a separatory funnel and dissolve the fat with 50 ml. of 3 + 1 light petroleum. Add 10 ml. of 72 percent ethyl alcohol and shake for two minutes. Allow the phases to separate, run off the alcoholic layer, filter and to the filtrate add 1 ml. of concentrated ammonium hydroxide. The appearance of a rose colour indicates the presence of propyl gallate, lauryl gallate or possibly other gallate esters. Less than one microgram of propyl gallate per ml. of solution can be detected by this test.

c. Quantitative Procedure

Dissolve 10 g. of fat with 50 ml. of purified light petroleum (3 + 1), in a 250 ml. separatory funnel. Extract the fat solution with three, 20 ml. volumes of aqueous 1.67 percent ammonium acetate solution by continuously inverting the separatory funnel for 2.5 minutes per extraction. Finally, extract the fat solution for one minute with 15 ml. of water. Allow time after each extraction for complete separation of the phases before running off the aqueous layer.

Combine the extracts which contain the propyl gallate, dilute to 80 ml. with water and filter. This solution now contains 1.25 percent of ammonium acetate. Pipette three different aliquots of the extract, not exceeding 20 ml. into 40 m.m. rectangular Coleman absorption cells and dilute all aliquots to 20 ml. with 1.25 percent ammonium acetate solution. Add 4 ml. of water and 1 ml. of fresh ferrous tartrate reagent to each cell. Stir the contents and after three minutes measure the absorbancy at 530 mμ in a Coleman Universal Spectrophotometer. Measure all absorbancies relative to a "blank" containing 20 ml. of 1.25 percent aqueous ammonium acetate solution, 4 ml. of water and 1 ml. of fresh ferrous tartrate reagent. If any visible turbidity is present in the extract, the appropriate correction is obtained by reading the absorbancy of the extract solution before adding the ferrous tartrate reagent relative to a water "blank". Any absorbancy measured, is multiplied by 24/25 and the appropriate fraction of this value is subtracted from the absorbancy measured after the addition of the ferrous tartrate reagent. The small amount of ferrous tartrate employed has no effect on the absorbancy at 530 mμ relative to a water "blank".

Place suitable portions (1 - 20 ml.) of a standard aqueous solution of propyl gallate (20 micrograms per ml.) in 40 m.m. absorption cells, add 2.5 ml. of 10. percent

aqueous ammonium acetate solution, dilute to 24 ml. with water, and add 1 ml. of ferrous tartrate reagent. Measure the absorbancy as previously described and plot a reference line over the range of 20 to 360 micrograms of propyl gallate and determine the "K-value" per microgram of propyl gallate.

To calculate the amount of propyl gallate in an unknown sample, divide the observed absorbancy by the "K-value" to obtain the concentration of propyl gallate in micrograms per aliquot taken for analysis.

In the case of certain shortenings, a strong tendency to emulsify was noted during the aqueous extraction of propyl gallate. To avoid this emulsification, add 1 ml. of capryl alcohol to the light petroleum - fat solution before beginning the extraction. Use a 1.67 percent ammonium acetate solution in 5 percent ethyl alcohol for the extraction in place of the usual aqueous ammonium acetate solution. Adopt this procedure only when the normal method fails. The use of capryl alcohol and 1.67 percent ammonium acetate in 5 percent ethyl alcohol yielded results of the same magnitude and accuracy as was obtained with the original method and the same "K-values" applied.

d. <u>More Sensitive Quantitative Procedure</u>

In the case of fat samples expected to contain less than 0.005 percent of propyl gallate, the following more

sensitive procedure has been developed. This procedure differs from the foregoing method as follows:-

Prepare the fat solution in light petroleum (3+ 1) as previously outlined. Extract this fat solution with three, 20 ml. portions of aqueous 1.3 percent ammonium acetate solution, extracting for three minutes each. Next, extract the fat solution for one minute with 8 ml. of water. Combine these four extracts, dilute to 70 ml. with water and filter. Pipette three different volumes of the extract, not exceeding 24 ml. into 40 m.m. Coleman absorption cells, dilute to 24 ml. with aqueous 1.1 percent ammonium acetate solution and add 1 ml. of ferrous tartrate reagent. Allow five minutes for maximum colour formation and measure the absorbancy as previously outlined. Employing this procedure, reasonably accurate results can be obtained for concentrations as low as 0.0014 percent propyl gallate in fat. At these low concentrations the correction for turbidity in the extract becomes very important, especially if the fat sample extracted is becoming rancid. This modified procedure is 1.37 times more sensitive than those previously described.

5. CHARACTERISTICS OF QUANTITATIVE METHODS

a. Recovery of Propyl Gallate from Fat

In order to obtain an accurate indication of the recovery of propyl gallate in lard or shortening employing the

foregoing procedures, it was necessary to prepare a fat, free of peroxides and other oxidizing materials. Oxidizing substances, if present in the fat, react with the propyl gallate and the result is lowered recovery.

Fat samples free from oxidizing materials were prepared by holding the fat containing approximately 0.05 percent of tocopherol at 60°C for ten minutes. If, on cooling, tocopherol was still present (see subsequent method) it was concluded that there was an excess of tocopherol over oxidizing materials. In the case of fresh shortenings there is usually natural tocopherol present and, therefore, less need be added. Four analyses of lard containing 0.01 percent of propyl gallate resulted in an average recovery of 98 percent of the propyl gallate added.

b. Reproducibility of Analytical Results

In order to ascertain the precision of the foregoing analytical procedures, six identical fat samples were analysed for their propyl gallate content, employing both variations of the extraction procedure. In every case the extract from a single sample was analysed at three concentration levels, and these results were averaged and reported as a single figure. The results of six such determinations by each method were analysed statistically and the results are shown in Table VI. In all cases the concentrations employed fall within the range normally to be expected

in foods.

Table VI

Reproducibility of the Methods for the Determination of Propyl Gallate

Extraction Procedure Employed	Mean of Six Determinations Percent	Standard Deviation	99 Percent Confidence Limits
Normal procedure	0.0104	0.00005	± 0.0002
Modified procedure employing capryl alcohol and 1.67% ammonium acetate in 5% ethyl alcohol	0.0068	0.00005	± 0.0002

These results indicate that the 99 percent confidence limits for a single determination of propyl gallate is ±2 to 3 percent of the amount present.

c. Analytical Range for Propyl Gallate Employing Five Different Colorimeters

Both the Canadian Food and Drug Regulations (17) and the American Meat Institute Foundation (122) limit the amount of BHA that can be added to "edible fat" to 0.02 percent by weight of the fat. It is, therefore, impossible to add more than 0.006 percent of propyl gallate employing an "AMIF-72-type" antioxidant formulation without exceeding the maximum amount of BHA permitted. Even so, a number of manufacturers add only half this amount of antioxidant to

their products, namely, 0.003 percent of propyl gallate, 0.01 percent of BHA and 0.002 percent of citric acid. Hence it was necessary to have an analytical procedure capable of determining less than 0.003 percent of propyl gallate in fat. This is especially necessary when conducting experiments on the disappearance of propyl gallate from fats or fatty foods during storage.

In order to determine the practical analytical range for propyl gallate using various instruments to measure the absorbancy; five commonly used spectrophotometers and colorimeters were investigated. Table VII gives the analytical range characteristic of each instrument employing the more sensitive method for propyl gallate.

Table VII

Analytical Range for Propyl Gallate Employing a Number of Instruments to Measure the Colour

Instrument	Width of Absorption Cell	Micrograms of Propyl Gallate/ 25 ml. to give Absorbancy of:-		Percent of Propyl Gallate per 10 g. fat corresponding to absorbancy of:-	
	m.m.	0.1	0.8	0.1	0.8
Beckman DU	10	175	1380	0.006%	0.031%
Beckman B	10	175	1380	0.006	0.031
Beckman B	50	35	280	0.001	0.007
Coleman 11	13	140	1120	0.004	0.026
Coleman 11	20	90	720	0.003	0.017
Coleman 11	40	45	360	0.0014	0.008
Coleman Junior	16 (tube)	115	920	0.004	0.021
Evelyn	20 (tube)	90	720	0.003	0.017

These results shows that only two of these instruments, namely, the Beckman Model B fitted with 50 m.m. cells and the Coleman 11 fitted with 40 m.m. cells can be used to analyse fat samples containing less than 0.03 percent of propyl gallate.

B. Butylated Hydroxyanisole; Method Using Ferric Chloride Plus α,α'-Bipyridine

1. INTRODUCTION

It has been demonstrated that the synthetic antioxidant known as butylated hydroxyanisole, also referred to as BHA, possesses very desirable properties (73). These include:- a high solubility in oils (73); a carry-through of its antioxidant properties into baked goods (8,73,129, 147); non-toxicity at the concentrations employed (147), as witnessed by its approval for use in foods (17,122); no colour, odour or taste imparted to the substrate to which the antioxidant is added at a concentration of 0.02 percent (125); low cost; and effectiveness at the low concentrations of 0.005 to 0.02 percent of the fat. It has been reported that BHA and mixtures containing this antioxidant more nearly meet the requirements of an ideal antioxidant than does any other compound studied (73).

BHA (125) is a trisubstituted benzene compound containing as substituents a tertiary-butyl group, a hydroxyl group, and a methoxy group. The antioxidant activity of BHA is not assured by the mere presence of these three substituents for it is essential that these groups be properly orientated in the ring. Rosenwald and Chenicek (125) investigated the relationship between structure and antioxidant activity for a number of hydroxyanisoles. These authors (125) evaluated

the antioxidant potencies of these substances by employing the Active Oxygen Method (69,123). All measurements were made employing a single lard with an initial stability of three hours. The relative potencies of the antioxidants tested were expressed as the "inhibitor ratio", namely:-

$$\text{Inhibitor Ratio} = \frac{L_x - L_c}{L_s - L_c}$$

where, L_x is the length of the induction period with the unknown, L_c the induction period of the pure lard, and L_s the induction period with the standard antioxidant at the same concentration as the unknown. Pure 3-tertiary-butyl-4-hydroxyanisole (3-BHA), was selected as the standard and was assigned an inhibitor ratio of 1.00.

Employing 0.02 percent of antioxidant in lard, Rosenwald and Chenicek (125) assigned inhibitor ratios of less than 0.1 to 2-hydroxyanisole (guaiacol), less than 0.1 to 3-hydroxyanisole, and 0.31 to 4-hydroxyanisole (methyl ether of hydroquinone). Methyl substitution of 4-hydroxyanisole increased its inhibitor ratio. These authors (125) assigned inhibitor ratios of 0.48 to 2-methyl-4-hydroxyanisole and 0.55 to 3-methyl-4-hydroxyanisole. Dimethyl, 3-n-propyl-, 3-allyl-, 3-isopropyl-, di-isopropyl-, 3-n-butyl-, and 3-iso-butyl-, substitutions of 4-hydroxyanisole produced no further increase in the inhibitor ratio. The inhibitor ratio of 3-secondary-butyl-4-hydroxyanisole was

0.60; for 2-tertiary-butyl-4-hydroxyanisole, 0.36; for 3-tertiary-butyl-4-hydroxyanisole, 1.00 the reference compound. Of the forty compounds tested only three exhibited inhibitor ratios greater than 1.0. These were 3-tertiary-butyl-5-methyl-4-hydroxyanisole, 1.10; monotertiary-butyl-hydroquinone, 2.65; and hydroquinone 3.1. Hydroquinone, although employed for a short period as an antioxidant in edible fats in the United States (95) is no longer permitted because of its high toxicity (96). Therefore, 3-BHA was judged to be the most satisfactory antioxidant in view of its relatively low order of toxicity and high antioxidant activity.

Commercial BHA is a mixture of the isomers, 2-BHA and 3-BHA (31) in which 3-BHA predominates (88). Since BHA is stable to heat and mild alkali (8) it is not destroyed during many cooking processes; and, therefore, extends the storage life of a variety of cooked foods (8,73,129,147).

2. TOXICITY OF BUTYLATED HYDROXYANISOLE

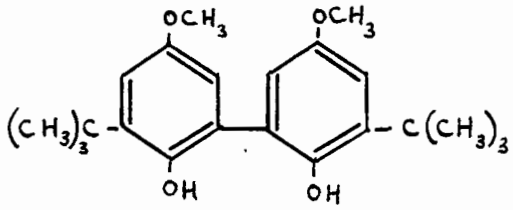
BHA is relatively non-toxic as indicated by the findings of Wilder and Kraybill (147), who reported that the LD-50 for BHA in acute toxicity tests was 4130 mg. per kg. of body weight when administered in corn oil to adult, non-fasted rats via stomach tube. The LD-50 for BHA was reported to be in excess of 5000 mg. per kg. when given to

adult fasted rats via stomach tube in the form of a water emulsion (147). Chronic toxicity studies in which rats were fed diets containing up to 2 percent of BHA for a period of six months resulted in no ill effects, except a lower rate of feed consumption (147). The hot peppery taste of the ration containing 2 percent of BHA was held responsible for the lowered feed intake. At the 2 percent level of BHA in the diet Wilder and Kraybill (147) calculate that the rats received over 1000 times as much BHA as they could eat if they consumed lard containing 0.02 percent of BHA, the maximum amount of BHA permitted (17,122). Also, each rat received more BHA per day than they would normally receive in a lifetime (147) of eating fats containing 0.02 percent BHA. Histological examination of the organs of the rats fed on a diet containing 2 percent BHA for six months showed no pathological conditions that could be attributed to this antioxidant. These and other published data (147) indicate a low order of toxicity for BHA fed to rats as judged by acute and chronic toxicity tests.

3. OXIDATION PRODUCTS

The question is often raised as to the fate of the antioxidant molecule as a result of functioning as an antioxidant. It is generally conceived that the antioxidant is oxidized during the stabilization process. Rosenwald and

Chenicek (125) attempted to determine any possible oxidation step which may be involved. For this purpose they subjected 3-BHA to mild oxidation by such reagents as potassium ferricyanide or oxygen in the presence of alkali. A colourless compound was formed, possessing a high melting point (225°C), low solubility and the carbon and hydrogen contents of a BHA dimer minus one mole of hydrogen. This oxidation product was believed to have the following structure:



(125)

This oxidation product still exhibited an inhibitor ratio of 0.5 at a concentration of 0.02 percent relative to 1.0 for the original 3-BHA. It was not established that this material actually is formed in fats during the inhibiting process and Rosenwald and Chenicek (125) considered it likely that other products may also be involved.

4. DEVELOPMENT OF ANALYTICAL METHOD

a. Extraction of Butylated Hydroxyanisole from Fat

Kraybill et al. (73) state that BHA is readily soluble in fats in all proportions, but nearly insoluble in water. These authors determined the solubility of BHA in water as 0.00154 percent at 25°C and 0.00165 percent at 70°C.

In addition these authors determined the extractability of BHA from lard with water by shaking 5 g. of lard containing 0.015 percent of BHA with 10 g. of water at 70°C for twenty-two hours and reported that the water failed to remove any measurable amount of BHA.

The foregoing findings of Kraybill et al. (73) indicated that the aqueous extraction of propyl gallate from fat - light petroleum solution should not remove any BHA. Experiments showed that if lard containing BHA was dissolved in light petroleum and extracted repeatedly with 1.67 percent aqueous ammonium acetate solution no BHA was present in the aqueous extract. Of course the extract was filtered to avoid possible entrainment of fat droplets containing BHA. Thus, it is possible to extract a light petroleum solution of a fat containing both propyl gallate and BHA with aqueous ammonium acetate solution while removing only the propyl gallate and leaving the BHA in the fat - light petroleum phase.

i. Effect of Ethyl Alcohol Concentration on the Recovery of Butylated Hydroxyanisole and Tocopherol from a Fat Solution

Lundberg and Halvorson (85) reported that a number of antioxidants with the exception of tocopherol could be quantitatively extracted from a light petroleum solution of fat with 80 percent ethyl alcohol. Investigations showed

that repeated extraction of a light petroleum solution of fat with 80 percent ethyl alcohol (by volume) quantitatively removed all propyl gallate, BHA and nordihydroguaiaretic acid (NDGA) present together with a proportion of the tocopherol.

Experiments were conducted in which pairs of 10 g. fat samples containing approximately 0.02 percent of BHA and approximately 0.18 percent of α-tocopherol were dissolved in 50 ml. of 3+1 light petroleum. These fat solutions were each extracted four times with 25 ml. aliquots of alcoholic solutions ranging from 40 to 90 percent ethyl alcohol by volume. Each sample was shaken for three minutes per extraction. The resulting alcoholic extracts were filtered and analysed for BHA and tocopherol respectively. The results of some of these experiments are given in Figure 2.

The results plotted in Figure 2 show that the amount of BHA extracted, increased as the concentration of alcohol in the extracting solution was increased. Above 75 percent ethyl alcohol no further increase in the recovery of BHA was obtained, therefore, the extraction secured with this concentration of alcohol was taken as 100 percent of BHA recovery. The results in Figure 2 show that four extractions with 72 percent ethyl alcohol recovered all of the BHA while leaving about 98.6 percent of the tocopherol behind in the light petroleum - fat solution. Therefore, a fat containing

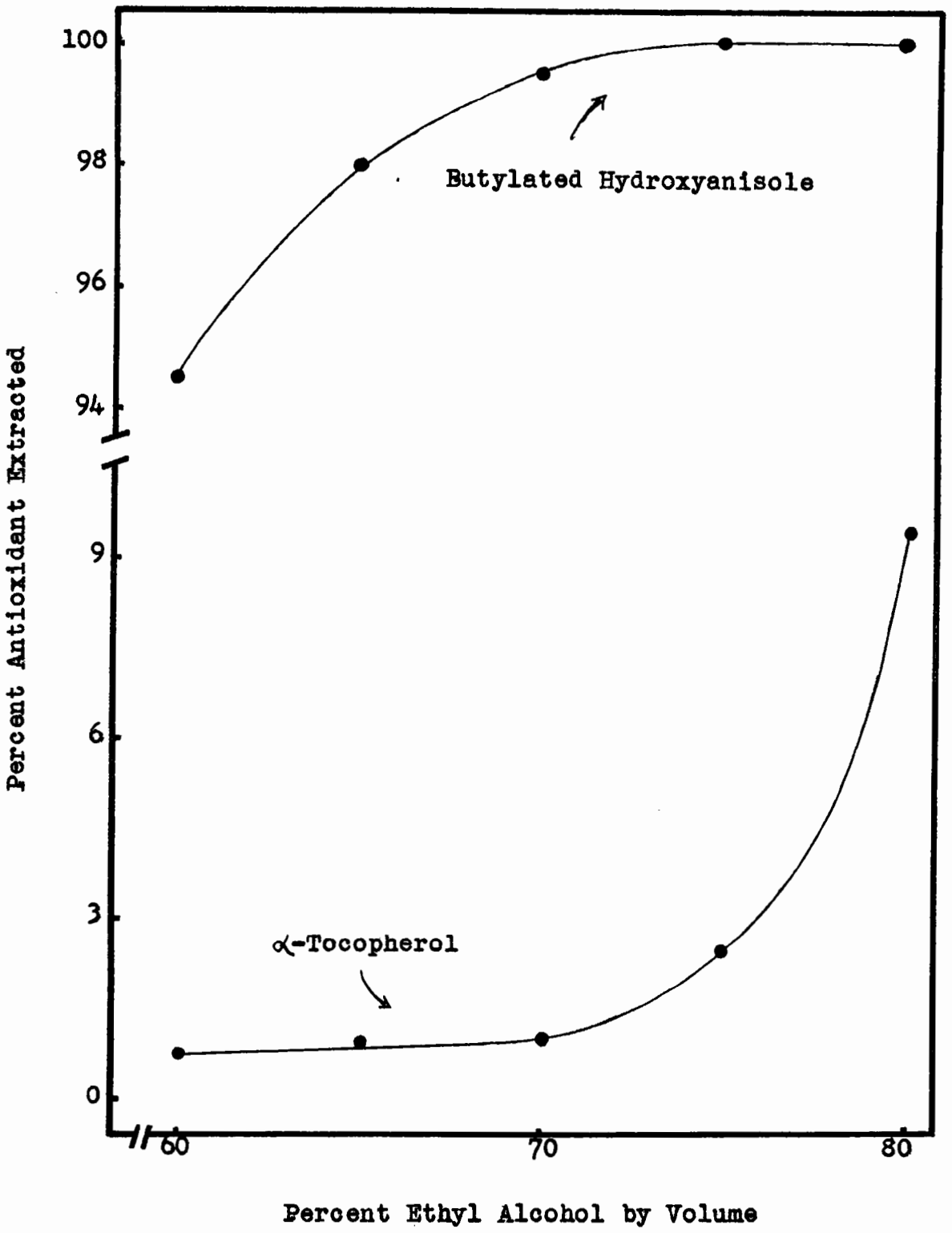


Figure 2 Effect of Alcohol Concentration Upon the Extraction of Butylated Hydroxyanisole and Tocopherol from Light Petroleum Solution of Fat

propyl gallate, BHA and tocopherol can be dissolved in light petroleum, the propyl gallate extracted with aqueous ammonium acetate, the BHA extracted next with 72 percent ethyl alcohol (by volume) while the tocopherol remains behind in the light petroleum together with the fat.

Because of the similar solubilities of propyl gallate and NDGA, these antioxidants cannot be separated when both are present in the same fat. However, since neither the Canadian Food and Drug Regulations (17) nor the American Meat Institute Foundation (122) permit the use of both propyl gallate and NDGA in the same sample, it is not likely that such a combination will be encountered. Again, whereas the use of BHA together with NDGA is permitted in Canada (17) and the United States (122), both these antioxidants are extracted by 72 percent ethyl alcohol.

ii. Effectiveness of Seventy-two Percent Ethyl Alcohol as Extractant of Butylated Hydroxyanisole from Lard in a Light Petroleum Solution

In order to determine the effectiveness of 72 percent ethyl alcohol as an extractant of BHA in lard, three 10 g. samples of lard containing approximately 0.02, 0.03 and 0.04 percent of BHA respectively, were dissolved in 50 ml. portions of 3+ 1 light petroleum. These solutions were extracted with three, four and five successive 25 ml. portions of 72 percent ethyl alcohol respectively, by continually inverting the separatory funnels for three minutes per

extraction. Each fat solution was then extracted with a further 75 ml. of 72 percent ethyl alcohol for one minute. Each of these alcoholic extracts was diluted separately to a convenient volume, filtered and analysed for BHA. Table VIII contains the results of these experiments.

Table VIII

BHA Recovered from Fat in Light Petroleum Solution by Successive Extractions with 72 percent Ethyl Alcohol

Duration of Extraction (Minutes)	Volume of 72% Ethyl Alcohol Employed (ml.)	Percent of Total BHA Extracted		
		0.02% BHA	0.03% BHA	0.04% BHA
3	25	41.9	34.9	30.1
3	25	32.3	27.2	24.3
3	25	20.6	19.9	20.7
3	25	-	14.1	12.6
3	25	-	-	9.4
1	75	3.9	2.7	1.8

The results in Table VIII show that four extractions recover over 98 percent of the BHA in the fat - light petroleum solution when less than 0.02 percent BHA is present in lard. If over 0.02 percent of BHA is present, five or more extractions will be required to recover 98 percent of the BHA.

b. Choice of Reagent Concentrations

It would seem that the only reagent previously employed for the determination of BHA was the ferric chloride plus

α,α'-bipyridine reagent originally described by Emmerie and Engel (35). Kraybill et al. (73) have described the use of an adaption of the Emmerie and Engel method for the colorimetric determination of BHA. Therefore, this reagent was the first to be considered for the determination of this compound in the present study. Lundberg and Halvorson (85) employed the Emmerie and Engel reaction for the colorimetric determinations of hydroquinone, guaiaretic acid, NDGA, gallic acid and propyl gallate. These authors reported that although this reagent is almost completely lacking in specificity, it is extremely useful when dealing with only one phenolic antioxidant whose identity is known. This reagent is applicable to a variety of phenolic antioxidants with no variation except in the length of the reaction time (85).

Since it was found that BHA reacts rather slowly with the ferric chloride plus α,α'-bipyridine reagent as employed by Lundberg and Halvorson (85), it was decided to investigate different amounts and relative proportions of these two reagents to determine whether the rate of reaction with BHA could be increased. For this purpose, 5 ml. portions of 72 percent ethyl alcohol containing 40 micrograms of BHA were placed in a number of opaque 25 ml. Erlenmeyer flasks. To these were added various volumes of 0.2 percent ferric chloride solution ($FeCl_3.6H_2O$) and 0.5 percent α,α'-bipyridine solution in absolute ethyl alcohol. In all cases the

contents of the flasks were diluted to 12 ml. with 100 percent ethyl alcohol. After exactly thirty minutes the content of each flask was transferred to a colorimeter tube and the absorbancy measured relative to the appropriate reagent "blank" held for thirty minutes employing an Evelyn colorimeter fitted with a No. 515 filter. The results of these experiments are given in Table IX.

Table IX

Effect of the Amount and Ratio of Ferric Chloride to α,α'-Bipyridine Upon the Absorbancy Formed with BHA in Thirty Minutes

Volume of 0.5% α,α'-Bipyridine	Absorbancy per Micrograms of BHA Measured After Thirty Minutes Reaction			
	Volume of 0.2% Ferric Chloride.$6H_2O$			
ml.	1	2	3	4 ml.
0.4	0.624	0.604	0.560	0.503
0.8	0.592	0.632	0.592	0.576
1.0	0.520	0.592	0.624	0.605
2.0	0.476	0.544	0.584	0.628

The results in Table IX revealed a striking symmetry. Namely, that essentially the same absorbancies were obtained for the combinations of ferric chloride plus α,α'-bipyridine that were represented along the diagonal from the top left corner to the bottom right corner of Table IX. Absorbancies ranging from 0.624 to 0.632 were obtained along this

principal diagonal. Apparently any of these combinations would have been equally satisfactory for the analysis of BHA. It is perhaps revealing to point out that the absorbancy of 0.628 (4 ml. 0.2 percent $FeCl_3$ + 2.0 ml. of 0.5 percent α,α'-bipyridine) was obtained by employing almost the exact concentrations and ratio of reagents as were originally employed by Lundberg and Halvorson (85). However, this reagent concentration was too great to be employed, as the "blank" was so strongly coloured as to render it impossible to determine the "centre setting" with an Evelyn colorimeter. Accordingly, a reagent consisting of 2 ml. of 0.2 percent ferric chloride ($FeCl_3.6H_2O$) together with 2 ml. of 0.2 percent α,α'-bipyridine (same as 0.8 ml. of 0.5 percent solution) was adopted for the analysis of BHA.

c. Characteristics of the Ferric Chloride - α,α'-Bipyridine-Butylated Hydroxyanisole Colour Reaction

i. Absorption Curve

Five ml. of 72 percent ethyl alcohol containing 40 micrograms of BHA were placed in a 25 ml. opaque Erlenmeyer flask and 3 ml. of 100 percent ethyl alcohol, 2 ml. of 0.2 percent ferric chloride and 2 ml. of 0.2 percent α,α'-bipyridine reagents added. After thirty minutes the absorption curve was determined relative to a reagent "blank", employing a Beckman B Spectrophotometer. The

absorption curve obtained is illustrated in Figure 3.

The results in Figure 3 indicate that the ferric chloride - α,α'-bipyridine - BHA colour exhibits a maximum absorption at 522 mμ. Because of the shape of the absorption curve a No. 515 or No. 520 filter gave the same absorbancy in an Evelyn colorimeter. A No. 515 filter was chosen for future measurements.

ii. Colour Stability

Since a mixture of ferric chloride plus α,α'-bipyridine in alcoholic solution is very photosensitive, it is necessary to protect this reagent from strong light. For this purpose 25 ml. glass-stoppered Erlenmeyer flasks were rendered impervious to light with black electric tape and the reaction with BHA conducted in these flasks.

Five ml. portions of 72 percent ethyl alcohol containing 40 micrograms of BHA were placed into each of a number of 25 ml. opaque flasks, followed by 3 ml. of 100 percent ethyl alcohol, 2 ml. of 0.2 percent ferric chloride and 2 ml. of 0.2 percent α,α'-bipyridine reagents. After different time intervals the resulting colour was measured in an Evelyn colorimeter fitted with a No. 515 filter relative to reagent "blanks" held for similar time intervals. Experiments were conducted with 2-BHA, 3-BHA and two commercial BHA preparations. The results of these experiments are given in Table X.

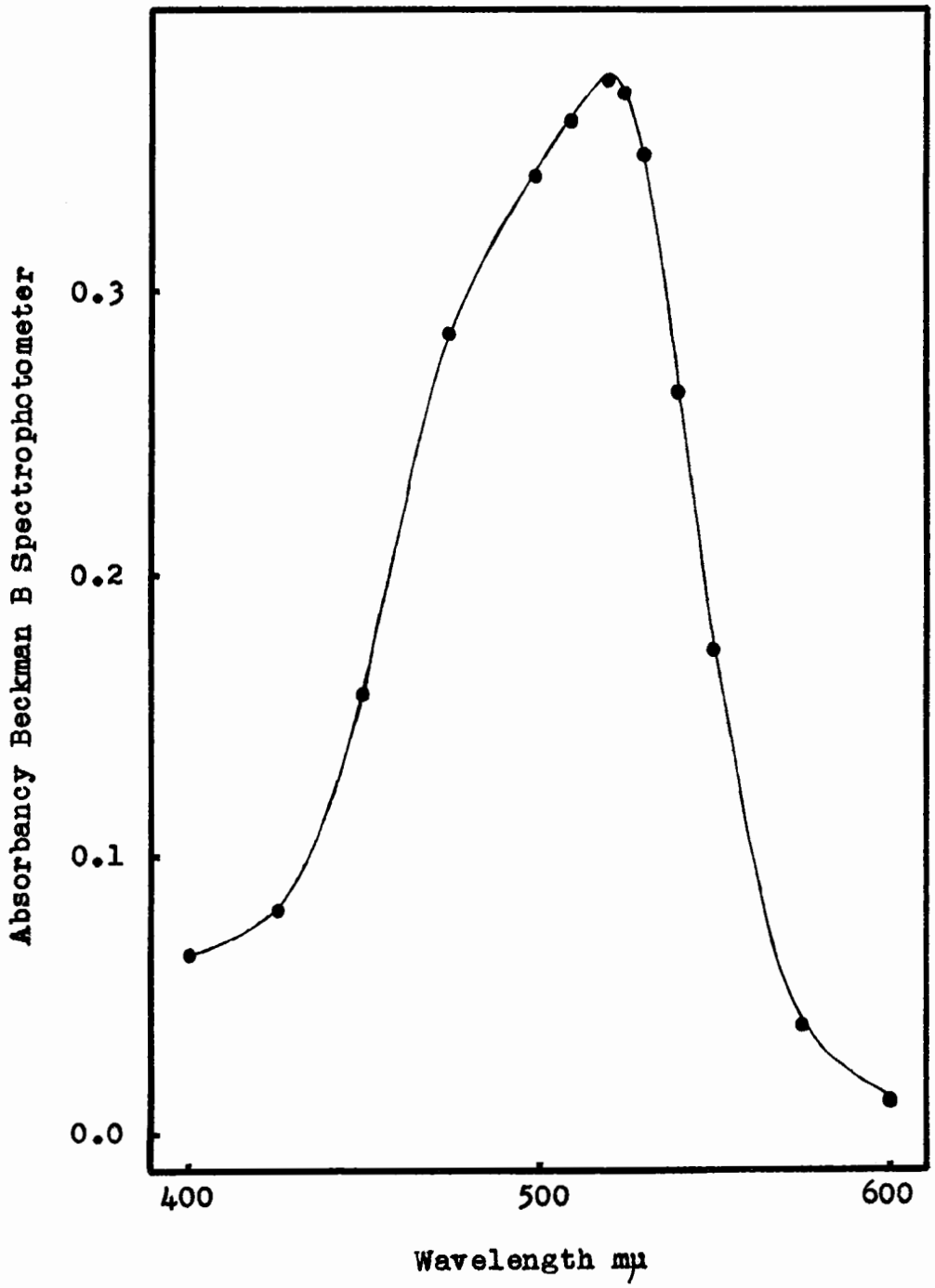


Figure 3 Absorption Curve of the Ferric Chloride - α,α'-Bipyridine - Butylated Hydroxyanisole Coloured Product

Table X

Effect of Time Upon the Absorbancy Produced by Commercial BHA, 2-BHA and 3-BHA on Reaction With the Ferric Chloride Plus α,α'-Bipyridine Reagent

BHA Employed	Absorbancy in Evelyn Colorimeter With a No. 515 Filter			
	Reaction Time in Minutes			
	15	30	45	60
Commercial BHA No.1	0.569	0.618	0.621	0.624
Commercial BHA No.2	0.552	0.624	0.626	0.629
Pure 2-BHA	0.604	0.698	0.710	0.714
Pure 3-BHA	0.608	0.610	0.615	0.621

Table X shows that 3-BHA reacts more rapidly with ferric chloride plus α,α'-bipyridine than 2-BHA, but that 2-BHA produces more colour per unit weight than 3-BHA. These results indicate that an effective maximum colour formation is obtained in fifteen minutes with 3-BHA, in forty minutes with 2-BHA and in approximately thirty minutes with the two commercial BHA preparations. Since it was found that commercial BHA preparations contain from 60 to 80 percent of 3-BHA, it was decided to employ thirty minutes for colour development between BHA and the ferric chloride plus α,α'-bipyridine reagent.

iii. Calibration Curves

The calibration curve for BHA on reaction with the ferric chloride plus α,α'-bipyridine reagent was

determined as follows:- Varying amounts of commercial BHA or pure BHA isomers contained in 72 percent ethyl alcohol were placed into 25 ml. (opaque) Erlenmeyer flasks. In all cases the BHA solution was diluted to 5.0 ml. with 72 percent ethyl alcohol. Then 3 ml. of 100 percent ethyl alcohol, 2 ml. of 0.2 percent ferric chloride and 2 ml. of 0.2 percent α,α'-bipyridine reagents were added to each flask. Exactly thirty minutes after adding the ferric chloride reagent the resulting absorbancies were measured relative to a reagent "blank". Measurements were made in an Evelyn colorimeter fitted with a No. 515 filter with the results given in Table XI.

Table XI

Calibration Curves for 2-BHA, 3-BHA and Commercial BHA Upon Reaction With Ferric Chloride Plus α,α'-Bipyridine Reagents

BHA Present	Absorbancy Measured in Evelyn Colorimeter With a No. 515 Filter		
Microgram	2-BHA	3-BHA	Commercial BHA
10	0.174	0.155	0.159
20	0.344	0.308	0.314
30	0.516	0.463	0.474
40	0.685	0.617	0.624
50	0.830	0.750	0.763

These demonstrate that the colour reaction of BHA with ferric chloride plus α,α'-bipyridine reagents obeys Beer's

Law over the range of 10 to 40 micrograms of BHA in 12 ml. of solution.

iv. Effect of the Proportion of 2-tertiary-butyl-4-hydroxyanisole and 3-tertiary-butyl-4-hydroxyanisole Isomers on the Absorbancy per Microgram, Employing the Ferric Chloride Plus α,α'-Bipyridine Reagents

The data in Table XI show that the "K-value", i.e. absorbancy per microgram in a volume of 12 ml., is 0.0154 for 3-BHA, 0.0172 for 2-BHA and 0.0158 for the commercial BHA tested. These differences prompted a further study of the relation between the "K-value" and the relative proportions of 2-BHA and 3-BHA in a mixture.

Accordingly, the absorbancy per microgram was determined for a series of samples comprising 2-BHA only, 3-BHA only and four mixtures of 2-BHA and 3-BHA in known proportions. This experiment was repeated six times. The results displayed close reproducibility and the average values are plotted in Figure 4. The scale of this Figure has been constructed so as to emphasize a peculiar feature of the results to wit that the absorbancy per microgram total BHA does not display a simple rectilinear or curvilinear relation to the composition of the BHA. The results strongly suggest that one linear relation holds over the range of 0 percent to 60 percent 2-BHA, while a second steeper relation holds over the range of 60 percent to 100 percent 2-BHA. No reason for these remarkable facts has

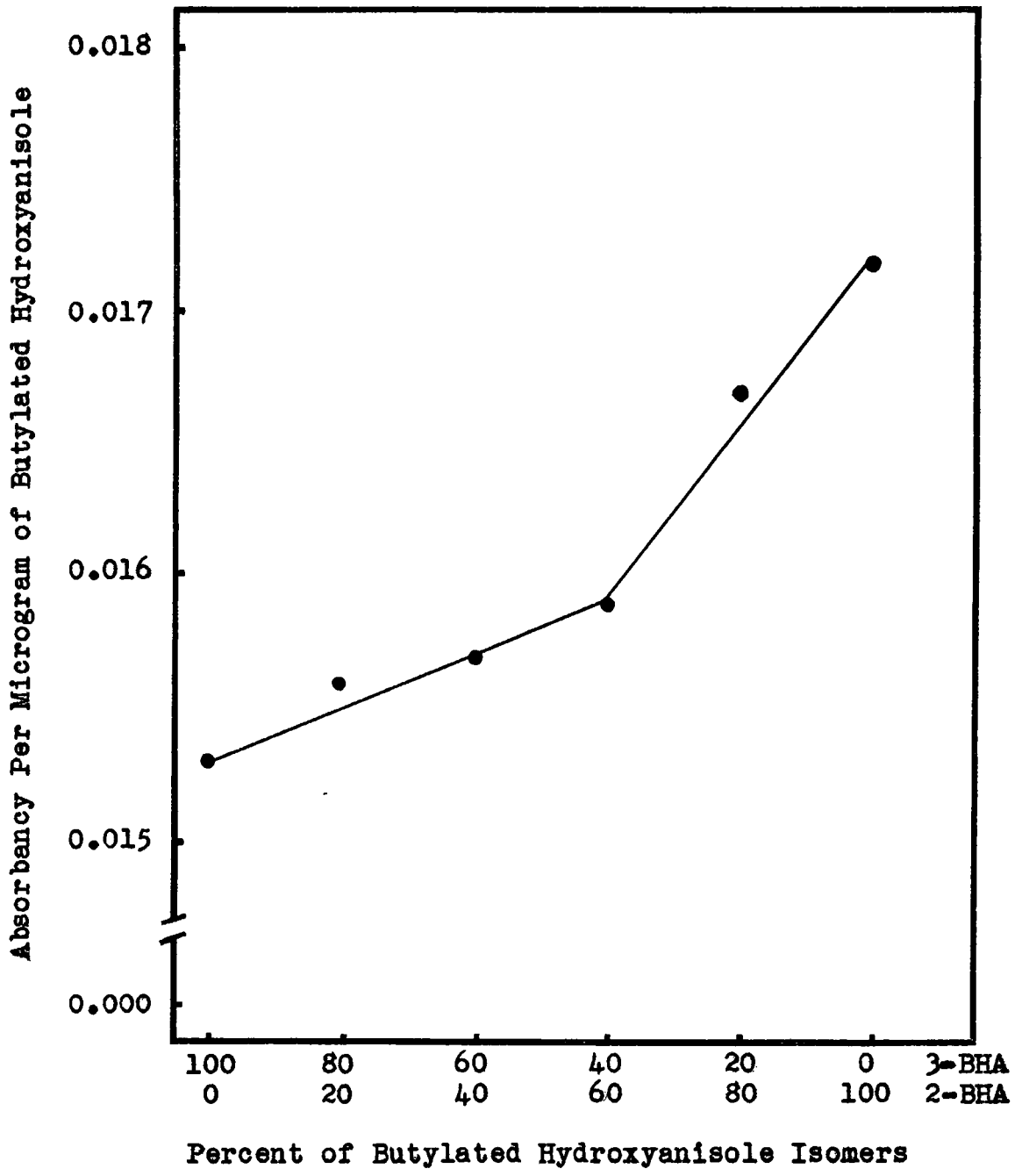


Figure 4 Effect of the Proportion of 2-Tertiary-butyl-4-hydroxyanisole and 3-Tertiary-butyl-4-hydroxyanisole Upon the Absorbancy per Micrograms Employing the Ferric Chloride - α,α'-Bipyridine Reagents

been found, but there is very little doubt as to the reality of the relationships depicted in Figure 4. The reproducibility of the results was such as to preclude the possibility of the relationships being due to fortuitous experimental variations. The slope of the curve over the range of 0 percent to 60 percent 2-BHA is greatly exaggerated by the intentional construction of the axes. From the standpoint of the determination of total BHA in commercial mixtures, little error is introduced by the use of an average "K-value" of 0.0156 since commercial BHA contains from 10 to 40 percent of the 2-BHA. The extreme range of "K-values" for such mixtures is approximately 0.0154 to 0.0157.

5. ANALYTICAL METHOD

a. Reagents

Light Petroleum (3 + 1) - As described for propyl gallate, page 35.

Ethyl Alcohol - Add approximately 0.1 percent of potassium hydroxide and 0.1 percent potassium permanganate to absolute ethyl alcohol. Distil in an all-glass apparatus. The distillate is used for 100 percent ethyl alcohol and is diluted to 72 percent alcohol by volume (Sp. gr. 0.8818 at 20°C) with water and used for the extraction of BHA.

Ferric Chloride - 0.2 percent ferric chloride

($FeCl_3.6H_2O$) in purified 100 percent ethyl alcohol. This reagent should be freshly prepared to avoid highly coloured blanks. In addition, this reagent should be kept in an opaque glass-stoppered bottle while being used.

<u>α,α'-Bipyridine</u> - 0.2 percent α,α'-bipyridine in 100 percent purified alcohol.

<u>2,6-Dichloroquinonechloroimide</u> - 0.01 percent of 2,6-dichloroquinonechloroimide in 100 percent ethyl alcohol. Prepare each week.

<u>Borax</u> - 2.0 percent borax ($Na_2B_4O_7.10H_2O$) in water.

b. <u>Qualitative Procedure</u>

Gibbs (47) employed 2,6-dibromoquinonechloroimide or 2,6-dichloroquinonechloroimide in the presence of a borax buffer for the analysis of phenol in river water. Since this is an extremely sensitive reagent, which is also specific for BHA among the commonly employed food anti-oxidants, it was used as a qualitative test for BHA in fat.

Place approximately 10 g. of the fat in a separatory funnel and dissolve the fat with 50 ml. of 3+1 light petroleum. Extract this solution for three minutes with 10 ml. of 72 percent ethyl alcohol, run off the alcoholic phase and filter. Add 0.5 ml. of 0.01 percent 2,6-dichloroquinone-chloroimide reagent and 1 ml. of 2 percent borax reagent to 5 ml. of the alcoholic extract. The appearance of a blue colour within fifteen minutes indicates the presence of BHA.

A mixture of the 2,6-dichloroquinonechloroimide and borax reagents produce a purplish colour which must not be confused with the blue colour produced if BHA is present. Only gum guaiac, among permitted food antioxidants, produces a blue colour. However, gum guaiac produces a blue colour before the addition of the borax buffer, while BHA produces no colour with the 2,6-dichloroquinonechloroimide reagent until the borax buffer (pH 9.4) is added; hence it is possible to differentiate between BHA and gum guaiac.

c. Quantitative Procedure

If propyl gallate is present, it must be extracted employing 1.67 percent aqueous ammonium acetate solution as previously described, before proceeding to the extraction of BHA from the remaining solution of fat in light petroleum. If the qualitative test for propyl gallate is negative, dissolve 10 g. of the fat in 50 ml. of 3 + 1 light petroleum in a 250 ml. separatory flask. Extract the BHA with three 25 ml. portions of 72 percent ethyl alcohol by continually inverting the separatory funnel for three minutes per extraction. Carry out a further extraction of the fat solution employing 60 ml. of 72 percent ethyl alcohol. If more than 0.02 percent BHA is expected in the sample, additional extractions may be required (see Table XI). Allow time after each extraction for complete separation of the phases.

Combine the extracts and make up to 150 to 300 ml. with 72 percent ethyl alcohol, depending upon the concentration of BHA expected and filter.

Place three different portions of the BHA extract, varying from 1 to 5 ml., into 25 ml. Erlenmeyer flasks provided with glass stoppers. These flasks must be rendered impervious to light with black electric tape, owing to the effect of light upon the reagents. Dilute the portions in all flasks to 5 ml. with 72 percent ethyl alcohol. Add 3 ml. of 100 percent ethyl alcohol, 2 ml. of 0.2 percent ferric chloride reagent, and 2 ml. of 0.2 percent α,α'-bipyridine reagent to each flask. Immediately stopper the flasks and gently swirl the contents. Thirty minutes after adding the ferric chloride reagent, transfer the coloured solutions to Evelyn colorimeter tubes and immediately measure the absorbancy with an Evelyn photoelectric colorimeter using a No. 515 filter. Make all measurements relative to a "blank" containing 5 ml. of 72 percent ethyl alcohol, 3 ml. of 100 percent ethyl alcohol, 2 ml. of 0.2 percent ferric chloride reagent, and 2 ml. of 0.2 percent α,α'-bipyridine reagent and held for thirty minutes.

Prepare a reference curve over the range of 8 to 40 micrograms of commercial BHA per flask. For general purposes employing an Evelyn colorimeter fitted with a No. 515 filter it was found that the absorbancy divided by a "K-value"

of 0.0157 yielded the concentration of BHA in micrograms per portion employed. However, a reference curve should be prepared at intervals to check the "K-value".

6. RECOVERY OF BUTYLATED HYDROXYANISOLE AND REPRODUCIBILITY OF THE ANALYTICAL RESULTS

To determine the recovery of BHA and the reproducibility of the foregoing analytical method, six identical fat samples containing added BHA were analysed for their BHA content. In all cases the extract solution from a single sample was analysed at three different concentrations, the results averaged and reported as a single figure. Another six fat samples containing propyl gallate and BHA were first extracted with 1.67 percent aqueous ammonium acetate solution to remove the propyl gallate. The remaining fat solution was extracted with 72 percent ethyl alcohol and this extract filtered and analysed for BHA. The results were analysed statistically and the results are reported in Table XII.

The results in Table XII show that approximately 97 percent of the BHA is recovered. The 3 percent loss of BHA may be due to incomplete extraction or to destruction of BHA in the fat. The 99 percent confidence limits for a single determination of BHA ranges from ± 0.5 to 5.0 percent of the BHA present.

Table XII

Recovery of Butylated Hydroxyanisole and Reproducibility of the Analytical Results

Treatment	BHA Added to Fat %	Recovery of BHA %	Standard Deviation	99 Percent Confidence Limits
BHA	0.02	97.4	0.00025	± 0.0010
BHA after Propyl Gallate Extraction	0.02	96.9	0.00003	± 0.0001

Butylated Hydroxyanisole; Method Using 2,6-Dichloroquinonechloroimide-Borax

1. INTRODUCTION

The ferric chloride plus α,α'-bipyridine reagent has been applied satisfactorily to the determination of butylated hydroxyanisole (BHA), but this reagent mixture is unstable to light in the presence of ethyl alcohol and is not specific for BHA. Accordingly, a further search for a possible reagent which did not exhibit these disadvantages was undertaken.

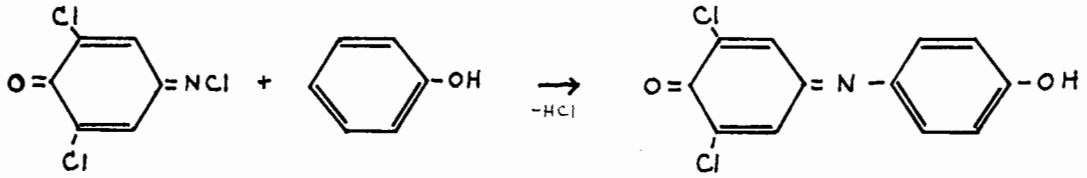
Gibbs (47) reported that 2,6-dichloroquinonechloroimide and 2,6-dibromoquinonechloroimide in the presence of alkaline buffer were sensitive reagents for phenol and that the resulting blue indophenol colour was stable. Gibbs (47) also reported that the reaction between phenol and the quinonechloroimides proceeded at an optimum rate at pH 9.4.

Investigation of Gibbs' reagent showed that it was specific for BHA among the antioxidants permitted for use in edible fats. Furthermore, the resulting coloured complex was not affected by light and was virtually stable. Therefore, Gibbs' reagent was investigated for possible application to the determination of BHA

2. DEVELOPMENT OF ANALYTICAL METHOD

a. Choice of Concentrations of Reagents

Gibbs (47) states that 2,6-dichloroquinonechloroimide reacted with phenol according to the following reaction:-



Since this reaction is analogous to that visualized for BHA, it would appear that the pH of the medium should exercise a profound effect upon the rate of such a reaction. To determine the effect of pH on the reaction between BHA and 2,6-dichloroquinonechloroimide, the following experiment was conducted.

Twelve ml. portions of 72 percent ethyl alcohol containing 40 micrograms of commercial BHA were placed into a number of Evelyn colorimeter tubes. To each tube was added 2 ml. of 0.01 percent 2,6-dichloroquinonechloroimide in 100 percent ethyl alcohol and 2 ml. of aqueous buffer solutions ranging from pH 6.8 to 10.0. The resulting absorbancies were measured after twenty minutes, in an Evelyn colorimeter fitted with a No. 620 filter relative to appropriate reagent "blanks". The resulting data are given in Figure 5.

Figure 5 shows that maximum colour formation is obtained over the pH range of 9.2 to 9.8. Also, no measurable

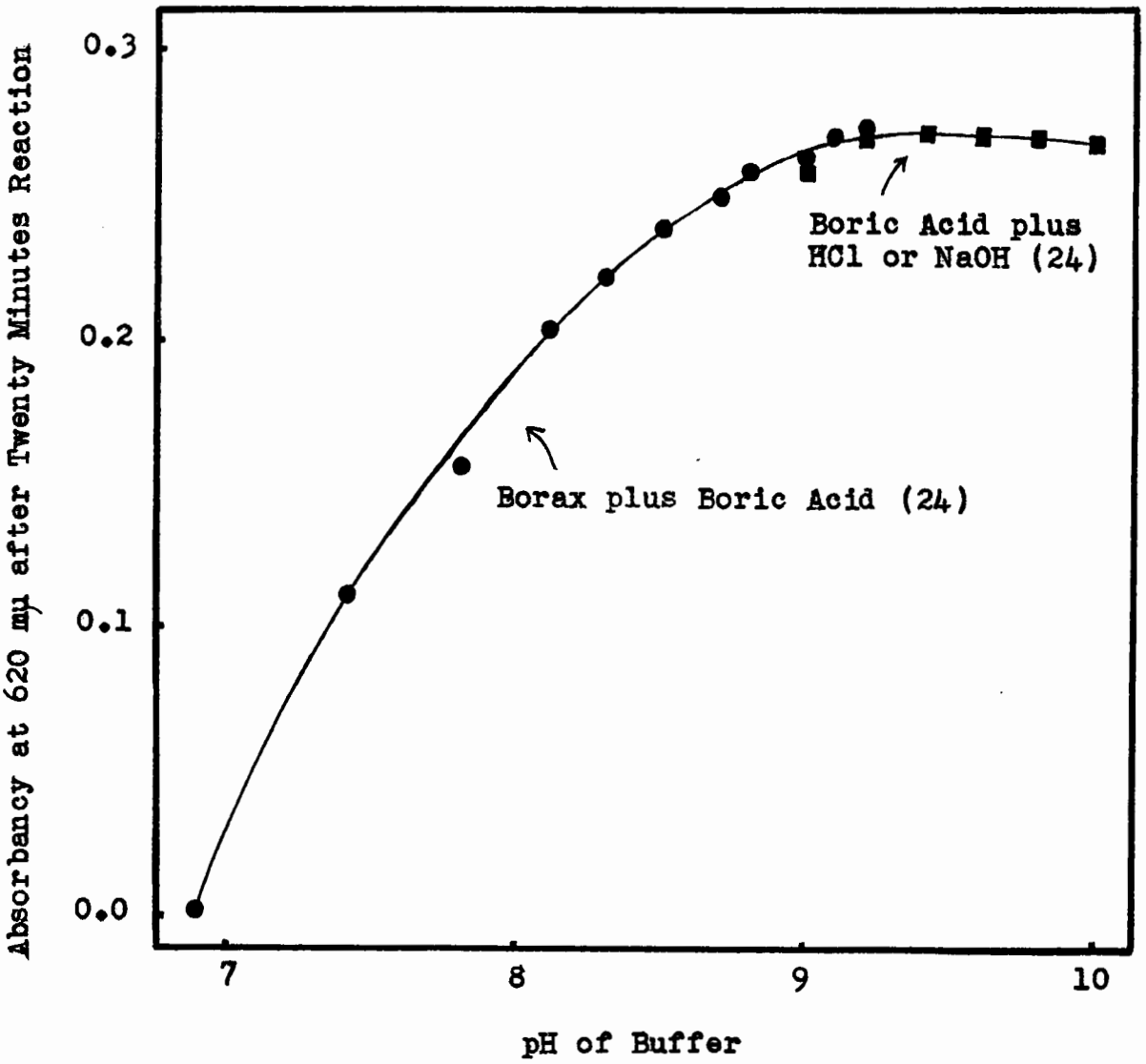
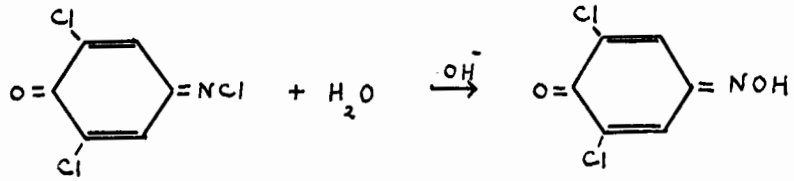


Figure 5 Effect of pH on the Colour Reaction Between Butylated Hydroxyanisole and the 2,6-Dichloroquinonechloroimide Reagent

absorbancy was produced at pH 6.8 in twenty minutes indicating that an alkaline pH is essential for this reaction with BHA to take place. The use of 2 ml. of aqueous 1.91 percent borax ($Na_2B_4O_7.10H_2O$) solution (pH 9.2) produced maximum colour formation. Therefore, to facilitate ease of preparing this reagent 2 ml. of aqueous 2.0 percent borax solution was employed to buffer the reaction medium at approximately pH 9.2.

According to Gibbs' equation, 2 ml. of 0.01 percent 2,6-dichloroquinonechloroimide contains sufficient reagent to react with approximately 170 micrograms of BHA. However, the 2,6-dichloroquinonechloroimide reagent undergoes hydrolysis on contact with the borax buffer producing a pale purple-brown colour which is found in the "blank". This colour is believed to be due to the following reaction:-



Because of this side reaction, 2 ml. of 0.01 percent 2,6-dichloroquinonechloroimide will probably react with less than the theoretical maximum of 170 micrograms of BHA. Therefore, if over 100 micrograms of BHA is present in an aliquot, it will be necessary to increase the concentration of the 2,6-dichloroquinonechloroimide reagent employed or take a smaller aliquot and dilute to the appropriate volume.

b. Characteristics of the Colour Reaction of 2,6-Dichloroquinonechloroimide with Butylated Hydroxyanisole in Borate Buffer

i. Absorption Curves

The absorption curves for the pure butylated hydroxyanisole isomers upon reaction with the 2,6-dichloroquinonechloroimide-borax reagent was determined as follows:- Two ml. of 0.01 percent 2,6-dichloroquinonechloroimide reagent and 2 ml. of 2 percent aqueous borax were added to 45 micrograms of 3-tertiary-butyl-4-hydroxyanisole (3-BHA) contained in 12 ml. of 72 percent ethyl alcohol. A similar experiment was conducted employing 40 micrograms of 2-tertiary-butyl-4-hydroxyanisole (2-BHA). After twenty minutes the absorption curves were determined employing a Beckman B spectrophotometer fitted with 1 cm. absorption cells. The resulting absorption curves are illustrated in Figure 6.

The absorption curves show that 3-BHA upon reaction with the 2,6-dichloroquinonechloroimide plus borax reagents, exhibits a maximum absorption at 614 mμ and that the maximum for 2-BHA is at 655 mμ. As previously stated, 3-BHA is the predominant isomer in commercial BHA preparations, it was decided to employ a wavelength of 620 mμ to measure the absorbancies due to commercial BHA.

ii. Colour Stability

The effect of time on the colour produced by

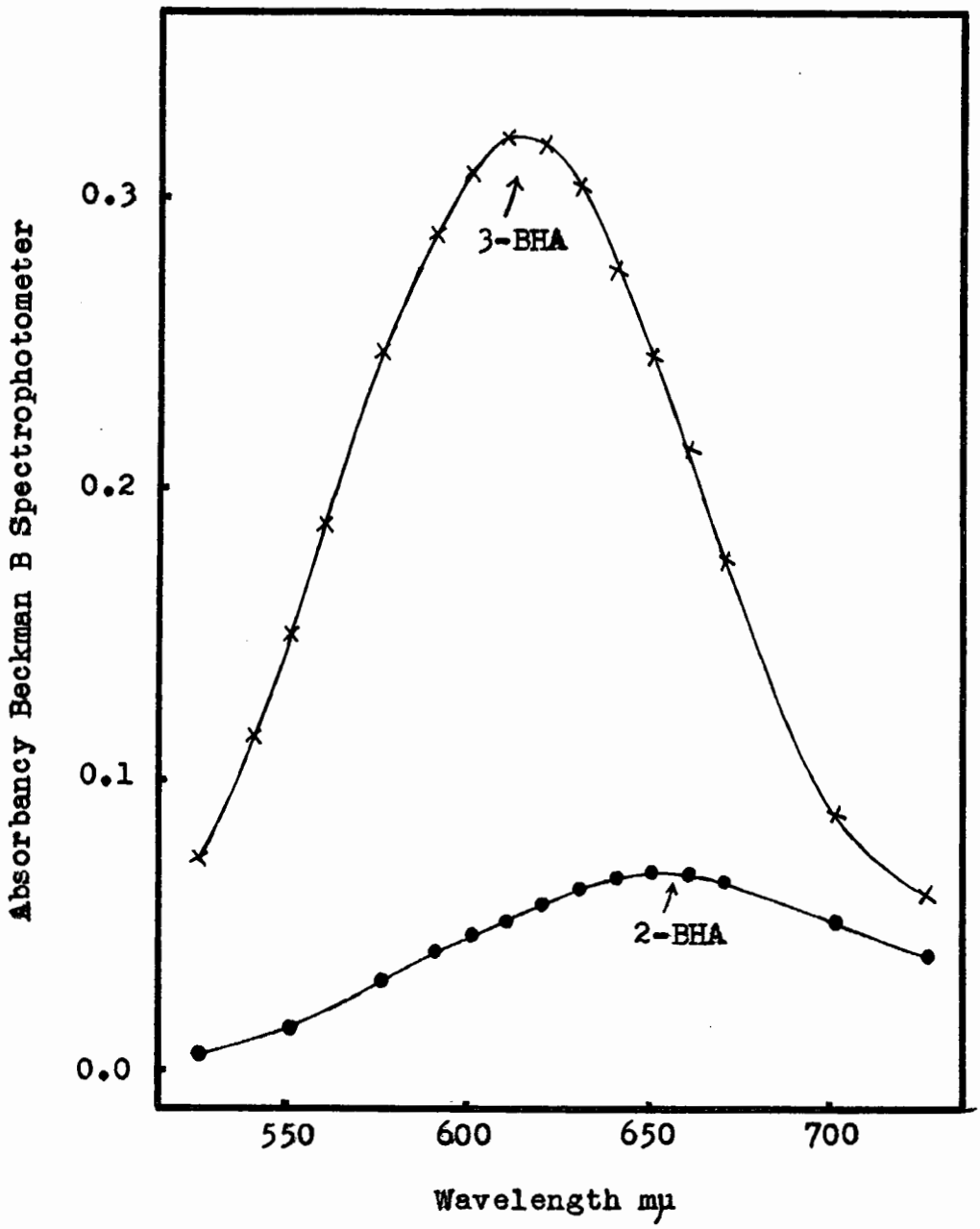


Figure 6 Absorption Curves for 2-Tertiary-butyl-4-hydroxyanisole and 3-Tertiary-butyl-4-hydroxyanisole Upon Reaction With the 2,6-Dichloroquinonechloroimide - Borax Reagents

butylated hydroxyanisole upon reaction with the 2,6-dichloroquinonechloroimide-borax reagent was investigated. Two ml. of the 2,6-dichloroquinonechloroimide reagent and 2 ml. of the borax reagent were added to 40 micrograms of commercial BHA contained in 12 ml. of 72 percent ethyl alcohol solution. The absorbancy of the resulting blue solution was measured at intervals in an Evelyn colorimeter fitted with a No. 620 filter relative to a reagent "blank". All colorimeter tubes were tightly stoppered to avoid evaporation of the alcohol. This experiment was also repeated employing 120 micrograms of pure 2-BHA and 40 micrograms of pure 3-BHA. The results of these experiments are given in Table XIII.

Table XIII

Effect of Time on the BHA Colour Reaction with the 2,6-Dichloroquinonechloroimide-Borax Reagents

Time in Minutes	Absorbancies Measured at 620 mμ		
	Commercial BHA 40 Micrograms	2-BHA, 120 Micrograms	3-BHA, 40 Micrograms
1	0.310	0.162	0.424
2	0.367	0.227	0.496
4	0.426	0.275	0.523
8	0.458	0.306	0.544
16	0.479	0.317	0.551
32	0.481	0.319	0.552
64	0.482	0.320	0.552
128	0.482	0.320	0.552
256	0.482	0.319	0.550
512	0.471	-	-
1024	0.467	-	-
2048	0.459	-	-
4096	0.435	-	-

The results show that the colour reaction between BHA and 2,6-dichloroquinonechloroimide attains an effective maximum in twenty minutes and thereafter remains essentially constant for four hours. After four hours the rate of colour fading was approximately 4 percent per day. Therefore, twenty minutes was allowed for complete colour development.

iii. Specificity

A number of antioxidant materials were treated with the 2,6-dichloroquinonechloroimide reagent as follows:- Twelve ml. portions of 72 percent ethyl alcohol containing equal weights of a number of antioxidants were reacted with 2 ml. of 0.01 percent 2,6-dichloroquinonechloroimide reagent and 2 ml. of 2 percent borax reagent. After twenty minutes the absorbancies were measured with an Evelyn colorimeter fitted with a No. 620 filter relative to a reagent "blank". In addition the wave length of maximum absorption was determined for each antioxidant relative to the "blank" employing a Beckman B spectrophotometer. The results of these experiments are summarized in Table XIV.

Of the materials studied in Table XIV, gum guaiac exhibited a maximum absorbancy at a wavelength close to that given by BHA. Although gum guaiac is permitted in edible fats (17,122), it is no longer employed commercially to any appreciable extent. In any event, the presence of gum guaiac is easily detected because it produces an instant

Table XIV

Characteristics of the Reaction Between the 2,6-Dichloroquinonechloroimide-Borax Reagent and Different Antioxidants

Antioxidant	Wavelength of Maximum Absorbancy mμ	Absorbancy/ Microgram at 620 mμ	Relative Absorbancy
Pure 3-BHA	616	0.0139	100.0
Commercial BHA No. 1	616	0.0127	91.4
Commercial BHA No. 2	618	0.0092	66.2
Pure 2-BHA	655	0.0027	19.4
Hydroquinone	460	0.0020	14.4
Gum Guaiac	635	0.0014	10.0
NDGA	430	0.0003	2.2
Propyl Gallate	435	0.0002	1.4
Lauryl Gallate	440	0.0001	0.7
Dithiopropionic Acid	No colour	0.0000	0.0
Dilauryldithiopropionate	No colour	0.0000	0.0
α-Tocopherol	No colour	0.0000	0.0

blue colour with the 2,6-dichloroquinonechloroimide reagent before the addition of the borax buffer. Hydroquinone, NDGA, propyl gallate and lauryl gallate exhibit maximum absorbancies between 430 and 460 mμ. These latter antioxidants can introduce small errors in the determination of BHA, because of their absorbancies at 620 mμ. Hydroquinone, which produces the largest error, is no longer permitted in edible fats (96). Of the remaining antioxidants, only propyl gallate, NDGA and tocopherol are permitted to be added to edible fats in Canada (17); of these, tocopherol produces no colour, while propyl gallate or NDGA would only cause a small

error in the determination of BHA.

One important fact to be noted here is that 3-BHA yields approximately five times as great an absorbancy per unit weight at 620 mμ as does 2-BHA. Here it is necessary to use as a standard the precise BHA preparation used as antioxidant if the amount of BHA present is to be estimated with adequate precision. The processor of edible oils will normally have access to the antioxidant preparation actually used, and can, therefore, use this procedure for industrial control purposes. The procedure has the advantage of being much easier to perform than the ferric chloride plus α,α'-bipyridine reaction.

iv Calibration Curves

The reaction between BHA and the 2,6-dichloroquinonechloroimide-borax reagent obeys Beer's Law within certain limits. Tables XV and XVI gives calibration data obtained for pure 2-BHA and 3-BHA.

It will be noted that the colour reaction with 3-BHA obeys Beer's Law up to 50 micrograms per 16 ml. of solution; for the 2-BHA isomer, Beer's Law applies up to 100 micrograms per 16 ml. of solution. If the concentration of the 2,6-dichloroquinonechloroimide reagent is increased, Beer's Law will apply above 100 micrograms of 2-BHA per 16 ml. of solution.

Table XV

Calibration Data for 3-BHA Upon Reaction With 2,6-Dichloroquinonechloroimide-Borax Reagent

Micrograms of 3-BHA	Absorbancy at 620 mμ	Absorbancy/ Microgram "K-Value"
10	0.140	0.0140
20	0.278	0.0139
30	0.419	0.0140
40	0.556	0.0139
50	0.689	0.0140
60	0.811	0.0135

Table XVI

Calibration Data for 2-BHA Upon Reaction With 2,6-Dichloroquinonechloroimide-Borax Reagent

Micrograms of 2-BHA	Absorbancy at 620 mμ	Absorbancy/Microgram "K-Value"
30	0.081	0.0027
60	0.164	0.0027
90	0.242	0.0027
120	0.307	0.0026 *
150	0.341	0.0023 *

* Low due to apparent lack of 2,6-dichloroquinonechloroimide reagent.

v. Effect of Propyl Gallate or Nordihydroguaiaretic Acid on the Analysis for Butylated Hydroxyanisole

Only three antioxidants, namely, propyl gallate, NDGA and tocopherol are permitted for use in combination

with BHA in Canada (17) and in the United States (122). Although 0.01 percent of NDGA or propyl gallate together with 0.02 percent BHA is permitted in the United States (122), 0.01 percent propyl gallate together with 0.02 percent BHA or 0.005 percent NDGA with 0.02 percent BHA are permitted in Canada (17). Actually, current industrial practice is to employ 0.003 percent propyl gallate or 0.001 percent NDGA together with 0.01 percent of BHA. In any case, one might expect to find propyl gallate equal to 30 percent or NDGA equal to 10 percent of the weight of BHA present. As previously stated, tocopherol produces no colour with this reagent and, therefore, cannot introduce any error.

In order to determine the effects of different proportions of propyl gallate or NDGA on the analysis of BHA the following experiments were conducted. BHA calibration curves were prepared over the range of 10 to 40 micrograms per 16 ml. and other calibration curves were prepared for BHA together with different proportions of propyl gallate or NDGA. The effect of the presence of propyl gallate or NDGA on the analysis of BHA is given in Table XVII.

The results in Table XVII show that the presence of propyl gallate equal to 30 percent of the BHA (the proportion frequently employed), results in an increase of 2.9 to 4.3 percent in the absorbancy measured at 620 mμ. Similarly, the presence of NDGA equal to 10 percent of the BHA, (the

Table XVII

Effect of Various Proportions of Propyl Gallate or NDGA on the Determination of BHA

Antioxidants Present	Relative Absorbancy Obtained at 620 mμ	
	Commercial BHA No.1	Commercial BHA No.2
BHA (10-40 Microgram Range)	100.0	100.0
BHA + 30% Propyl Gallate	102.9	104.3
BHA + 50% Propyl Gallate	103.3	105.5
BHA + 10% NDGA	103.2	104.1
BHA + 25% NDGA	104.1	106.2
BHA + 50% NDGA	105.3	108.0

proportion frequently employed), results in an increase of 3.2 to 4.1 percent in the absorbancy measured at 620 mμ. However, if the same BHA preparation containing the propyl gallate or NDGA was used in the construction of the calibration curve, no error would result from this source. Therefore, there is no need to extract propyl gallate and BHA separately from the fat sample. Furthermore, since the antioxidant preparation employed will have a fixed ratio of say, propyl gallate to BHA, then if the amount of BHA was determined, it would be possible to estimate the amount of propyl gallate present, and similarly for NDGA in the case of antioxidant preparations which contained BHA plus NDGA. The net effect of this procedure would represent a considerable saving in time required for the industrial routine analysis

of fats containing BHA plus propyl gallate or BHA plus NDGA.

3. ANALYTICAL METHOD

a. Reagents

Light Petroleum (3+1) - as described for the analysis of propyl gallate, page 35.

Ethyl Alcohol - as described for the analysis of BHA, page 64.

Borax Buffer - 2.0 percent aqueous solution of $Na_2B_4O_7 \cdot 10H_2O$.

2,6-Dichloroquinonechloroimide - 0.01 percent solution of 2,6-dichloroquinonechloroimide in absolute ethyl alcohol. For optimum results this reagent should be freshly prepared.

b. Quantitative Procedure

Place 10 g. of the fat in a 250 ml. separatory flask and dissolve with 50 ml. of 3+1 light petroleum. Extract the light petroleum solution of the fat by shaking the contents of the flask with three separate 25 ml. portions of 72 percent ethyl alcohol for three minutes per extraction. Make a fourth extraction employing 75 ml. of 72 percent ethyl alcohol and shaking for one minute. Allow about five minutes between extractions for complete separation of the phases before running off the alcoholic layer. Combine these four extracts, dilute to a suitable volume (150 to 300 ml.)

with 72 percent ethyl alcohol, and filter through two Whatman No. 54 filter papers. This clear alcoholic extract contains the BHA plus propyl gallate or NDGA. Place three suitable portions (1 to 12 ml.) of the extract in separate Evelyn colorimeter tubes and dilute to 12 ml. with 72 percent ethyl alcohol. Add to each tube 2 ml. of freshly prepared 0.01 percent 2,6-dichloroquinonechloroimide reagent. Mix the contents of the tubes, add 2 ml. of 2 percent aqueous borax solution, and mix the contents again. Prepare a "blank" containing 12 ml. of 72 percent ethyl alcohol and the reagents. After twenty minutes measure the absorbancy relative to the "blank" in an Evelyn photoelectric colorimeter fitted with a No. 620 filter. Calculate the amount of BHA per tube employing the "K-value" obtained with the same BHA preparation that was added to the fat. Prepare a new calibration curve for each type or batch of antioxidant preparation used. Report a single average value derived from the three levels of extract analysed.

4. CHARACTERISTICS OF QUANTITATIVE PROCEDURE

a. Recovery of Butylated Hydroxyanisole from Fat and the Reproducibility of Analytical Results

Three samples of shortening were prepared containing 0.020, 0.015 and 0.010 percent of BHA respectively. In all cases, the BHA was added as a commercial antioxidant

preparation containing 20 percent BHA, 6 percent propyl gallate and 4 percent citric acid in propylene glycol. Each of these fat samples were analysed four times for BHA, employing the foregoing procedure. The BHA content of the sample was calculated by means of calibration curves prepared employing the same antioxidant preparation as added to the fat. The recovery of BHA and the estimated reproducibility of the analytical data are shown in Table XVIII.

Table XVIII

Recovery of BHA Added to Shortening and the Reproducibility of Analytical Results

BHA Added Percent	Average BHA Recovered Percent	Estimated Standard Deviation for Single Analysis
0.0200	0.0198	± 0.0002
0.0150	0.0146	± 0.0002
0.0100	0.0097	± 0.0001

It will be noted that 97 to 99 percent of the BHA added to shortening was recovered. The apparent loss of 1 to 3 percent of the added BHA might be attributed to incomplete extraction and to the destruction of some of the BHA by small amounts of fat peroxides present in the fat.

b. Advantages of the 2,6-Dichloroquinonechloroimide-Borax Reagent

The 2,6-dichloroquinonechloroimide-borax reagent has been found superior to the ferric chloride plus α,α'-bipyridine reagent for the routine determination of BHA for the following reasons:- It is unaffected by strong light, therefore, opaque glassware is not required. Maximal colour formation is attained in fifteen to twenty minutes. The colour maximum is stable for five hours at room temperature. The reagent is relatively specific for BHA among the antioxidants currently added to lard or shortening. The reagent permits the determination of BHA in the presence of propyl gallate or NDGA, with no error, provided the original antioxidant preparation can be employed to construct a calibration curve. Extraneous reducing agents are unlikely to introduce serious errors with this reagent. Rigid purification of the light petroleum and ethyl alcohol used is not necessary, as is the case when employing the ferric chloride plus α,α'-bipyridine reagent.

D. 2-Tertiary-butyl-4-hydroxyanisole and 3-Tertiary-butyl-4-hydroxyanisole Isomers

1. INTRODUCTION

As has been mentioned already, commercial butylated hydroxyanisole (BHA) is a mixture of the 2-tertiary-butyl-4-hydroxyanisole (2-BHA) and 3-tertiary-butyl-4-hydroxyanisole (3-BHA) isomers (73), while the literature contains evidence to the effect that 3-BHA is a more effective antioxidant than 2-BHA when added to lard. In this connection Rosenwald and Chenicek (125) found that a concentration of 0.02 percent, 3-BHA was 2.8 times more effective than 2-BHA and at a concentration of 0.005 percent 3-BHA was 5.9 times more effective than 2-BHA. Dugan et al. (31) investigated the relative effectiveness of these isomers singly and in mixtures of known composition and found that 3-BHA was 1.5 times as effective as 2-BHA when added to lard at a concentration of 0.01 percent. It was felt, therefore, that a procedure for the estimation of the proportions of these isomers should be useful in evaluating the potential effectiveness of commercial BHA preparations. In addition such a method should prove useful in following the relative rates of loss of the 2-BHA and 3-BHA isomers during the storage of fats or baked products.

It has been shown in the foregoing section that 3-BHA on reaction with the 2,6-dichloroquinonechloroimide-borax

reagent produces 5.1 times more absorbancy per unit weight measured at 620 mμ than does 2-BHA. On the other hand, it has been shown in a previous section that 2-BHA on reaction with the ferric chloride plus α,α'-bipyridine reagents produces 1.1 times more absorbancy per unit weight measured at 515 mμ than does 3-BHA. Therefore, it appeared that the ratio of absorbancies measured at 620 and 515 mμ, employing the appropriate reagents, might provide a basis for estimating the proportions of these isomers in BHA preparations.

2. DEVELOPMENT OF ANALYTICAL METHOD

a. Absorbancy per Unit Weight for Mixtures of 2-Tertiary-butyl-4-hydroxyanisole and 3-Tertiary-butyl-4-hydroxyanisole Employing the Ferric Chloride Plus α,α'-Bipyridine and 2,6-Dichloroquinonechloroimide-Borax Reagents

Mixtures were prepared containing known proportions of 2-BHA and 3-BHA in 72 percent ethyl alcohol. These mixtures were treated with the 2,6-dichloroquinonechloroimide-borax reagent as previously described, the absorbancies measured at 620 mμ and the absorbancy per microgram of BHA calculated. Similar BHA mixtures were also treated with the ferric chloride plus α,α'-bipyridine reagents as previously described, the absorbancies being measured at 515 mμ and the absorbancy per microgram of BHA calculated. The results of these experiments are given in Figure 7.

Since the curves in Figure 7 are sharply divergent, it

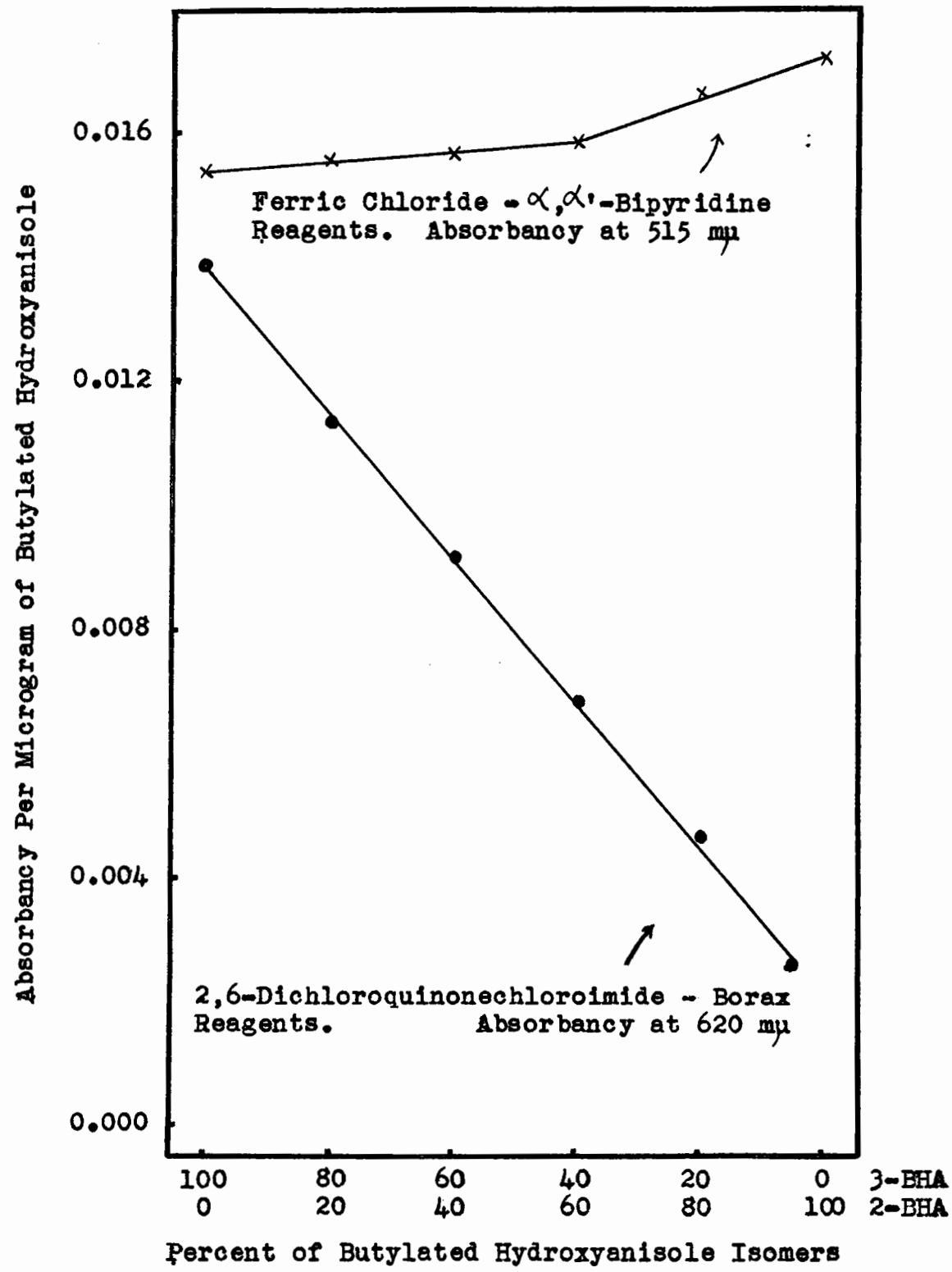


Figure 7 Absorbancy per Microgram for Known Mixtures of 2-Tertiary-butyl-4-hydroxyanisole and 3-Tertiary-butyl-4-hydroxyanisole

was felt that the ratio of the absorbancy per microgram of the BHA mixture, measured at 620 mμ to the absorbancy per microgram measured at 515 mμ, employing the appropriate reagents, would be characteristic of the proportions of the 2-BHA and 3-BHA isomers in a mixture.

b. Ratio of Absorbancies at 620 mμ/515 mμ Employing the Appropriate Reagents for Mixtures of 2-Tertiary-butyl-4-hydroxyanisole and 3-Tertiary-butyl-4-hydroxyanisole

The ratio of the absorbancies at 620 mμ/515 mμ for known mixtures of 2-BHA and 3-BHA employing the appropriate reagents were calculated and are illustrated in Figure 8.

Figure 8 indicates that a straight-line relationship exists between the proportions of these BHA isomers and the ratio of absorbancies at 620 mμ/515 mμ except when the mixture contains less than 20 percent of 3-BHA. Therefore, it appeared that the determination of the 620 mμ/515 mμ ratio of absorbancies employing the appropriate reagents might constitute a suitable basis for estimating the proportions of the 2-BHA and 3-BHA isomers in commercial BHA.

3. ANALYTICAL METHOD

a. Reagents

Light petroleum (3+1) - as described for the analysis of propyl gallate, page 35.

Ethyl alcohol - as described for the analysis of BHA, page 64.

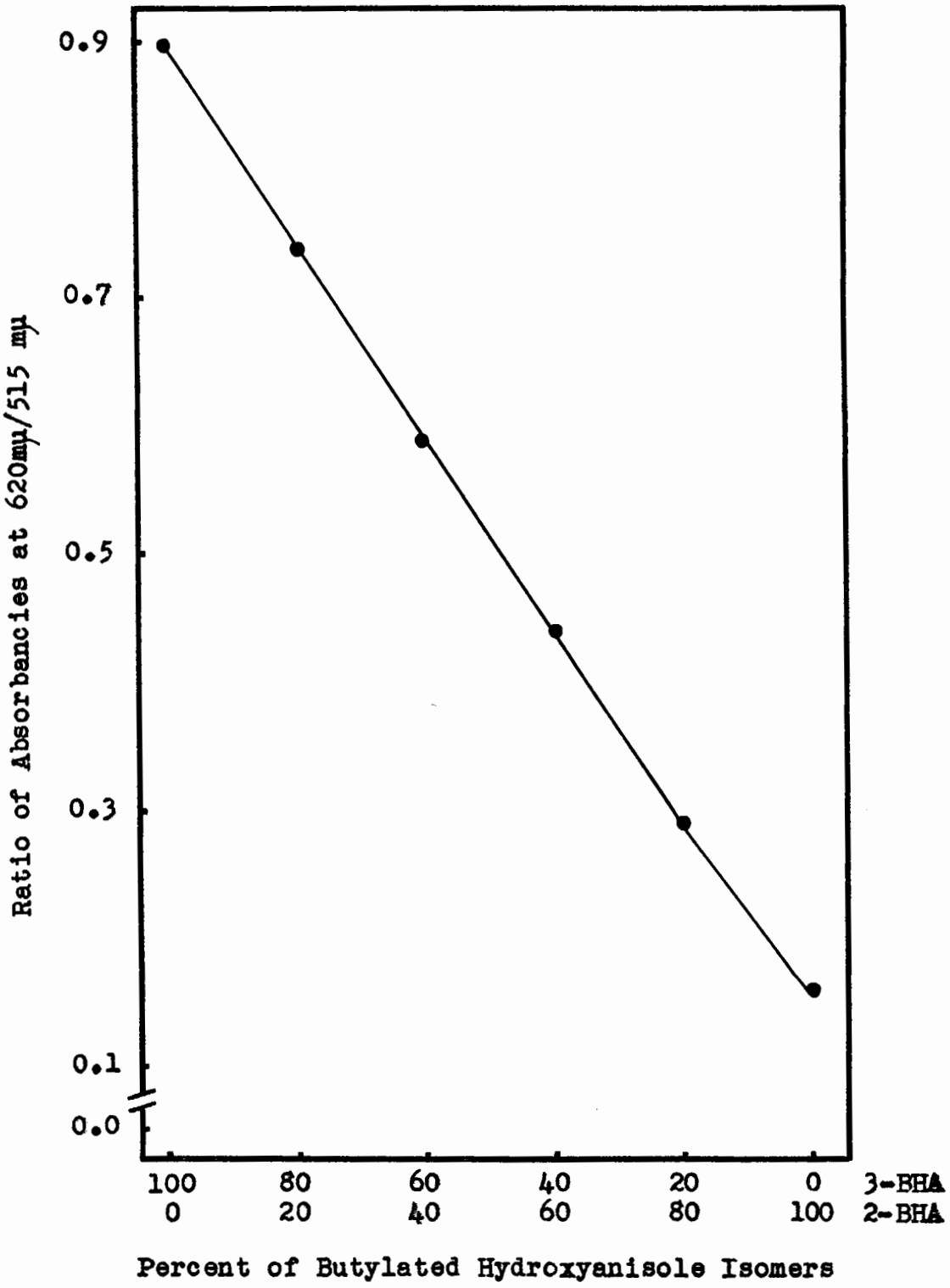


Figure 8 Absorbancy Ratios at 620 mμ/515 mμ for Mixtures of 2-Tertiary-butyl-4-hydroxyanisole and 3-Tertiary-butyl-4-hydroxyanisole Employing the Appropriate Reagents

Ferric Chloride - as described for the analysis of BHA, pages 64 and 65.

α,α'-Bipyridine - as described for the analysis of BHA, page 65.

2,6-Dichloroquinonechloroimide - as described for the analysis of BHA, page 65.

Borax - as described for the analysis of BHA, page 65.

Ammonium Acetate - as described for propyl gallate, page 36.

b. Extraction of Butylated Hydroxyanisole from Fat

BHA is frequently used together with propyl gallate, and hence these two antioxidants may be encountered in the same sample. Since the presence of propyl gallate would lead to serious errors in the estimation of the proportion of 2-BHA and 3-BHA, a qualitative test for propyl gallate must be made. If present, the propyl gallate must be extracted from the light petroleum - fat solution with 1.67 percent aqueous ammonium acetate solution, as previously described, before proceeding with the extraction of BHA employing 72 percent ethyl alcohol. The proportions of 2-BHA and 3-BHA are estimated in the alcoholic extract.

c. Quantitative Analysis of Total Butylated Hydroxyanisole and the Proportion of 2-Tertiary-butyl-4-hydroxyanisole and 3-Tertiary-butyl-4-hydroxyanisole

Place 10 g. of the fat, or a suitable weight of antioxidant preparation, in a 250 ml. separatory flask and dissolve with 50 ml. of 3+1 light petroleum. If propyl gallate is present, extract this solution with three 20 ml. portions of aqueous 1.67 percent ammonium acetate solution for three minutes per extraction. Allow the phases to separate completely before drawing off the lower aqueous layer. Make a fourth extraction by shaking the contents of the flask with 20 ml. of water for one minute.

These aqueous extracts contain the propyl gallate and can either be discarded or employed for the quantitative analysis of propyl gallate as previously described.

Extract the BHA from the light petroleum solution with three 25 ml. portions of purified 72 percent ethyl alcohol by shaking for three minutes per extraction. Allow the phases to separate completely and draw off the lower alcoholic layer. Make a fourth extraction using 75 ml. of 72 percent ethyl alcohol and shaking for one minute. Combine the four alcoholic extracts, dilute to a suitable volume (150 to 300 ml.) with 72 percent ethyl alcohol, and filter through double Whatman No. 54 filters.

Place three portions (1 to 5 ml.) of the alcoholic BHA solution in three separate glass-stoppered 25 ml. Erlenmeyer

flasks that have been rendered opaque with black tape. Dilute all portions to 5 ml. with 72 percent ethyl alcohol. Add 3 ml. of 100 percent ethyl alcohol, 2 ml. of 0.2 percent ferric chloride reagent, and 2 ml. of 0.2 percent α,α'-bipyridine reagent to each flask and mix the contents. Prepare a blank containing 5 ml. of 72 percent ethyl alcohol, 3 ml. of 100 percent ethyl alcohol, and 2 ml. portions of the reagents. Measure the absorbancy relative to the "blank" after exactly thirty minutes employing an Evelyn photoelectric colorimeter fitted with a No. 515 filter. Calculate the average absorbancy at 515 mμ per ml. of the alcoholic BHA extract.

Place three other portions (1 to 12 ml.) of the BHA extract in separate Evelyn colorimeter tubes and dilute to 12 ml. with 72 percent ethyl alcohol. Add 2 ml. of the 0.01 percent 2,6-dichloroquinonechloroimide reagent and 2 ml. of 2 percent aqueous borax buffer, mixing the contents of the tubes after each addition. Prepare a "blank" containing 12 ml. of 72 percent ethyl alcohol and 2 ml. portions of the reagents. After twenty minutes measure the absorbancy relative to the "blank" in an Evelyn photoelectric colorimeter fitted with a No. 620 filter. Calculate the average absorbancy at 620 mμ per ml. of the alcoholic BHA extract.

Calculate the ratio of the average absorbancies at 620 mμ/515 mμ per ml. of the BHA-containing extract. By

reference to Figure 8 estimate the proportions of 2-BHA and 3-BHA in the mixture. With this datum refer to Figure 7 and ascertain the appropriate factor to calculate the total weight of BHA from the average absorbancy per ml. of extract measured at 515 mμ. Determine the actual weight of the 2-BHA and 3-BHA isomers in the sample by multiplying the total weight of BHA by the respective percentage of these isomers in the mixture as previously estimated.

4. REPRODUCIBILITY OF ANALYTICAL RESULTS AND THE EFFECT OF THE PRESENCE OF OTHER ANTIOXIDANTS

Six fat samples containing known proportions of 2-BHA and 3-BHA were analyzed employing the foregoing analytical procedures. These results were analyzed statistically and indicate that the 95 percent confidence limits for the determination of the proportion of 3-BHA in a mixture of the 2-BHA and 3-BHA isomers is ± 3 percent units of 3-BHA. Since the percentage of 2-BHA is determined by difference from 100 percent, the 95 percent confidence limits for 2-BHA is also ±3 percent units.

The 95 percent confidence limits for the determination of the total BHA content, calculated from the average absorbancy per ml. measured at 515 mμ, was less than ± 1 percent of the total BHA.

Nordihydroguaiaretic acid (NDGA), gum guaiac, lauryl gallate or other reducing agents that cannot be separated

from BHA, will react strongly with the ferric chloride plus α,α'-bipyridine reagents. On the other hand, such materials will produce relatively little absorbancy with the 2,6-dichloroquinonechloroimide-borax reagent (see Table XIV). Therefore, the presence of these antioxidants or other reducing agents would result in the overestimation of total BHA and the underestimation of the proportion of the 3-BHA isomer.

5. ANALYSIS OF COMMERCIAL BUTYLATED HYDROXYANISOLE PREPARATIONS

In order to ascertain the variation in composition of commercial BHA preparations, three commercial BHA preparations were analyzed and the proportion of 2-BHA and 3-BHA isomers were estimated. The results of these analyses are given in Table XIX.

Table XIX

Proportion of 2-BHA and 3-BHA Isomers in Commercial BHA Preparations

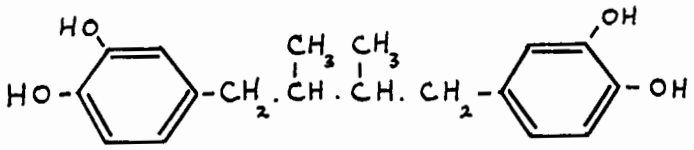
Commercial BHA Preparation	Percent 3-BHA Found	Percent 2-BHA Found
No. 1	95	5
No. 2	81	19
No. 3	63	37

The results for these three samples suggest that the proportions of the two isomers in commercial BHA preparation may vary widely. It may be noted, at the same time, that the predominant isomer in the three preparations analyzed was 3-BHA.

E. Nordihydroguaiaretic Acid

1. INTRODUCTION

Nordihydroguaiaretic acid (NDGA), was first synthesized by Schroeter et al. (130) from hydroguaiaretic acid and was later assigned the following structure by Waller and Gisvold (145).



During cooperative investigations by the United States Department of Agriculture and the University of Minnesota, NDGA was found to occur in the creosote bush (Larrea divaricata Cav.), a desert plant which grows commonly in the Southwestern United States (145). Pure NDGA was prepared by crystallization from a crude extract of this plant material. Stull et al. (138) state that crystalline NDGA is practically odourless, but has a slight astringent flavour.

NDGA has been employed successfully for retarding the development of rancidity in lard (57,73,83,84,85,143), bacon (134), cured fish (132), carotene solutions (9,10), vegetable oils (92) and dairy products (65,75,137,138,139,140).

NDGA is permitted to be added to edible fats in the United States at a maximum level of 0.01 percent of the fat (122). On the other hand, NDGA is permitted to be added to edible fats in Canada (17) at a maximum level of 0.005 percent of the fat.

2. TOXICITY OF NORDIHYDROGUAIARETIC ACID

Cranston et al. (26) estimated the LD_{50} for NDGA by acute toxicity tests with mice as more than 2000 mg. per kg. and with rats as more than 2000 mg. per kg. of body weight. However, the LD_{50} for NDGA injected intraperitoneally into mice was reduced to 550 mg. per kg.

Cranston et al. (26) also conducted chronic toxicity tests with NDGA over a two-year period employing rats and mice as the test animals. Mice were fed diets containing 0.25 and 0.5 percent of NDGA. At these levels, NDGA exerted little effect on the growth rate or feed intake. Rats were fed diets containing 0.5 and 1.0 percent of NDGA and no effect on body growth or feed intake was noted in the rats at the 0.5 percent level of NDGA in the diet. However, at the 1 percent level, NDGA caused an initial small decrease in growth rate which was attributed to decreased food intake. As soon as the animals became accustomed to the diet their food intake increased and eventually their weight equaled that of the control rats.

These results support the belief that NDGA can safely be employed at a level of 0.01 percent NDGA in edible fats.

3. DEVELOPMENT OF ANALYTICAL METHOD

a. Extraction of Nordihydroguaiaretic Acid from Fat

Stull et al. (138) state that NDGA is only slightly

soluble in water, while being soluble to the extent of 50 g. per 100 g. in ethyl alcohol, 20 g. per 100 g. in ethylene glycol and from 0.3 to 3.0 g. per 100 g. in fats and oils at ordinary temperatures.

Lundberg and Halvorson (85) determined the solubility of NDGA in lard and cottonseed oil at temperatures between 30 and 150°C. Their values for solubilities are cited in Table XX for convenience.

Table XX

Minimum Solubilities of NDGA in Lard and Cottonseed Oil at Temperatures Between 30 and 150°C (85).

Temperature °C	Percent Solubility in Lard g./100 g.	Percent Solubility in Cottonseed Oil g./100 g.
30	-	0.71
45	0.52	-
75	0.66	0.81
125	1.94	2.43
150	3.44	4.32

Since NDGA is slightly soluble in water (138) it is extracted at least in part during the aqueous extraction of the fat - light petroleum solution for the removal of propyl gallate. Therefore, propyl gallate and NDGA cannot be separated on the basis of their differential solubilities. Also, since NDGA is highly soluble in ethyl alcohol (138),

it is easily extracted from a solution of fat in light petroleum with this solvent. The extraction procedure for BHA employing 72 percent ethyl alcohol is even more effective for the extraction of NDGA. To determine the extractability of NDGA contained in fat, the following experiment was conducted. Ten g. of lard containing 0.01 percent NDGA was dissolved in 50 ml. of 3 + 1 light petroleum. This solution was extracted with three, 25 ml. portions of 72 percent ethyl alcohol, the mixture being shaken for three minutes per extraction. The light petroleum solution of lard was extracted a fourth time for one minute with 75 ml. of 72 percent ethyl alcohol. Each alcoholic extract was diluted to a convenient volume, filtered and analyzed for NDGA as described below. The results of these experiments are given in Table XXI.

Table XXI

Proportion of NDGA Extracted from a Lard in Light Petroleum Solution per Extraction with 72 Percent Ethyl Alcohol

No. of Extraction	Volume of 72% Ethyl Alcohol	Duration of Extraction Minutes	NDGA Recovered per Extraction %	Total NDGA Extracted %
1	25 ml.	3	91.8	91.8
2	25	3	4.8	96.6
3	25	3	1.1	97.7
4	75	1	0.6	98.3

These results indicate that four extractions of a fat in light petroleum solution employing 72 percent ethyl alcohol recovered over 98 percent of the NDGA present.

b. Characteristics of the Ferric Chloride - α,α'-Bipyridine - Nordihydroguaiaretic Acid Colour Reaction

The ferric chloride plus α,α'-bipyridine reagents previously employed for the analysis of BHA were found satisfactory for the analysis of NDGA.

i. Absorption Curve

Lundberg and Halvorson (85) determined the absorption curve for the ferric chloride - α,α'-bipyridine solution upon reduction by NDGA and reported that the absorption maximum was close to 510 mμ.

The absorption curve for the NDGA - ferric chloride - α,α'-bipyridine colour, employing the foregoing reagents was determined as follows:- Five ml. of 72 percent ethyl alcohol containing NDGA were employed and 3 ml. of 100 percent ethyl alcohol, 2 ml. of ferric chloride reagent and 2 ml. of the α,α'-bipyridine reagent were added. A "blank" reference solution was prepared employing 5 ml. of 72 percent ethyl alcohol in place of the NDGA solution. After five minutes the absorption curve of the NDGA-containing solution was determined relative to the "blank", employing a Beckman B spectrophotometer. The resulting data are given in Figure 9.

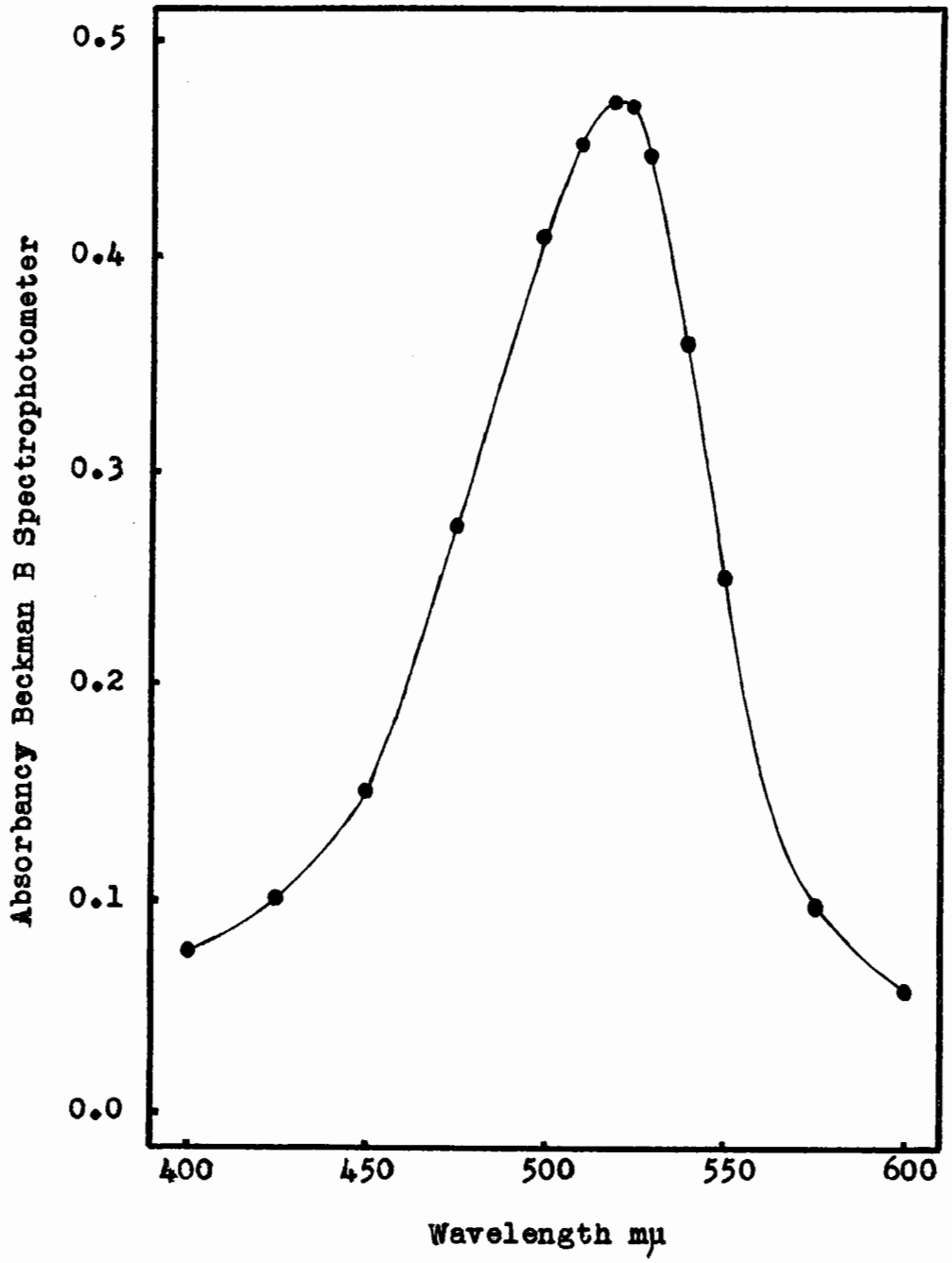


Figure 9 Absorption Curve for Nordihydroguaiaretic Acid on Reaction with Ferric Chloride - α,α'-Bipyridine Reagents

Figure 9 indicates that the NDGA - ferric chloride - α,α'-bipyridine coloured product exhibits a maximum absorption at approximately 520 mμ and, therefore, the absorbancy was measured employing a No. 515 filter in an Evelyn photoelectric colorimeter.

ii. Colour Stability

The effect of time upon the absorbancy produced by NDGA upon reaction with the ferric chloride plus α,α'-bipyridine reagents was determined as previously described for BHA and the results are given in Table XXII.

Table XXII

Effect of Time Upon the Absorbancy Produced by NDGA Upon Reaction with the Ferric Chloride Plus α,α'-Bipyridine Reagents

Time of Reaction Minutes	Absorbancy Measured with No. 515 Filter	Absorbancy/Microgram of NDGA "K-value"
0.33	0.572	0.0143
0.67	0.577	0.0144
1	0.583	0.0146
2	0.613	0.0153
3	0.620	0.0155
4	0.622	0.0156
5	0.621	0.0155
10	0.624	0.0156
30	0.644	0.0161

These results indicate that an effective maximum absorbancy was produced within three minutes and thereafter there was only a slow increase in absorbancy. Therefore, three

minutes was adopted for colour development in the analysis of NDGA.

iii. Calibration Curve

A calibration curve for NDGA was prepared exactly as described for BHA except that the resulting absorbancy was measured three minutes after adding the ferric chloride reagent. An Evelyn colorimeter fitted with a No. 515 filter was employed to measure the absorbancies. Typical results for construction of a calibration curve for NDGA are presented in Table XXIII.

Table XXIII

Calibration Data for NDGA on Reaction with Ferric Chloride - α,α'-Bipyridine Reagents for Three Minutes

NDGA Present Micrograms	Absorbancy with No. 515 Filter	Absorbancy/Microgram of NDGA "K-Value"
5	0.080	0.0160
10	0.156	0.0156
20	0.310	0.0155
30	0.468	0.0156
40	0.622	0.0155
50	0.712	0.0142

The values reported in Table XXIII suggest that the NDGA calibration curve obeys Beer's Law over the range of 10 to 40 micrograms NDGA per 12 ml. of solution.

4. ANALYTICAL METHOD

a. Reagents

Light Petroleum (3 + 1) - As described for propyl gallate, page 35.

Ethyl Alcohol - As described for BHA, page 64.

Ferric Chloride - As described for BHA, pages 64 and 65.

α,α'-Bipyridine - As described for BHA, page 65.

b. Quantitative Procedure

No specific qualitative test for microgram quantities of NDGA has been found or is reported in the literature. Therefore, if propyl gallate is absent, it is possible that NDGA may be present, since these two antioxidants are not employed together.

The extraction procedure for NDGA is identical with that previously described for BHA. However, it should be remembered that NDGA cannot be determined after the extraction of propyl gallate with aqueous 1.67 percent ammonium acetate solution, because this extraction also removes a considerable portion of the NDGA.

The analytical procedure for NDGA is identical to that previously described for BHA, except that the absorbancy is measured three minutes after the addition of the ferric chloride reagent. The concentration of NDGA in micrograms per aliquot is obtained by dividing the observed absorbancy

at 515 mμ by a "K-value" of 0.0155.

5. RECOVERY OF NORDIHYDROGUAIARETIC ACID FROM FAT AND REPRODUCIBILITY OF ANALYTICAL RESULTS

Six identical fat samples containing approximately 0.01 percent of added NDGA were analyzed. In all cases the extract from a single sample was analyzed employing three different portions, the results averaged and reported as a single figure. These results were analyzed statistically with the results reported in Table XXIV.

Table XXIV

Reproducibility of NDGA Analyses

Average NDGA Recovery Percent	Standard Deviation	99 Percent Confidence Limits
98.4	0.0001	± 0.0005

The values in Table XXIV indicate that a single analysis of a fat containing 0.01 percent of NDGA would be subject to an error of no more than ± 5 percent of the NDGA present.

F. Determination of Butylated Hydroxyanisole and Nordihydroguaiaretic Acid in the Same Sample

1. INTRODUCTION

The use of 0.02 percent butylated hydroxyanisole (BHA) in combination with 0.01 percent nordihydroguaiaretic acid (NDGA) is permitted in edible fats in the United States (122). However, the Canadian Food and Drug Regulations (17) permit the use of 0.005 percent NDGA, either alone or in combination with 0.02 percent BHA in edible fats.

The analytical method for mixtures of BHA and NDGA here described is based upon the fact that NDGA reacts much more rapidly with the ferric chloride plus α,α'-bipyridine reagents than does BHA. As previously stated, NDGA attains an effective maximum colour formation in three minutes, while BHA requires thirty minutes. Therefore, by measuring the absorbancy produced after two different time intervals employing duplicate portions, it is possible to estimate the amount of NDGA and BHA in a mixture.

2. DEVELOPMENT OF ANALYTICAL METHOD

a. Extraction of Butylated Hydroxyanisole plus Nordihydroguaiaretic Acid from Fats

If both NDGA and BHA are present in the same fat sample, they will both be extracted by 72 percent ethyl alcohol. Therefore, to extract these antioxidants, dissolve ten g. of the fat in 50 ml. of 3+1 light petroleum and extract with

72 percent ethyl alcohol as previously described for the determination of BHA (pages 66 and 67).

b. Effect of Time on the Absorbancy Produced by Butylated Hydroxyanisole and Nordihydroguaiaretic Acid Upon Reaction with the Ferric Chloride plus α,α'-Bipyridine Reagents

Five ml. portions of 72 percent ethyl alcohol, containing 40 micrograms of BHA or 40 micrograms of NDGA were placed into opaque Erlenmeyer flasks followed by 3 ml. of 100 percent ethyl alcohol, 2 ml. of 0.2 percent ferric chloride and 2 ml. of 0.2 percent α,α'-bipyridine reagents. After intervals of one and of thirty minutes, the resulting absorbancies were measured in an Evelyn colorimeter fitted with a No. 515 filter relative to reagent "blanks" held for equal intervals of time. The results of these experiments are summarized in Table XXV.

Table XXV

Effect of Time on the Absorbancy Produced by BHA and NDGA Upon Reaction with the Ferric Chloride plus α,α'-Bipyridine Reagents

Antioxidant	Reaction Time Minutes	Absorbancy/Microgram of Antioxidant "K-Value"
BHA	1	0.0016
BHA	30	0.0156
NDGA	1	0.0146
NDGA	30	0.0162

It will be noted that after one minute BHA has produced

approximately 10 percent of the absorbancy finally obtained after thirty minutes, while NDGA has produced approximately 90 percent of the absorbancy obtained after thirty minutes. Therefore, it is possible to set up two simultaneous equations on the assumption that the absorbancies are additive.

Absorbancy at one minute = 0.9 N + 0.1 B

Absorbancy at thirty minutes = 1.0 N + 1.0 B

Where "N" and "B" are the absorbancies due to NDGA and BHA respectively after a reaction time of thirty minutes.

c. Additivity of the Butylated Hydroxyanisole and Nordihydroguaiaretic Acid Absorbancies Obtained with the Ferric Chloride plus α,α'-Bipyridine Reagents

Solutions containing BHA, NDGA and BHA plus NDGA were analyzed in duplicate at three concentration levels employing the ferric chloride plus α,α'-bipyridine reagents. The absorbancy of one of each pair was measured relative to appropriate reagent "blanks" after one minute and the other after thirty minutes of reaction. All measurements were made with an Evelyn colorimeter fitted with a No. 515 filter. The results of this experiment are given in Table XXVI.

The results given in Table XXVI were then evaluated employing the following formulae:-

Absorbancy after one minute = 0.9 N + 0.1 B

Absorbancy after thirty minutes = 1.0 N + 1.0 B

where "N" and "B" are the respective absorbancies due to

Table XXVI

Additivity of the BHA and NDGA Absorbancies Obtained with the Ferric Chloride plus α,α'-Bipyridine Reagents

Antioxidants/Aliquot		Absorbancy Measured with No. 515 Filter	
BHA Micrograms	NDGA Micrograms	After 1 Minute Reaction	After 30 Minutes Reaction
0	45	0.642	0.703
0	36	0.527	0.575
0	27	0.389	0.452
9	18	0.282	0.444
12	24	0.364	0.602
15	30	0.463	0.703
18	9	0.169	0.440
24	12	0.219	0.564
30	15	0.277	0.703
27	0	0.063	0.445
36	0	0.086	0.575
45	0	0.110	0.706

NDGA and BHA after thirty minutes reaction. The calculated value of "N" divided by 0.0161 and of "B" divided by 0.0156 yield the concentrations of NDGA and BHA respectively, expressed as micrograms per aliquot employed. The results of these calculations are given in Table XXVII.

The values in Table XXVII show that analytical values for BHA ranged from 96.2 to 103.7 percent of the BHA present while for NDGA these values ranged from 98.7 to 106.0 percent of the NDGA present. On the other hand, estimates of total antioxidant (BHA plus NDGA) ranged from 96.9 to 101.8

Table XXVII

Results of the Analysis of Mixtures of BHA and NDGA

Antioxidants Present		Antioxidants Found		Total Antioxidant Found
Micrograms/ml. of Solution		Micrograms/ml. of Solution		Micrograms/ml. of Solution
BHA	NDGA	BHA	NDGA	BHA plus NDGA
0.0	9.0	-0.03	8.88	8.85
3.0	6.0	3.11	6.05	9.16
6.0	3.0	5.85	3.18	9.03
9.0	0.0	8.66	0.06	8.72

percent of the amounts present. In view of the large number of manipulations required and the small amount of antioxidant present, these results were considered satisfactory. Since the use of BHA together with NDGA was not permitted in Canada at the time this investigation was made, further study of this procedure was not undertaken. In its present form this method should give at least a good estimate of the BHA and NDGA present as a mixture in fats.

3. ANALYTICAL PROCEDURE

a. Reagents

Light Petroleum (3+1) - As described for propyl gallate, page 35.

Ethyl Alcohol - As described for BHA, page 64.

Ferric Chloride - As described for BHA, pages 64 and 65.

α,α'-Bipyridine - As described for BHA, page 65.

b. Qualitative Procedure for Butylated Hydroxyanisole plus Nordihydroguaiaretic Acid in Fat

In order to extract propyl gallate, NDGA and BHA present in the sample, place 10 g. of fat in a separatory funnel and dissolve with 50 ml. of 3 + 1 light petroleum. Shake this fat solution for two minutes with 20 ml. of 72 percent ethyl alcohol, run off the alcoholic layer and filter. To 3 ml. of extract add a few drops of concentrated ammonium hydroxide. If a rose colour does not appear the sample does not contain propyl gallate. Next, test another 3 ml. of the extract for the presence of BHA using the 2,6-dichloroquinonechloroimide-borax reagent as previously described (page 65). The appearance of a blue colour within fifteen minutes is indicative of BHA. If BHA is found but propyl gallate is not, then it is possible that BHA plus NDGA might be present. Since there is no specific test for NDGA, the only characteristics of NDGA that can be employed are, firstly, that it is easily extracted from a light petroleum solution of fat with 72 percent ethyl alcohol and secondly, that it reacts almost instantaneously with the ferric chloride plus α,α'-bipyridine reagents to produce a red colour. However, BHA also reacts with these reagents to produce a red colour. Hence, if the qualitative test for BHA is positive, then it becomes necessary to perform the following test to ascertain whether NDGA is also present.

Place duplicate portions of the 72 percent ethyl alcohol extract into opaque 25 ml. Erlenmeyer flasks and dilute to 5 ml. with 72 percent ethyl alcohol, add 3 ml. of 100 percent ethyl alcohol followed by 2 ml. of the ferric chloride and 2 ml. of the α,α'-bipyridine reagents. Determine the absorbancies measured after one and ten minutes respectively, relative to reagent "blanks" held for the same time intervals employing an Evelyn colorimeter with a No. 515 filter. Portions should be so chosen that the absorbancy measured after ten minutes does not exceed 0.7. Next, calculate the ratio of the absorbancies measured at one minute to that measured at ten minutes and compare this value with those in Table XXVIII.

Table XXVIII

Ratio of Absorbancies Measured at One and Ten Minutes as Related to the Proportion of BHA and NDGA Present

Percent NDGA	Percent BHA	Ratio of Absorbancies at 1 Minute per 10 Minutes
100	0	0.99
80	20	0.85
60	40	0.71
40	60	0.54
20	80	0.35
0	100	0.27

In the United States where the use of BHA together with

NDGA was first permitted (122), these antioxidants were initially employed in the ratio of two parts of BHA to one part of NDGA. In such a mixture there would be 33 percent of NDGA and, therefore, the ratio of the absorbancies at one minute per ten minutes would be approximately 0.47, which is significantly different from the ratio of 0.27 for BHA alone. At present, the trend in Canada and the United States appears to be towards the use of a smaller proportion of NDGA. In the antioxidant formulation known as "Tenox N" (Tennessee Eastman Corporation) there are ten parts of BHA to one part of NDGA. In such a mixture there is only 9 percent of NDGA and consequently the ratio of absorbancies at one minute per ten minutes would be approximately 0.30, which is not distinguishable from the ratio of 0.27 for pure BHA. Consequently, the application of this qualitative procedure, although effective a few years ago, is now quite limited in its application. In actual fact it is almost as simple to conduct the following quantitative procedure for the analysis of BHA plus NDGA if BHA is present, propyl gallate is absent and the presence of NDGA is suspected.

c. Quantitative Procedure for Butylated Hydroxyanisole plus Nordihydroguaiaretic Acid in Fat

Place 10 g. of fat in a separatory funnel and dissolve with 50 ml. of 3+1 light petroleum. The BHA and NDGA are extracted together from this fat solution with 72 percent

ethyl alcohol as described for the extraction of BHA. Place three different portions of the alcoholic extract ranging from 1 to 5 ml. into each of a duplicate series of opaque 25 ml. Erlenmeyer flasks. Dilute the contents of each flask to 5 ml. with 72 percent ethyl alcohol. Add 3 ml. of 100 percent ethyl alcohol, 2 ml. of 0.2 percent ferric chloride reagent and 2 ml. of 0.2 percent α,α'-bipyridine reagent to each flask. Measure the resulting absorbancies for one series after one minute and for the other series after thirty minutes relative to reagent "blanks" held for similar time intervals. Employ an Evelyn colorimeter fitted with a No. 515 filter to measure the absorbancies. Apply the following simultaneous equations to the absorbancies measured.

Absorbancy after one minute = 0.9 N + 0.1 B

Absorbancy after thirty minutes = 1.0 N + 1.0 B

where "B" is the absorbancy due to BHA and "N" is the absorbancy due to NDGA at thirty minutes.

Solve the foregoing equations and calculate the respective absorbancies due to NDGA and BHA. The value of "N" divided by a "K-value" of 0.0161 (see Table XXV) and the value of "B" divided by 0.0156 gives the concentrations of NDGA and BHA respectively expressed in micrograms per portion employed.

4. RECOVERY OF BUTYLATED HYDROXYANISOLE PLUS NORDIHYDRO-GUAIARETIC ACID FROM FAT AND REPRODUCIBILITY OF THE ANALYTICAL RESULTS

Six, 10 g. fat samples containing 0.020 percent of BHA together with 0.010 percent of NDGA were extracted and analyzed for these antioxidants as previously described. The average recovery for BHA was 97.0 percent and for NDGA was 96.4 percent. It is not clear why the recovery figures for BHA plus NDGA in the same sample should be lower than those obtained for BHA and NDGA present separately. However, in this case there was a total of 0.03 percent antioxidant in the samples and perhaps an additional extraction with 72 percent ethyl alcohol should have been employed. Recoveries might have been one or two percent higher if this had been done.

A fat sample containing approximately 0.02 percent of BHA and 0.01 percent of NDGA was analyzed six times employing the preceding method. In all cases the extract from a single sample was analyzed at three concentration levels, and the results averaged and reported as a single value. The results of six such determinations were analyzed statistically and are shown in Table XXIX.

Data in Table XXIX indicate that the 99 percent confidence limits for NDGA was approximately ± 2.5 percent and for BHA was approximately ± 1.3 percent of the amount present.

Table XXIX

Reproducibility of the Analytical Values for BHA and NDGA

Antioxidant	Mean of Six Determinations Percent	Standard Deviation	99 Percent Confidence Limits
NDGA (with BHA)	0.01059	0.00006	± 0.00025
BHA (with NDGA)	0.01942	0.00006	± 0.00024

G. Lauryl Gallate

1. INTRODUCTION

As has been stated already, gallic acid and propyl gallate are powerful antioxidants for lard and other dry fats (102), but their solubility in fats is low (85,102) and in addition their activity is not carried over into baked goods (102). Morris et al. (105) synthesized the octyl-, dodecyl-, tetradecyl-, hexadecyl- and octadecyl- esters of gallic acids and stated that these higher esters are readily soluble in fats and oils (105) which is a very important factor facilitating the commercial stabilization of fats. Morris et al. (105) state that these gallate esters, exhibit about the same order of antioxidant activity in lard as does NDGA or gallic acid as measured by the active oxygen method. These workers (105) also determined the carry-over of the antioxidant properties of these higher gallate esters into baked pie crust by means of storage tests at 38° and 63°C. Their results show that the higher gallates have good protective action in baked pie crust and are far superior to gallic acid or propyl gallate in this respect. Of the higher gallate esters, lauryl gallate has received a considerable amount of attention. Therefore, it was decided to try to devise an analytical method for dodecyl- (lauryl) gallate in fat.

2. TOXICITY OF LAURYL GALLATE

With respect to the possible toxicity of lauryl gallate Allen and De Eds (1) made the following assumptions:- If it is assumed that lauryl gallate is hydrolyzed in the digestive tract, there is little reason to believe that the resulting lauryl alcohol and gallic acid would exert any toxic actions. Lauryl alcohol might be oxidized to lauric acid, which occurs as a glyceride in many vegetable fats, for example coconut oil, and is presumably harmless. Gallic acid is widely distributed in the form of gallo-tannin in vegetable foods and is stated to occur in the free state in tea (124) and in the pomegranet (107). Gallo-tannin is not absorbed from the gastrointestinal tract but is hydrolyzed to gallic acid, which is readily absorbed and oxidized in the body. Although such speculation suggests that the repeated daily ingestion of reasonable amounts of lauryl gallate would not produce chronic toxicity, prolonged feeding experiments with albino rats were conducted for a period of eight months. Allen and De Eds (1) state that long-term feeding experiments with albino rats involving concentrations of lauryl gallate as high as 0.5 percent of the daily diet produced no deleterious effects as judged by food intake, rate of growth, gross observations at time of autopsy and histopathological examination of the tissues. Diets containing 1.0 percent of lauryl gallate definitely inhibited growth of male rats and

retarded the growth of females slightly. Levels of 2.5 and 5.0 percent of lauryl gallate in the diet caused no histopathological changes in the various organs but decreased food intake to the point where starvation was an obvious factor contributing to the death of the animals. There is no evidence that ingestion of slight amounts, such as might be encountered in edible fats and oils protected against oxidation, would cause harmful effects.

3. SYNTHESIS OF LAURYL GALLATE

The direct esterification of gallic acid with the lower alcohols containing up to six carbon atoms has been accomplished by various workers (22,94,127). Morris and Riemenschneider (105) describe a procedure for the indirect esterification of gallic acid with the higher fatty alcohols. Ault et al. (3) described the direct esterification of gallic acid with the normal aliphatic alcohols having even numbers of carbon atoms from eight to eighteen with the exception of decyl-gallate.

The synthesis of lauryl gallate was based on the direct method of esterification described by Ault et al. (3) and was as follows:-

Technical grade gallic acid was decolourized with activated carbon, recrystallized twice from glass distilled water and then heated to 125°C for four hours to remove water

of crystallization.

The lauryl alcohol employed was purified by distillation through a fractionating column under reduced pressure and the middle-portion of the distillate was used.

Other reagents employed were not subjected to additional purification.

One hundred and two g. of anhydrous gallic acid and 224 g. of lauryl alcohol were refluxed slowly in 1070 ml. of anisole and 62 ml. of nitrobenzene in the presence of 5 g. of naphthalene-β-sulfonic acid for twenty hours. The refluxing solvents were circulated through a Barrett-type distilling receiver to separate the water produced during the esterification. After the reaction was completed the solvent mixture was subjected to steam distillation until a considerable portion of the unreacted lauryl alcohol had been removed. The reaction product was then dissolved in two liters of benzene and washed with water to remove the excess gallic acid and naphthalene-β-sulfonic acid. The lauryl gallate was precipitated by the addition of light petroleum. To ensure complete removal of gallic acid, the lauryl gallate was dissolved in warm absolute ethyl alcohol and the lauryl gallate precipitated by the addition of cold glass distilled water. This precipitate was filtered, sucked dry of solvent and dried in vacuo. This purification step was repeated until there was no further change in the

infra-red absorption spectra of the precipitate. This product was employed for all subsequent experiments.

4. DEVELOPMENT OF ANALYTICAL METHOD

a. Choice of Reagents

As previously stated, propyl gallate, lauryl gallate and perhaps other higher gallate esters react with ammonia to produce an intense rose colour. Because of the high sensitivity of this qualitative test for these gallates, considerable effort was expended to devise a quantitative method for lauryl gallate based on this reaction. In the course of preliminary studies, it was found that other alkalis such as sodium, or potassium hydroxide failed to produce this pink colour with propyl gallate. On the other hand, the addition of sodium methylate in anhydrous methanol produced an intense transitory rose colour with propyl gallate. After investigating other combinations of alkalis and organic solvents it was found that propyl gallate in the presence of 10 to 70 percent acetone produced an intense rose colour with sodium carbonate, and the hydroxides of sodium and potassium. Lauryl gallate reacted in an analagous manner as described for propyl gallate. In the absence of acetone no rose colour was produced unless ammonium hydroxide was employed as the alkali. These reactions were studied in some detail as described in the following sections.

i. The Effect of the Concentrations of Acetone and Sodium Carbonate Upon the Colour Reaction with Lauryl Gallate

A number of 1 ml. portions of acetone containing 200 micrograms of lauryl gallate were placed into colorimeter tubes. Different amounts of acetone and aqueous sodium carbonate solutions were added to each tube to produce a final volume of 10 ml. The resulting colour formation was followed in an Evelyn colorimeter fitted with a No. 515 filter, all measurements were made relative to appropriate "blanks". The results of these experiments are summarized in Table XXX.

Table XXX

Effect of Acetone and Sodium Carbonate Concentrations on the Absorbancy Produced with Lauryl Gallate

Percent Acetone	Percent Aqueous Sodium Carbonate	Maximum Absorbancy Obtained with a No. 515 Filter within 15 Minutes					
		Concentration of Sodium Carbonate Solution Employed					
		0.025%	0.10%	0.30%	0.50%	1.00%	1.50%
10	90	0.063	0.100	0.053	0.000	0.000	0.000
20	80	-	-	-	-	0.201	0.245
30	70	0.213	0.370	0.471	0.462	0.495	-
40	60	-	-	-	0.463	0.541	0.550
50	50	0.235	0.359	0.458	0.481	0.555	T
70	30	0.100	0.255	T	T	T	T

T Solution turbid, due to the high concentration of acetone which precipitates the higher concentrations of sodium carbonate.

Results in Table XXX indicate that 40 to 50 percent acetone together with 50 to 60 percent of a 1.0 to 1.5 percent sodium carbonate solution produced maximum colour absorbancy with lauryl gallate. Therefore, 50 percent acetone and 50 percent of a 1.0 percent aqueous solution of sodium carbonate were chosen as the reagents for the analysis of lauryl gallate. The higher the concentration of sodium carbonate or the lower the acetone concentration, the faster is the colour formation. Low acetone concentrations (10 percent) and relatively high sodium carbonate concentrations (0.5 percent or over) produce yellow colours with lauryl gallate.

b. Characteristics of the Lauryl Gallate -Acetone - Sodium Carbonate Colour Reaction

i. Absorption Curve for the Colour Formed by the Lauryl Gallate - Acetone-Sodium Carbonate Colour Reaction

Five ml. of an acetone solution containing approximately 22 micrograms of lauryl gallate was added to 5 ml. of aqueous 1.0 percent sodium carbonate reagent. The resulting colour was allowed to reach maximum intensity and the absorption curve determined relative to a "blank" employing a Beckman B spectrophotometer fitted with a one cm. cell. The resulting data are given in Figure 10.

These results show that lauryl gallate in the presence of acetone and sodium carbonate exhibits an

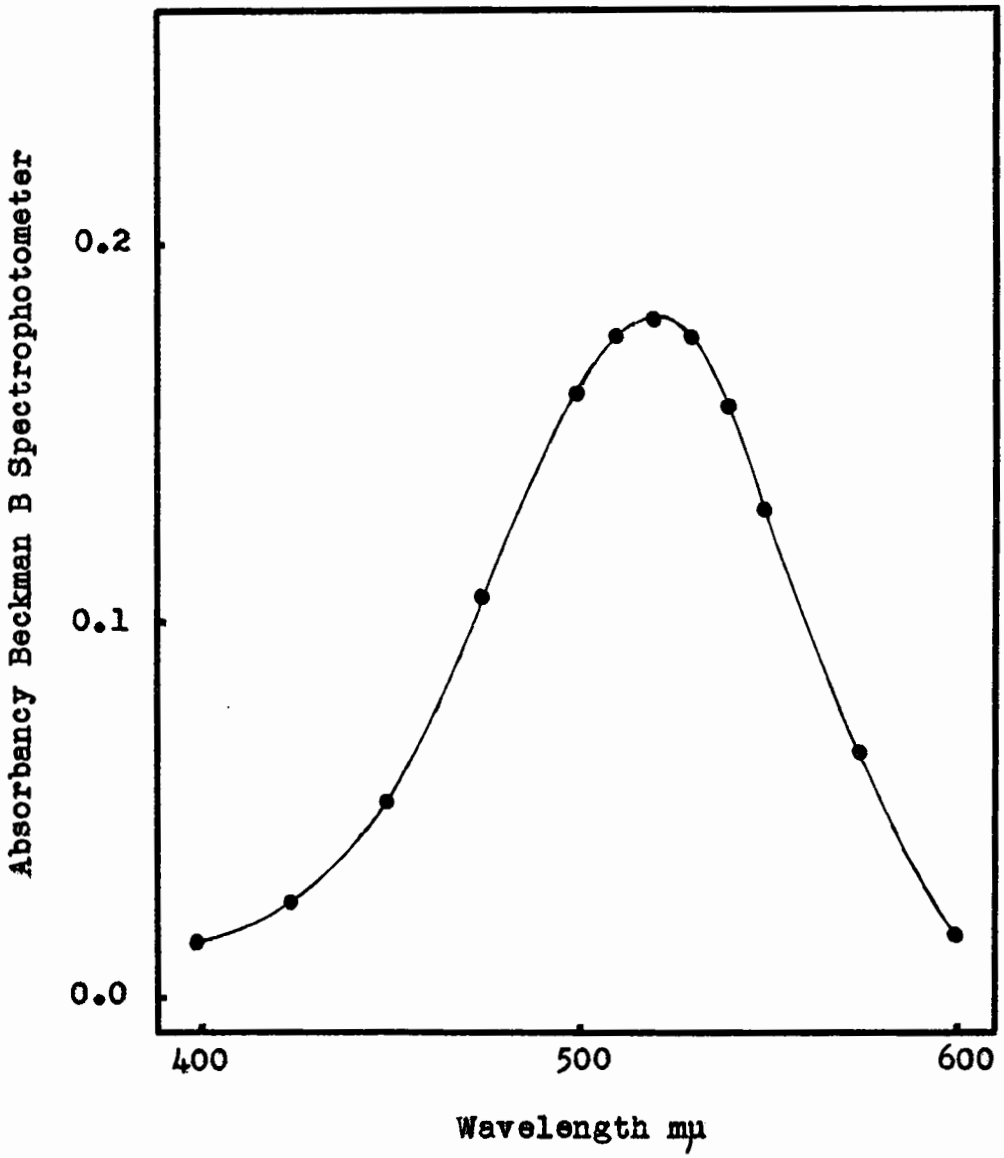


Figure 10 Absorption Curve for Lauryl Gallate in the Presence of Acetone and Sodium Carbonate

absorption maximum at 520 mμ. Therefore, when employing an Evelyn colorimeter, a No. 520 filter was employed to measure the absorbancy produced by lauryl gallate.

ii. Calibration Curve for Lauryl Gallate in Acetone Solution

Different volumes of a standard acetone solution of lauryl gallate were placed in Evelyn colorimeter tubes and diluted to 5 ml. with acetone. Five ml. of 1.0 percent aqueous sodium carbonate reagent was added to each tube and the maximum colour intensity was determined with an Evelyn colorimeter fitted with a No. 520 filter. The resulting data are given in Table XXXI.

Table XXXI

Calibration Curve for Lauryl Gallate in Acetone Solution

Lauryl Gallate Micrograms	Absorbancy with a No. 515 Filter	Absorbancy/Microgram of Lauryl Gallate "K-Value"
80	0.230	0.00288
160	0.451	0.00282
180	0.520	0.00288
200	0.562	0.00281
200	0.570	0.00285
240	0.693	0.00287
300	0.856	0.00285

Results in Table XXXI shows that the lauryl gallate colour reaction obeys Beer's Law over the range of 80 to 300

micrograms per 10 ml.

iii. Extraction of Lauryl Gallate From Fats

Previous investigations showed that lauryl gallate exhibited maximum colour formation with sodium carbonate in the presence of 40 to 50 percent acetone. In view of this fact it was necessary to try acetone solutions for the extraction of lauryl gallate from fat. This approach was doomed to failure, because, lauryl gallate is very soluble in fat and insoluble in water. Therefore, aqueous acetone solutions would not be expected to act as efficient extractants of lauryl gallate from fat in light petroleum solution. When high concentrations of acetone were employed these solutions dissolved in the fat - light petroleum solution. After a few attempts this extractions procedure was abandoned.

Instead, lard containing lauryl gallate was dissolved in a mixture containing equal quantities of acetone and light petroleum. Aqueous 1 percent sodium carbonate reagent was then added to this solution in sufficient quantity to separate an aqueous phase containing 40 to 50 percent of acetone. This aqueous-acetone phase contains all the rose-coloured reaction product of lauryl gallate, and may be run off, filtered and the absorbancy measured at 520 mμ. Employing this procedure it was possible to extract the lauryl gallate and develop the coloured product in a single step.

iv. Colour Stability

Ten g. of lard containing 0.016 percent lauryl gallate were dissolved in 110 ml. of a solvent mixture containing 50 percent acetone and 50 percent 60 - 100°C light petroleum. Thirty ml. of this solution containing approximately 400 micrograms of lauryl gallate were placed in a 20 x 150 m.m. test tube and 10 ml. of aqueous 1.0 percent sodium carbonate reagent was added. The contents of the test tube were shaken for five seconds and the phases allowed to separate. After approximately one minute the light petroleum - fat phase was removed with the help of a suction tube and the aqueous - acetone phase filtered through a 9 cm. Whatman No. 42 filter. The filtrate was collected in an Evelyn colorimeter tube and the absorbancy measured relative to a "blank", employing an Evelyn colorimeter fitted with a No. 520 filter. The effect of time on the lauryl gallate - acetone - sodium carbonate reaction is illustrated in Table XXXII.

Table XXXII shows that maximum colour intensity was obtained five minutes after adding the sodium carbonate solution.

Table XXXII

Effect of Time on the Lauryl Gallate - Acetone - Sodium Carbonate Colour Reaction

Reaction Time in Minutes	Absorbancy with a No. 520 Filter
2	0.540
3	0.562
4	0.570
5	0.574
6	0.572
8	0.570
10	0.565
12	0.562
15	0.552

v. Calibration Data for Lauryl Gallate in Lard

Ten g. of lard containing 0.016 percent lauryl gallate were dissolved in 110 ml. of a solvent mixture containing 50 percent acetone and 50 percent light petroleum by volume. Different portions of this solution were placed in 20 x 150 m.m. test tubes and the colour developed with sodium carbonate as previously described. The results are given in Table XXXIII.

Results in Table XXXIII indicate that the lauryl gallate - acetone - sodium carbonate colour reaction obeys Beer's Law over the range of 80 to 320 micrograms of lauryl gallate in 18 ml. of aqueous acetone solution.

Table XXXIII

Calibration Data for Lauryl Gallate in Lard

Lauryl Gallate Micrograms	Absorbancy with a No. 520 Filter for Aqueous-Acetone Phase (18 ml.)	Absorbancy/Micrograms of Lauryl Gallate "K-Value"
80	0.125	0.00156
160	0.248	0.00155
240	0.368	0.00153
320	0.490	0.00153
400	0.572	0.00143

5. ANALYTICAL METHOD

a. Reagents

Light Petroleum - 60 to 100°C boiling range - (Skellysolve H) - redistilled in all-glass equipment.

Acetone - Acetone distilled in all-glass apparatus.

Sodium Carbonate - 1.0 percent aqueous solution of anhydrous sodium carbonate.

b. Quantitative Procedure

Dissolve 10 g. of fat containing lauryl gallate in 55 ml. of light petroleum plus 55 ml. of acetone. Place three portions of this solution, ranging from 3 to 30 ml. into 20 x 150 m.m. test tubes and dilute to 30 ml. with a similar fat solution not containing lauryl gallate. Add 10 ml. of 1.0 percent aqueous sodium carbonate solution and mix the

contents. After one minute remove the upper fat - light petroleum layer with a suction tube. Filter the aqueous - acetone layer through a 9 cm. Whatman No. 42 filter and collect the filtrate in a colorimeter tube. Five minutes after adding the sodium carbonate reagent, measure the absorbancy with an Evelyn colorimeter fitted with a No. 520 filter relative to a "blank". Under these conditions it was found that the observed absorbancy divided by a "K-value" of 0.00154 yielded the concentration of lauryl gallate expressed as micrograms per portion employed.

c. Reproducibility of Analytical Results

Lauryl gallate is not permitted to be added to edible fats in either Canada or the United States. Therefore, this procedure was merely devised to estimate the lauryl gallate content of experimental fats. For the above reasons no data on the reproducibility of this analytical method were obtained. It is felt, however, that the deviations of this simple procedure are unlikely to exceed ± 5 percent at a level of 0.016 percent lauryl gallate in lard.

H. Total Tocopherol in Lard and Shortening

1. INTRODUCTION

Little or no tocopherol is removed from a fat in a light petroleum solution upon being shaken with aqueous ammonium acetate or 72 percent ethyl alcohol employed for the extraction of propyl gallate, nordihydroguaiaretic acid, (NDGA), or butylated hydroxyanisole (BHA) in the foregoing procedures. Therefore, the fat solution after the extraction of these antioxidants can be employed for the estimation of total tocopherol in lard or shortening. Since both lard and the vegetable oils used in shortenings contain one or more of the α-, β-, γ- or δ- tocopherols, no attempt was made to determine the individual tocopherols. Instead, the total tocopherol content was estimated and expressed in terms of α-tocopherol.

There is an extensive literature on the chemical determination of tocopherol; only a few leading publications exemplifying major steps in the development of present-day methods will be mentioned. The Emmerie-Engel method (35,36), generally used for the determination of total tocopherol, is based upon the quantitative reduction of ferric ions by the tocopherols and the reaction of the ferrous ions formed to produce a coloured complex with α,α'-bipyridine. A large number of modifications of this procedure have been developed, most of them involving the preliminary treatment of the

sample for the removal of interfering substances. This is necessary because the reduction of ferric ion is not specific for the tocopherols and also because the different tocopherols differ in their rates of reaction (72).

Quaife et al. (116,117) introduced mild hydrogenation of tocopherol-containing lipids, to eliminate interference from vitamin A, carotenoids and other components in the Emmerie-Engel reaction. Later, as Dam et al. had done before (28, 49) Quaife and Harris (118) employed molecular distillation to concentrate the tocopherols from lipids of low tocopherol content and also to eliminate minor interfering constituents. These procedures, although highly effective, are not applicable to rapid routine analysis.

Fox and others (18,45) extracted the fat in light petroleum solution with 60 percent v/v sulphuric acid to remove vitamin A. Parker and McFarlane (40,113) employed 85 percent v/v sulphuric acid to remove carotenoids from wheat germ oil. It has since been shown by Chapman and Campbell (18) that the use of 85 percent v/v sulphuric acid results in the destruction of non-α-tocopherols, while 60 percent v/v sulphuric acid does not destroy tocopherol. Kjolhede (71) employed adsorption on floridin earth to remove interferences. However, there is considerable risk of tocopherol destruction during adsorption or chromatographic procedures. Meunier and Vinet (100) passed a benzene solution of the fat

through a column of montmorillonite clay and claimed that the carotenoids, vitamin A, sterols, phospholipids, chlorophyll, pyrroles and bilirubin were retained on the column while the tocopherols and neutral fat passed through.

Saponification has been employed as a means of removing the fat and concentrating the unsaponifiable. This approach has been studied extensively. Results reported vary from statements that saponification cannot be used, to statements that it is necessary in order to get reliable results (37,38, 102,118). Studies with the pure tocopherols (34,135), show that they are unstable in the presence of alkali unless oxygen is rigidly excluded. Therefore, any method employing saponification must necessarily provide some means of protecting the saponification mixture from oxidation.

Quaife et al. (119) employed a correction factor, namely, absorbancy of the fat solution at 460 mμ to correct for errors due to carotene in the Emmerie and Engel procedure. This is a rapid and apparently accurate procedure. Furter and Meyer (46) oxidized tocopherol with nitric acid and employed the resulting red-coloured quinoid form as a measure of tocopherol. This method, however, is not very sensitive but avoided errors due to other reducing impurities. Tocopherol may also be estimated by titration with ceric sulphate in the presence of p-ethoxy-chrysoidine (131) as an indicator and auric chloride may also be employed (133).

Meunier and Vinet (98,99) employed the reduction of ferric chloride in the presence of potassium ferricyanide for the estimation of tocopherol. Ramachandran and Rau (120) reported close agreement between the Meunier and Vinet and the Emmerie and Engel procedures for the determination of total tocopherol.

In addition to the foregoing methods there are a large number of methods designed for the estimation of one or more of the four known tocopherols. However, these methods will not be reviewed here.

The Emmerie and Engel (35) method was chosen for the estimation of total tocopherol because of its rapidity and relatively high sensitivity. Parker and McFarlane (113), employed a modification of the Emmerie and Engel procedure, to determine total tocopherol in wheat germ oil. This method consisted of adding an alcoholic solution of ferric chloride and α,α'-bipyridine to a 1 or 2 percent solution of wheat germ oil in light petroleum. Total tocopherol was estimated by measuring the absorbancy at 515 mμ in the ethyl alcohol - light petroleum - fat solution. This procedure is satisfactory for wheat germ oil which contains as much as 0.55 percent tocopherol (4). However, when the Parker and McFarlane method was applied to lard, which contains only 0.003 percent tocopherol (20), virtually no absorbancy was produced. In order to increase the sensitivity of this

method it was necessary to increase the concentration of the fat solution employed. However, since fats and oils such as lard, butterfat, coconut, soybean, and cottonseed exert a depressing effect on the intensity of colour produced by tocopherol on reaction with ferric chloride and α,α'-bipyridine (67) there was little scope for increasing the sensitivity of the method by increasing the concentration of fat employed. The colour depressing effect of fats and hydrocarbons is attributed to interaction between these compounds and the ferrous and ferric ions of the reagent (68).

In order to overcome the effect of high fat concentrations upon colour formation, it was decided to add water to the light petroleum - ethyl alcohol - fat solution in order to reduce the ethyl alcohol concentration to less than 90 percent. This causes an ethyl alcohol phase to separate carrying with it all of the coloured reaction products. At the same time, about 98 percent of the fat remains behind in the light petroleum phase. Thus freed of much of the fat, the resulting colour intensity is not suppressed to such an extent and, therefore, higher fat concentrations can be employed. A more sensitive method for estimating total tocopherol in lard or shortening was developed on the basis of these observations.

2. DEVELOPMENT OF ANALYTICAL METHOD

a. Choice of Reagent Concentrations

A 1.5 percent solution of hydrogenated soybean oil which contains the α-, β-, γ- and δ- tocopherols (116) was prepared in 60 - 100°C light petroleum. Ten ml. portions of this solution were placed into 125 ml. low-actinic-glass separatory funnels. Into a similar series of separatory funnels were placed 10 ml. portions of 60 - 100°C light petroleum to be employed as "blanks". Different volumes of 0.2 percent ferric chloride ($FeCl_3.6H_2O$) and 0.5 percent α,α'-bipyridine reagents in 100 percent ethyl alcohol were added. In all cases a complementary volume of 100 percent ethyl alcohol was added to adjust the total volume of ethyl alcohol to 10 ml. Six minutes after adding the ferric chloride reagent, 1.2 ml. of water was added to reduce the ethyl alcohol concentration to approximately 90 percent and the separatory funnel inverted twice to mix the contents. After nine minutes the alcoholic layer was run off, filtered and the absorbancy measured at ten minutes relative to an appropriate "blank" employing an Evelyn colorimeter fitted with a No. 515 filter. Some of these results are given in Table XXXIV.

The results shown in Table XXXIV indicate that the resulting absorbancy obtained with the tocopherols of

Table XXXIV

Effect of the Amount of Ferric Chloride and α,α'-Bipyridine Upon the Absorbancy Produced with the Tocopherols of Hydrogenated Soybean Oil

Volume of 0.5% α,α'-Bipyridine	Absorption Measured with a No. 515 Filter							
	Volume of 0.2 Percent Ferric Chloride ml.							
ml.	0.25	0.5	0.75	1.0	2.0	3.0	4.0	5.0
1.0	-	-	0.910	0.735	0.396	0.179	-	-
2.0	-	0.901	0.928	0.910	0.842	0.642	0.461	0.237
3.0	0.870	0.955	0.939	0.936	0.918	0.850	-	-
4.0	0.907	0.963	0.966	0.961	0.965	0.967	0.876	-
5.0	0.921	0.961	0.966	0.959	0.962	0.962	0.947	-

hydrogenated soybean oil is influenced by the amount and ratio of the ferric chloride to α,α'-bipyridine reagents. In all cases, increasing the amount of α,α'-bipyridine resulted in increased absorbancy. When 6 ml. of 0.5 percent α,α'-bipyridine solution was employed, the resulting alcoholic phase was turbid. In the presence of 4 or 5 ml. of 0.5 percent α,α'-bipyridine, maximum absorbancies were obtained with 0.5 to 3.0 ml. of 0.2 percent ferric chloride solution. Apparently, any reagent combination within these limits would prove equally satisfactory. Therefore, 1 ml. of 0.2 percent ferric chloride (2 mg. of $FeCl_3.6H_2O$) together with 4 ml. of 0.5 percent α,α'-bipyridine (20 mg.) were chosen for the analysis of tocopherol.

b. Characteristics of the Tocopherol - Ferric Chloride - α,α'-Bipyridine Reaction

i. Effect of the Volume of Light Petroleum, Containing the Fat Sample, Upon the Resulting Absorbancy

A series of experiments were conducted to determine the effect of the volume of light petroleum employed to dissolve the fat sample, upon the resulting absorbancy. One hundred mg. portions of hydrogenated soybean oil contained in 5 to 60 ml. of 60 - 100°C light petroleum were reacted with 5 ml. of 0.04 percent ferric chloride (2 mg. $FeCl_3.6H_2O$) and 5 ml. of 0.4 percent α,α'-bipyridine (20 mg.) as previously described and the resulting absorbancies measured with a No. 515 filter in an Evelyn colorimeter. The results of these experiments are given in Table XXXV.

Table XXXV

Effect of the Volume of Light Petroleum Employed to Dissolve the Fat Upon the Resulting Absorbancy

Volume Light Petroleum Containing Fat Sample ml.	Absorbancy Measured with No. 515 Filter	Volume of Alcoholic Phase ml.	Relative Sensitivity Absorbancy x Volume
5	0.703	14.6	104.0
10	0.725	13.6	100.0
15	0.745	12.5	94.5
20	0.735	12.3	91.9
25	0.742	11.0	82.9
40	0.764	9.0	69.7
60	-	5.5	Colour not completely extracted from the light petroleum phase

Results in Table XXXV show that the larger the volume of light petroleum employed to dissolve the fat sample, the lower became the relative sensitivity of the method. If more than 25 ml. of light petroleum was employed, the red ferrous bipyridine colour was not completely extracted into the alcoholic phase. Therefore, 10 ml. of 60 - 100°C light petroleum was retained for dissolving the fat sample, because the use of 5 ml. would necessitate the use of still higher fat concentrations which are undesirable.

ii. Effect of Varying the Time Interval Between the Addition of the Reagents and the Separation of the Alcoholic Phase

Ten ml. portions of a 1.0 percent solution of hydrogenated soybean oil in 60 - 100°C light petroleum were placed in 125-ml. low-actinic-glass separatory funnels. Five ml. of 0.04 percent ferric chloride and 5 ml. of 0.4 percent α,α'-bipyridine reagents were added. After intervals of two to fifteen minutes, 1.2 ml. of water was added and the separatory funnels inverted twice. After a further three minutes the alcoholic phase was run off, filtered and the absorbancy measured one minute later in an Evelyn colorimeter fitted with a No. 515 filter relative to a similarly treated "blank". The results of these experiments are reported in Table XXXVI.

It is evident that increasing the time interval between the addition of the reagents and the separation of the

Table XXXVI

Effect of the Time Interval Between the Addition of the Reagents and the Separation of the Alcoholic Phase

Time Between Addition of Reagents and Separation of the Alcoholic Phase Minutes	Absorbancy Measured with No. 515 Filter	Relative Absorbancy %
2	0.645	94.0
4	0.670	97.7
6	0.686	100.0
10	0.695	101.3
15	0.693	101.0

alcoholic phase from six to ten or fifteen minutes only increased the resulting absorbancy by 1 percent. Therefore, an interval of six minutes was retained between the addition of the reagents and the separation of the alcoholic phase.

iii. Effect of Varying the Time Interval Between the Separation of the Alcoholic Phase and the Measurement of the Absorbancy

Ten ml. portions of a 1 percent solution of hydrogenated soybean oil in 60 - 100°C light petroleum were placed in 125-ml. low-actinic-glass separatory flasks and 5 ml. of 0.04 percent ferric chloride and 5 ml. of 0.4 percent α,α'-bipyridine reagent added. Six minutes after adding the ferric chloride reagent 1.2 ml. of water was added and the flasks inverted twice. After intervals of one to fourteen minutes the alcoholic phase was run off, filtered and the

absorbancy measured one minute later at 515 mμ relative to an appropriate "blank". The results of these experiments are given in Table XXXVII.

Table XXXVII

Effect of Time Interval Between Separation of the Alcoholic Phase and Measurement of the Absorbancy

Time Between Separation of Alcoholic Phase and Measurement of Absorbancy Minutes	Absorbancy Measured with No. 515 Filter	Relative Absorbancy %
2	0.706	96.0
4	0.736	100.0
6	0.736	100.0
10	0.733	99.6
15	0.738	100.3

Results in Table XXXVII show that almost identical results were obtained when there was a time interval of four to fifteen minutes between the separation of the alcoholic phase and the measurement of the absorbancy. For future work an interval of six minutes was adopted between the separation of the alcoholic phase and the measurement of the absorbancy with a No. 515 filter.

iv. Effect of the Volume of Water Employed to Cause the Separation of the Alcoholic Phase

Ten ml. portions of a 0.8 percent solution of hydrogenated soybean oil in 60 - 100°C light petroleum were

placed in 125-ml. low-actinic-glass separatory flasks and 5 ml. of 0.04 percent ferric chloride and 5 ml. of 0.4 percent α,α'-bipyridine reagent added. Six minutes after adding the ferric chloride, 0 to 5 ml. of water were added and the flasks inverted twice. After a further five minutes the alcoholic phase was run off, filtered and the absorbancy measured one minute later in an Evelyn colorimeter fitted with a No. 515 filter, relative to an appropriate "blank". The volume of the alcoholic phase was also measured. The results of the experiments are given in Table XXXVIII.

Table XXXVIII

Effect of the Volume of Water Employed to Cause the Separation of the Alcoholic Phase

Volume of Water Added ml.	Volume of Alcoholic Phase ml.	Absorbancy with a No. 515 Filter
0.0	* 20.0	0.414
1.0	14.3	0.553
1.2	13.5	0.582
1.4	13.1	0.583
2.0	12.8	0.574
3.0	13.2	0.555
4.0	13.8	0.532
5.0	14.7	0.498

* In this case no water was added and the absorbancy was measured in the ethyl alcohol - light petroleum - fat solution.

Results in Table XXXVIII indicate that the addition of

2 ml. of water resulted in the minimum volume of alcoholic phase. In the vicinity of 2 ml., the effect of different volumes of water added, resulted in the smallest difference in the volume of the alcoholic phase. For integral volumes of added water, the maximum absorbancy with a No. 515 filter was obtained with 2 ml. Therefore, 2.0 ml. of water was selected to cause the separation of the alcoholic phase. Although slightly larger absorbancies were obtained when 1.4 ml. of water was added, it appeared desirable to employ a volume of 2.0 ml. because of the greater ease and accuracy of measurement.

v. Absorption Curve for the Tocopherol - Ferric Chloride - α,α'-Bipyridine Colour

The absorption curve for the tocopherol - ferric chloride - α,α'-bipyridine coloured reaction product was determined relative to a reagent "blank" employing a Beckman B spectrophotometer. The resulting absorption curve is given in Figure 11.

These results show that the maximum absorption occurred at 522 mμ. Therefore, either a No. 515 or No. 520 filter was employed to measure the absorbancy with an Evelyn colorimeter.

vi. Calibration Data for α-Tocopherol

Typical calibration data for α-tocopherol employing the foregoing conditions are illustrated in Table XXXIX.

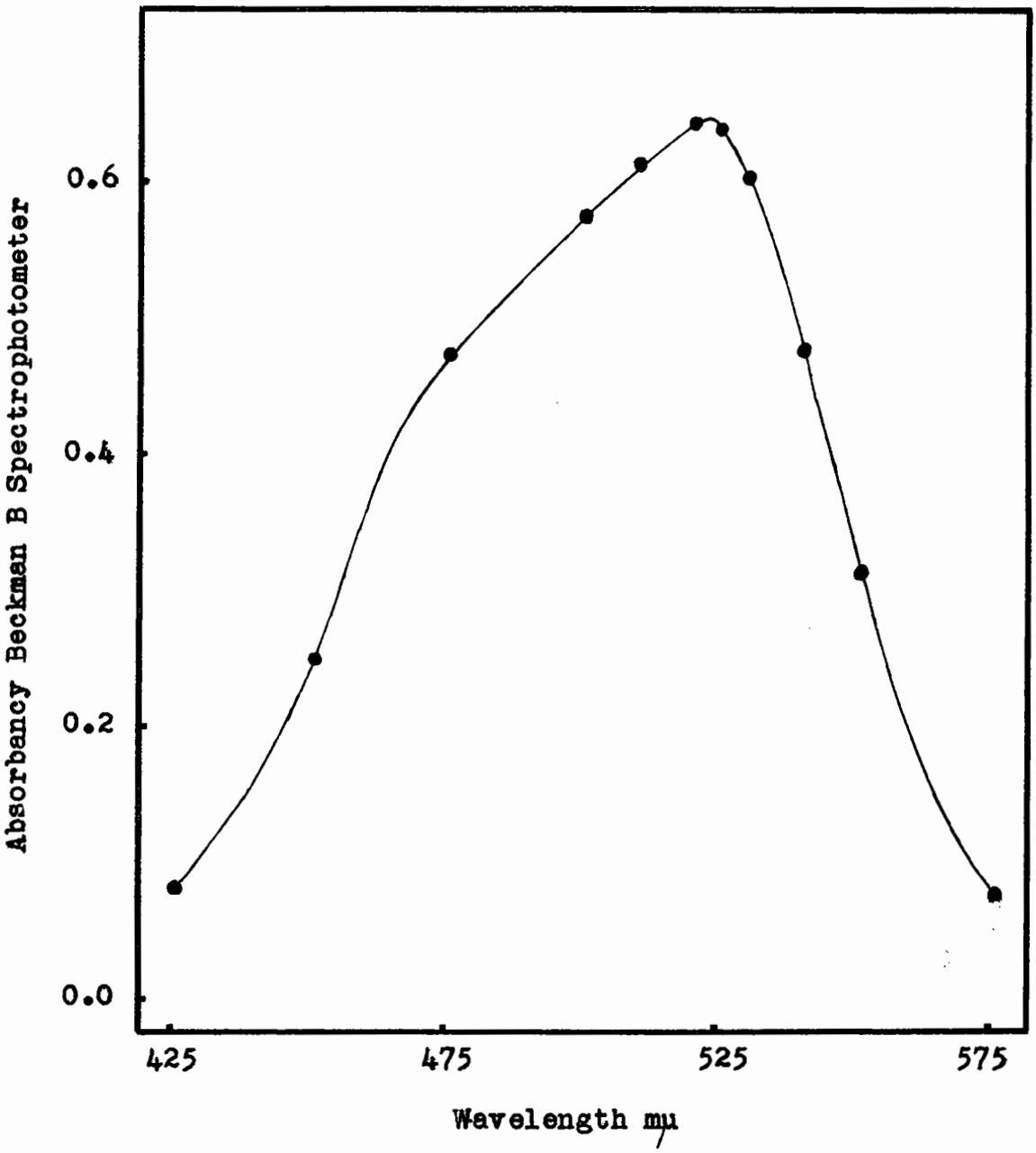


Figure 11 Absorption Curve for Tocopherol on Reaction with Ferric Chloride - α,α'-Bipyridine Reagents

Table XXXIX

Calibration Data for α-Tocopherol

α-Tocopherol/10 ml. Micrograms	Absorbancy with No. 515 Filter	Absorbancy per Microgram Tocopherol "K-Value"
20	0.100	0.00500
30	0.155	0.00517
40	0.204	0.00510
50	0.249	0.00498
60	0.303	0.00505
70	0.355	0.00507
80	0.403	0.00504
120	0.593	0.00494
160	0.776	0.00485
200	0.951	0.00475

Table XXXIX indicates that Beer's law applies over the range of 20 to 80 micrograms of α-tocopherol per 10 ml. Within this range the "K-value" was 0.00506 ± 0.0001. In excess of 100 micrograms of tocopherol per 10 ml. the resulting "K-value" decreases slightly.

3. ANALYTICAL METHOD

a. Reagents

Light Petroleum - Shake 60 - 100°C boiling range light petroleum (Skellysolve H), with one-fifth its volume of concentrated sulphuric acid, wash with water, then with dilute sodium hydroxide, until free of acid and redistilled in all-glass equipment.

Ethyl Alcohol - Distill absolute ethyl alcohol over potassium permanganate and potassium hydroxide in all-glass equipment.

Ferric Chloride - Prepare 0.04 percent $FeCl_3.6H_2O$ in redistilled 100 percent ethyl alcohol.

α,α'-Bipyridine - Prepare 0.04 percent α,α'-bipyridine in redistilled 100 percent ethyl alcohol.

α-Tocopherol - Prepare a solution containing 10 micrograms of α-tocopherol per ml. of light petroleum.

b. Quantitative Procedure for Total Tocopherol in Fats and Oils

The fat in light petroleum solution, from which the propyl gallate, BHA and/or NDGA have been extracted, is employed for the determination of tocopherol. Transfer this fat solution (containing 10 g. of fat) to a 200 ml. volumetric flask and wash the separatory funnel several time with light petroleum. Combine the washings and dilute to volume with light petroleum. If qualitative tests have shown that the fat does not contain propyl gallate, BHA, or NDGA, a 1 to 5 percent solution of the fat in light petroleum can be employed without any previous extraction. A treatment to remove interfering substances appears to be unnecessary when analyzing lard and shortening.

Place three duplicate portions of the fat solution, varying from 1 to 8 ml., in a duplicate series of 125-ml.

low-actinic-glass-stoppered separatory funnels. If it is desired to employ an internal standard add 2 ml. portions of a light petroleum solution containing 10 micrograms of α-tocopherol per ml. to one series of tubes. Dilute the fat solution in all separatory funnels to 10 ml. with light petroleum. Add 5 ml. of 0.04 percent ferric chloride reagent and 5 ml. of 0.4 percent α,α'-bipyridine reagent. These reagents should be blown from the pipettes into the fat - light petroleum solution to ensure rapid mixing. Six minutes after adding the ferric chloride reagent, add 2 ml. of water and invert the separatory funnel twice. At eleven minutes run off the alcoholic layer, filter through a 9 cm. Whatman No. 54 filter and measure the absorbancy employing an Evelyn colorimeter fitted with a No. 515 filter at exactly twelve minutes. Measure all absorbancies relative to a "blank" prepared by using 10 ml. of light petroleum in place of the fat solution.

If an internal standard was employed the tocopherol content of the portion can be determined by the following formula:-

$$\frac{S}{ST - S} \times 20 = \text{micrograms of tocopherol per portion}$$

where, "S" is the absorbancy due to the tocopherol in the fat portion, and "ST" is the absorbancy due to the tocopherol in the fat portion plus 20 micrograms of α-tocopherol. It

is desirable to employ an internal standard when an unknown fat is being analyzed.

The most accurate range was from 20 to 80 micrograms of tocopherol per portion. Under these circumstances it was found that the measured absorbancy employing an Evelyn colorimeter divided by a "K-value" of 0.00506 gave the concentration of tocopherol in micrograms per portion.

4. CHARACTERISTICS OF THE QUANTITATIVE ANALYTICAL PROCEDURE

a. Calibration Data for the Tocopherols in Six Different Fats and Oils

Calibration curves were prepared with six different fats and oils in order to ascertain the depressing effect of these oils on the Emmerie-Engel colour reaction employing the foregoing analytical method. The results of these experiments are given in Table XL.

The data in Table XL illustrate the effects of different fats and oils on the reaction between tocopherol (present in these oils) and the ferric chloride - α,α'-bipyridine colour reaction. In all cases the absorbancy per mg. of oil tends to diminish slightly at the higher concentrations of each oil. However, this effect was also observed at the higher levels of the tocopherol in light petroleum (see Table XXXVIII). Therefore, fat apparently does not effect this colour reaction to any appreciable extent employing this procedure.

Table XL

Calibration Data for Tocopherol in Six Fats and Oils

Type of Oil	Weight Oil in 10 ml. mg. Portion	Absorbancy with No. 515 Filter	Absorbancy per mg. Oil
Hydrogenated Soybean Oil	24	0.164	0.0068
	48	0.328	0.0068
	72	0.496	0.0069
	96	0.662	0.0069
	120	0.804	0.0067
Corn Oil - "Mazola"	40	0.180	0.0045
	80	0.358	0.0045
	120	0.535	0.0045
	160	0.698	0.0044
	200	0.830	0.0042
Mixed Hydrogenated Vegetable Oils "Biscot"	30	0.122	0.0041
	60	0.240	0.0040
	120	0.474	0.0040
	180	0.674	0.0037
	240	0.880	0.0037
Cottonseed Oil	120	0.221	0.0018
	240	0.400	0.0017
	360	0.616	0.0017
	480	0.760	0.0016
Sunflowerseed Oil	80	0.119	0.0015
	160	0.240	0.0015
	240	0.377	0.0016
	320	0.506	0.0016
	400	0.630	0.0016
Butter Oil	100	0.033	0.00033
	300	0.091	0.00030
	500	0.145	0.00029
	700	0.205	0.00029
	900	0.263	0.00029

In the case of the routine analysis of the same fat employing the same concentration, it is possible to determine the "K-value" for added α-tocopherol in the presence of the fat and thereafter employ this "K-value" to estimate the total tocopherol content of the fat from the absorbancy measured with a No. 515 filter.

I. Total Tocopherol in Butter Oil

1. INTRODUCTION

The foregoing procedure described for the determination of total tocopherol in lard and shortening employing the ferric chloride plus α,α'-bipyridine reagents cannot be applied directly to butter oil. Because of the non-specific nature of these reagents, a number of substances present in butter oil contribute to excessively high "apparent" tocopherol values. The chief interfering substances are carotene, vitamin A and the synthetic colours Oil Yellow AB (1-phenylazo-2-naphthylamine) and Oil Yellow OB (1-ortho-tolylazo-2-naphthylamine). It is, therefore, necessary to remove these interfering substances or correct for their presence in order to obtain an accurate estimate of the tocopherol.

2. DEVELOPMENT OF ANALYTICAL METHOD

Fox and Mueller (45) state that all the vitamin A is removed by extracting fat in light petroleum solution with 60 percent v/v sulphuric acid. Chapman and Campbell (18) have shown that none of the tocopherols are lost during this procedure. Therefore, the effect of extracting the fat solution with 60 percent v/v sulphuric acid was investigated for the removal of interfering substances.

Before investigating the effect of sulphuric acid extractions on the interfering substances, it was necessary to ascertain the maximum concentration of these materials

that are likely to occur in butter oil. From these data it was possible to calculate the amount that might be found in a 5 percent solution of butter oil, which is the concentration employed for the analysis for total tocopherol.

Vladimir (144) gives the range of carotene in butter fat as from 1.8 to 30 micrograms per g. Therefore, a 5 percent solution of butter fat should contain no more than 1.5 micrograms of carotene per ml.

The Food and Drug Regulations (15) limit the amount of synthetic coal tar colours that may be added to a food to one part by weight in 3500 parts by weight of food. Since butter must contain at least 80 percent of butter fat (16), a maximum addition of synthetic colour would be equivalent to one part in 2800 parts of butter oil. Therefore, a 5 percent solution of butter oil would contain a maximum of 17.8 micrograms of synthetic colour per ml. In actual practice, the butter industry employs only a fraction of the maximal amount of synthetic colour permitted.

Butter contains very little vitamin A, and therefore if all the vitamin A activity of butter was due to vitamin A alcohol, a 5 percent solution of butter oil would contain no more than 0.6 micrograms of vitamin A per ml. However, a considerable portion of the vitamin A activity in butter is due to its carotene content, so that a 5 percent solution of butter oil should never contain as much as 0.6 micrograms of

vitamin A alcohol per ml.

a. Effect of Carotene, Oil Yellow AB, Oil Yellow OB and Vitamin A Alcohol Upon the Estimation of Total Tocopherol

Solutions were prepared containing 1.5 micrograms per ml. of crystalline 90 percent β-, 10 percent α-carotene; 17.8 micrograms per ml. of Oil Yellow AB; 17.8 micrograms per ml. of Oil Yellow OB; 0.6 micrograms per ml. of vitamin A alcohol; and 1.5 micrograms per ml. of α-tocopherol in 60 - 100°C light petroleum. Fifty ml. portions of these solutions were shaken in separatory funnels for fifteen seconds with 10 ml. portions of 60 percent v/v sulphuric acid. After standing for fifteen minutes the acid layer was run off and the light petroleum solution washed twice with 25 ml. portions of water accompanied by a few seconds shaking. Ten ml. portions of these solutions, before and after extraction with sulphuric acid, were reacted with the ferric chloride plus α,α'-bipyridine reagents as previously described for the analysis of tocopherol in lard and shortening. The resulting absorbancies were measured in an Evelyn colorimeter fitted with a No. 515 filter, relative to a "blank" employing a similarly treated portion of light petroleum. The measured absorbancies were expressed in terms of apparent α-tocopherol with the results given in Table XLI.

Results in Table XLI indicate that a single extraction of the light petroleum solutions with 60 percent sulphuric

Table XLI

Effect of Extracting Light Petroleum Solutions of Carotene, Oil Yellow AB, Oil Yellow OB, Vitamin A Alcohol and α-Tocopherol with 60 Percent v/v Sulphuric Acid, Upon the Apparent Tocopherol Content

Materials Present	Micrograms per 10 ml. portion	Apparent α-Tocopherol Before Acid Extraction Micrograms	Apparent α-Tocopherol After Acid Extraction Micrograms
90% β-, 10% α-Carotene	15	22.7	22.6
Oil Yellow AB	178	31.8	0.1
Oil Yellow OB	178	38.7	0.0
Vitamin A Alcohol	6	1.0	0.2
α-Tocopherol	15	14.9	14.9

acid removed all the vitamin A and synthetic butter colours. Since carotene is not removed by extraction with 60 percent v/v sulphuric acid, it is responsible for the chief interference in the determination of total tocopherol in the extracted solution of butter oil. Quaife et al. (119) employed the absorbancy of a fat solution at 460 mμ to form the basis of a correction for the error due to carotene. Employing crystalline 90 percent β-, 10 percent α-carotene, the nature of this correction factor was established and applied to the results obtained from the acid extracted solution of butter oil.

b. Determination of the Appropriate Correction Factor for Carotene

i. Carotene Absorption Curve

A solution was prepared containing 0.4 micrograms per ml. of crystalline 90 percent β-, 10 percent α-carotene in 60 - 100°C light petroleum. This solution and a similar volume of light petroleum to be used as a reference "blank" were extracted with 60 percent v/v sulphuric acid for fifteen seconds and washed with water, as previously described. The absorption curve for carotene in light petroleum solution was determined employing a Beckman B spectrophotometer, relative to a light petroleum "blank". The resulting data are given in Figure 12.

Figure 12 shows that the maximum absorption for crystalline 90 percent β-, 10 percent α-carotene in a light petroleum solution was located at 448 mμ with a second lower maximum at 475 mμ. Therefore, an Evelyn colorimeter fitted with a No. 440 filter was employed to measure the absorbancy due to carotene in light petroleum solution.

ii. Carotene Calibration Curve

Solutions were prepared containing 0.2 to 1.0 micrograms per ml. of crystalline 90 percent β-, 10 percent α-carotene in 60 - 100°C light petroleum. The absorbancies of these solutions were measured in an Evelyn colorimeter fitted with a No. 440 filter relative to a light petroleum

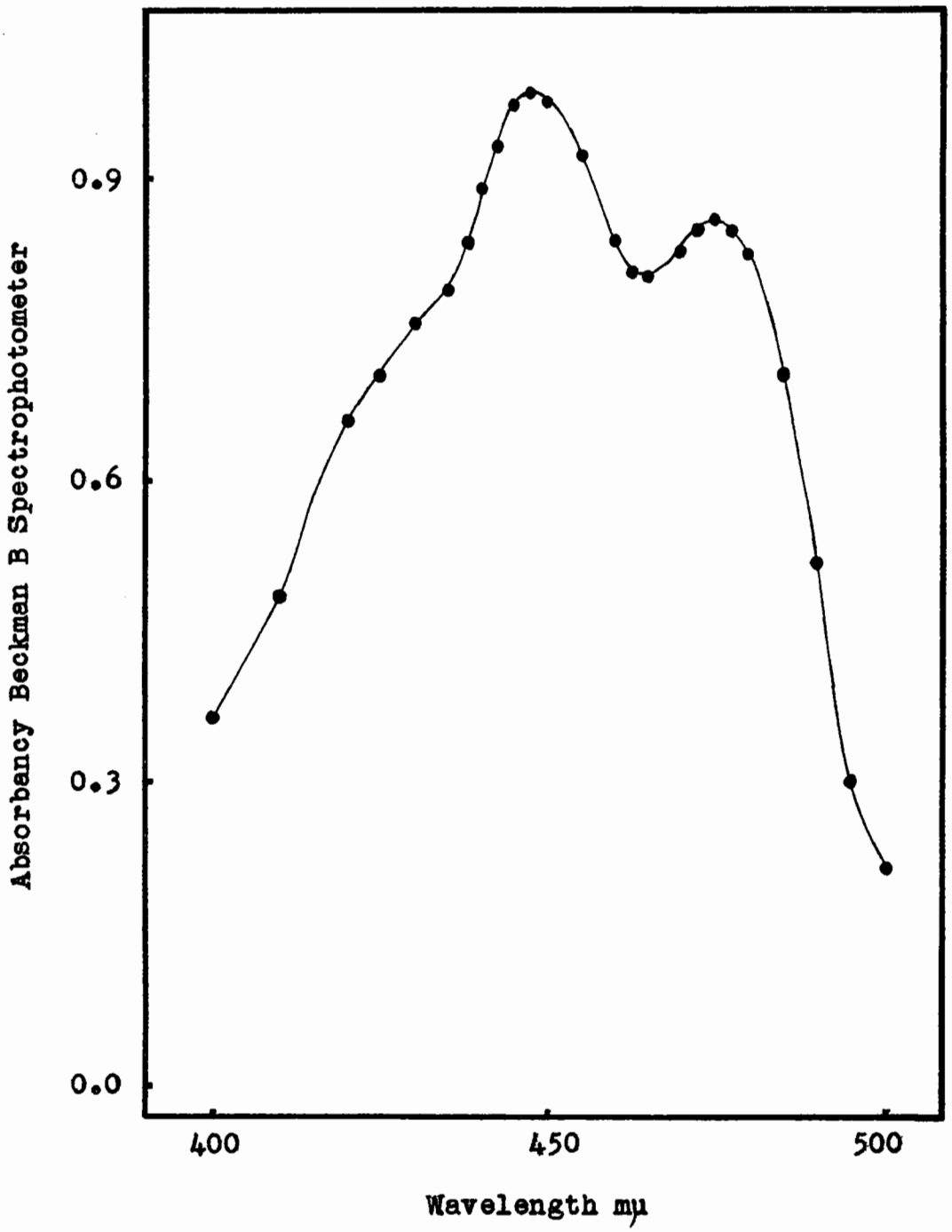


Figure 12 Absorption Curve for Crystalline 90 Percent β-, 10 Percent α-Carotene in Light Petroleum Solution

"blank" with the results given in Table XLII.

Table XLII

Calibration Data for Carotene in Light Petroleum Solution

90% β-, 10% α-Carotene/10 ml. Micrograms	Absorbancy with a No. 440 Filter	Absorbancy/Microgram of Carotene in 10 ml. "K-Value"
2	0.070	0.0350
4	0.142	0.0355
6	0.212	0.0353
8	0.282	0.0353
10	0.350	0.0350

Data in Table XLII indicate that the "K-value" for 90 percent β-, 10 percent α-carotene in 60 - 100°C light petroleum is approximately 0.0352 per microgram in 10 ml. of solution.

iii. Carotene Calibration Curve Employing the Ferric Chloride Plus α,α'-Bipyridine Reagents

The solutions previously employed for determining the calibration curve for 90 percent β-, 10 percent α-carotene in light petroleum were used. Ten ml. portions of these solutions were reacted with the ferric chloride plus α,α'-bipyridine reagents as previously described for the estimation of total tocopherol in lard and shortening. The resulting absorbancies were measured in an Evelyn colorimeter fitted with a No. 515 filter relative to an appropriate

"blank" prepared with light petroleum. The results of this experiment are given in Table XLIII.

Table XLIII

Carotene Calibration Data Employing the Ferric Chloride Plus α,α'-Bipyridine Reagents

90% β-, 10% α-Carotene/10 ml. Micrograms	Absorbancy with a No. 515 Filter	Absorbancy/Microgram of Carotene in 10 ml. "K-Value"
2	0.017	0.0085
4	0.032	0.0080
6	0.056	0.0093
8	0.071	0.0089
10	0.090	0.0090

Results in Table XLIII are satisfactory in view of the small absorbancies being measured. Assuming that the larger absorbancies are the more accurate, an average "K-value" of 0.0091 per microgram of carotene in 10 ml. was accepted.

iv. Correction for the Carotene Error in the Determination of Total Tocopherol

The procedure for correcting for the presence of carotene involves the estimation of the amount of carotene present by means of the absorbancy with a No. 440 filter. From this value it is possible to calculate the error that will be caused with a No. 515 filter after reaction with the ferric chloride plus α,α'-bipyridine reagents. This correction for carotene is subtracted from the apparent total

tocopherol value which is calculated from the absorbancy measured with a No. 515 filter.

Carotene in light petroleum solution exhibited a "K-value" of 0.0351 with a No. 440 filter, while carotene upon reaction with the ferric chloride plus α,α'-bipyridine reagents exhibited a "K-value" of 0.0091 with a No. 515 filter. Therefore, an absorbancy equal to $\frac{0.0091}{0.0352}$, that is 0.26 multiplied by the absorbancy measured with a No. 440 filter should be produced upon reaction with the ferric chloride plus α,α'-bipyridine reagents when the resulting absorbancy is measured with a No. 515 filter. Therefore, the formula for correcting for the presence of carotene is as follows:-

Corrected Absorbancy = Absorbancy with a No. 515 filter
- (0.26 x Absorbancy with a No. 440 filter)

The corrected absorbancy is employed to calculate the total tocopherol content of the sample fat.

3. ANALYTICAL METHOD

a. Reagents

Sulphuric Acid - Add 60 ml. of concentrated sulphuric acid sp. Gr. 1.84 to water and when cool dilute to 100 ml. with water.

Light Petroleum - As described for tocopherol, page 148.

Ethyl Alcohol - As described for tocopherol, page 149.

Ferric Chloride - As described for tocopherol, page 149.

α,α'-Bipyridine - As described for tocopherol, page 149.

b. Quantitative Procedure

i. Separation of Butter Oil from Butter

Cut the butter sample into one-cm. cubes, place in a beaker and heat in a 60°C-oven for the least possible time necessary to separate the butter oil. Decant the butter oil into a dry filter paper and filter at approximately 35°C. The butter oil should be perfectly clear, if not it must be refiltered.

ii. Removal of Oil Yellow AB, Oil Yellow OB and Vitamin A from Butter Oil

Dissolve 2.5 g. of butter oil in light petroleum and dilute to 50 ml. Transfer this solution to a separatory funnel, add 10 ml. of 60 percent sulphuric acid and shake for fifteen seconds. If synthetic butter colours are present the acid layer becomes pinkish. Stand for fifteen minutes, run off the acid layer and wash the light petroleum - fat solution for a few seconds with two 25 ml. portions of water. Fifty ml. of light petroleum are similarly extracted and serves as a "blank" in the subsequent procedures.

iii. Determination of Total Tocopherol, Correcting for the Interference Due to Carotene

Transfer 10 ml. of the acid extracted light petroleum - fat solution to a colorimeter tube. Add 0.2 ml.

of ethyl alcohol, to remove any possible turbidity due to entrained water in the light petroleum and measure the absorbancy relative to a light petroleum "blank", employing an Evelyn colorimeter fitted with a No. 440 filter. This absorbancy forms the basis of the correction for carotene present.

Transfer duplicate 10 ml. portions of the acid extracted fat solution (0.5 g. of butter oil) to 125-ml. low-actinic-glass separatory funnels. Add 5 ml. of 0.04 percent ferric chloride and 5 ml. of 0.40 percent α,α'-bipyridine reagents and swirl the contents of the flasks. Six minutes after adding the ferric chloride, add 2 ml. of water and invert the separatory funnel twice. At eleven minutes, run off and filter the alcoholic layer through a 9 cm. Whatman No. 54 filter. Measure the absorbancy at twelve minutes employing an Evelyn colorimeter fitted with a No. 515 filter, relative to a similarly treated light petroleum "blank".

Calculate the corrected absorbancy as follows:-

Corrected Absorbancy = Absorbancy with a No. 515 filter
- (0.26 x Absorbancy with a No. 440 filter).

The corrected absorbancy with a No. 515 filter divided by a "K-value" of 0.00506 yields the concentration of tocopherol in micrograms per portion employed (0.5 g. butter oil). Due to the low absorbancies obtained with genuine butter, the duplicate absorbancies per sample should be averaged and

employed to calculate the total tocopherol content of the butter.

4. VALIDITY OF ANALYTICAL RESULTS

a. Removal of Oil Yellow AB, Oil Yellow OB and Vitamin A Alcohol

Solutions containing Oil Yellow AB and/or α-tocopherol; Oil Yellow OB and/or α-tocopherol; Vitamin A alcohol and/or α-tocopherol were analyzed for apparent total tocopherol, before and after extraction with 60 percent v/v sulphuric acid, employing the foregoing analytical procedures. The results of these analyses are given in Table XLIV.

The data in Table XLIV illustrate the large errors that the synthetic colours Oil Yellow AB and Oil Yellow OB can cause in the estimation of total tocopherol in butter oil, while vitamin A alcohol causes little or no error. Extraction of the light petroleum solutions with 60 percent v/v sulphuric acid effectively removes the interferences due to these three materials.

Table XLIV

Removal of Errors Due to Oil Yellow AB, Oil Yellow OB and Vitamin A Alcohol

Materials Present Micrograms per 10 ml.	Sample Treatment	"Apparent" Tocopherol Micrograms
180.0 Oil Yellow AB	None	31.6
180.0 Oil Yellow AB	Acid Extracted	0.1
17.5 α-Tocopherol	None	17.45
17.5 α-Tocopherol 180.0 Oil Yellow AB	None	46.9
17.5 α-Tocopherol 180.0 Oil Yellow AB	Acid Extracted	17.45
180.0 Oil Yellow OB	None	38.8
180.0 Oil Yellow OB	Acid Extracted	0.0
17.5 α-Tocopherol	None	17.65
17.5 α-Tocopherol 180.0 Oil Yellow OB	None	54.2
17.5 α-Tocopherol 180.0 Oil Yellow OB	Acid Extracted	17.45
6.0 Vitamin A Alcohol	None	1.0
6.0 Vitamin A Alcohol	Acid Extracted	0.2
6.0 Vitamin A Alcohol 17.5 α-Tocopherol	Acid Extracted	17.60

b. Correction for the Interference Due to Carotene

Solutions containing 90 percent β-, 10 percent α-carotene and/or α-tocopherol were analyzed for apparent total tocopherol before and after extraction with 60 percent v/v

sulphuric acid. All measurements were corrected for the interference due to carotene based on the absorbancy measured with a No. 440 filter with the results given in Table XLV.

Table XLV

Correction for the Interference Due to Carotene

Materials Present Micrograms per 10 ml.	Sample Treatment	Apparent Total Tocopherol	
		Uncorrected for Carotene Interference Micrograms	Corrected for Carotene Interference Micrograms
15.0 Carotene	None	22.6	- 1.6
15.0 Carotene	Acid Extracted	22.6	- 0.4
17.5 α-Tocopherol	None	17.7	17.7
17.5 α-Tocopherol 15.0 Carotene	None	40.6	16.7
17.5 α-Tocopherol 15.0 Carotene	Acid Extracted	40.1	17.2

Results in Table XLV indicate that the analysis of α-tocopherol in the presence of carotene gave analytical results within ±2 percent of the microgram quantities of α-tocopherol added.

ANTIOXIDANT CONTENT OF FATS AND OILS

A. Propyl Gallate, Butylated Hydroxyanisole and Tocopherol Content of Commercial Lards and Shortenings

A number of lards and shortenings were analyzed during 1950 and again during 1953 to determine their content of propyl gallate, butylated hydroxyanisole (BHA), and total tocopherol. The results of these analyses are contained in Tables XLVI and XLVII.

Table XLVI

Analyses of Lard and Shortenings in 1950 for Antioxidant Content

Shortenings	Propyl Gallate %	Butylated Hydroxyanisole %	Total Tocopherol %
"Bakeasy"	0.0000	0.0000	0.000
"Crisco"	0.0000	0.0000	0.137
"Crispy Flake"	0.0000	0.0000	0.041
"Domestic"	0.0031	0.0104	0.049
"Domestic"	0.0028	0.0109	-
"Fluffo"	0.0000	0.0000	0.102
"Jewel"	0.0000	0.0000	0.038
"Swiftening"	0.0036	0.0000	0.008
Lards			
"Crispycrust"	0.0000	0.0000	-
"Maple Leaf"	0.0053	0.0206	-
"Royal"	0.0009	0.0086	-
"Royal"	0.0007	0.0076	-
"Silverleaf"	0.0000	0.0000	-

Table XLVII

Analysis of Lards and Shortenings in 1953 for Antioxidant Content

Shortenings	Propyl Gallate %	Butylated Hydroxyanisole %	Percent of 3-BHA Isomer in BHA	Total Tocopherol %
"Bakeasy"	0.0009	0.0063	80	0.002
"Coleman"	0.0014	0.0094	79	0.002
"Crisco"	0.0000	0.0000	-	0.116
"Crispy Flake"	0.0028	0.0089	79	0.008
"Domestic"	0.0020	0.0081	81	0.013
"Fluffo"	0.0000	0.0000	-	0.079
"Puritan"	0.0000	0.0000	-	0.000
"Swiftening"	0.0025	0.0000	-	0.003
Lards				
"Aylmer"	0.0000	0.0000	-	0.001
"Laurel"	0.0000	0.0000	-	0.000
"Royal"	0.0005	0.0058	81	0.001
"Maple Leaf"	0.0021	0.0104	79	0.001
"Shamrock"	0.0018	0.0078	78	-
"Silverleaf"	0.0021	0.0000	-	-
"Union"	0.0000	0.0000	-	-
"Wilsil"	0.0000	0.0000	-	-

The Canadian Food and Drug Regulations (17) prior to June, 1953, only permitted the use of two synthetic phenolic antioxidants, namely 0.01 percent of propyl gallate and/or 0.02 percent of BHA in lard and shortening. Since, a number of commercial antioxidant preparations contain 6 percent of propyl gallate, 20 percent of BHA, 4 percent of anhydrous citric acid and 70 percent of propylene glycol. If such a preparation is employed, the maximum concentrations of these

antioxidants encountered in lard or shortening should be 0.006 percent of propyl gallate and 0.02 percent of BHA. In June, 1953 the Food and Drug Regulations (17) were amended to permit the addition of 0.005 percent of nordihydroguaiaretic acid (NDGA), but propyl gallate cannot be used in combination with NDGA. Since the analyses reported in Tables XLVI and XLVII were made prior to this amendment of the Food and Drug Regulations, none of the samples were found to contain NDGA.

Inspection of the analytical results in Table XLVI (1950) indicate that only one sample contained the maximum content of propyl gallate and BHA. Four samples contained approximately half of the maximum amount of propyl gallate and BHA. One sample contained only added propyl gallate, while two lards and five of the shortenings contained neither propyl gallate nor BHA. Some of the shortenings contained appreciable quantities of tocopherol by virtue of their content of vegetable oils.

Data in Table XLVII (1953) illustrate a reduction in the maximum concentration of antioxidants found. The majority of samples contained one or more of the synthetic antioxidants, however, the maximum amount of propyl gallate found was 0.0028 percent or only 28 percent of the amount permitted by the Food and Drug Regulations (17). The maximum amount of BHA found was 0.0104 percent or just 52 percent of the

amount permitted by the Food and Drug Regulations (17). These low concentrations attest to the great effectiveness of these synthetic antioxidants. Nevertheless, four lards and three shortenings contained no propyl gallate or BHA. It is considered significant that the shortenings in 1953 contained less tocopherol than was found in 1950. This fact may result from the increased use of animal fats in shortenings and may also explain the more widespread use of antioxidants in shortenings in order to protect such mixtures from becoming rancid, due to the greater instability of the animal fats as compared to hydrogenated vegetable oils. The commercial BHA found in lard and shortening in 1953 consisted of approximately 80 percent 3-tertiary-butyl-4-hydroxyanisole (3-BHA) isomer.

B. Total Tocopherol Content of Genuine Butter Oils

Samples of genuine butter oil produced during the Summer and Winter were analyzed for total tocopherol employing the analytical procedure described in Section I. The results of these analyses are given in Table XLVIII.

Data in Table XLVIII agrees closely with values in the literature for total tocopherol. Lange (77) quotes five separate authorities to the effect that the total tocopherol content of butter oil ranges between 17 to 42 micrograms per g. butter oil. In addition no difference was found between

the tocopherol content of Summer and Winter butter oil.

Table XLVIII

Total Tocoperol Content of Genuine Butter Oils

Source of Sample	Total Tocopherol Micrograms/g. Butter Oil
New Zealand	37
University of British Columbia	29,24
Fraser Valley Dairy, British Columbia	23,24
University of Alberta	25
University of Manitoba	26,29,27
Ontario Agricultural College	28
Central Government Experimental Farm	26
St. Hyacinthe Dairy School	38
Average Total Tocopherol	30
Range of Total Tocopherol	23 to 43

C. Total Tocopherol Content of Commercial Vegetable and Animal Fats and Oils

Twenty-four assorted vegetable oils, hydrogenated vegetable oils, margarine oils, shortenings and lards were analyzed for total tocopherol. The results of these data are given in Table XLIX.

Where analytical values were obtainable in the literature, the analytical values found were in close agreement, except in the case of sunflower oil which was found to contain more tocopherol than reported in the literature. In view of the good agreement between results obtained by this

Table XLIX

Total Tocopherol Content of Animal and Vegetable Fats and Oils

Material Analyzed	Total Tocopherol Micrograms /g. fat or oil	Range of Tocopherol from Literature (77)
Crude Soybean Oil	1580	1520-2120
Refined, Hydrogenated Soybean Oil	1510	920-1900
"Crisco" All vegetable shortening	1160	1042
"Nucoa" Margarine oil	1050	1005
"Mazola" Corn Oil	1050	1020-1190
"Jewel" Sunflowerseed Oil	1020	700
Refined, Deodorized Soybean Oil	990	920-1900
"Biscot" Crude, Mixed Oils	860	-
"Allsweet" Margarine Oil	830	-
"Monarch" Margarine Oil	820	-
"Good Luck" Margarine Oil	790	-
"Fluffo" Shortening	785	-
"Margine" Margarine Oil	760	-
"Biscot" Refined, Hydrogenated Oils	680	-
Refined, Deodorized Cottonseed Oil	660	830-1100
Refined, Deodorized Palm Oil	480	440-560
"Planters" Peanut Oil	310	220-510
Crude Coconut Oil	42	50-83
"Kokoheart" Hydrogenated Coconut Oil	32	30
Butter Oil	23-43	17-42
"Bakeasy" Shortening	15	-
Edible Tallow	14	-
"Maple Leaf" Lard	6	12-27
Refined, Deodorized Coconut Oil	3	30
"Swiftening" Shortening	0	-

rapid analytical procedure and values reported, it is felt that this new analytical procedure for total tocopherol is accurate and reliable.

DETECTION OF BUTTER ADULTERATION

A. By Means of the Total Tocopherol Content of Butter Oil

The Reichert-Meissl and Polenske values have been employed for many years as the most effective routine procedures for detecting the adulteration of butter with small quantities of coconut oil or larger amounts of other fats and oils. The foregoing procedure for determining the total tocopherol content of butter oil is proposed as a means of detecting the addition of small quantities of vegetable oils, other than coconut oil. Thus, the simultaneous use of these three procedures should detect the addition of approximately 10 percent of any vegetable oil. Calculated values are given in Table L to illustrate the effect of various amounts of margarine added to butter, upon the Reichert-Meissl value, Polenske value and total tocopherol content.

Table L

Calculated Reichert-Meissl Values and the Tocopherol Content of Butter Adulterated with Margarine

Percent Margarine Oil in the Mixture	Reichert-Meissl Value	Polenske Value	Total Tocopherol Micrograms/g.Oil
0 (Pure Butter)	28.8	2.1	30
5	27.0	2.0	70
10	26.1	1.9	110
15	24.3	1.8	150
20	23.4	1.7	190
100 (Margarine)	1.8	0.5	830

The data in Table L indicate that the addition of 17 percent margarine is required to reduce the Reichert-Meissl value of an average butter to 24.0, the widely accepted limit between "genuine" and "adulterated" butter. On the other hand, the addition of only 5 percent of margarine would increase the tocopherol content of the mixture from 30 to 70 micrograms per g. of oil, an increase of 40 micrograms of tocopherol per g. of oil. This increase in tocopherol content is twice the range of tocopherol found in genuine butter oils (see Table XLIX) and consequently would constitute firm reason to suspect adulteration.

B. By Means of the Antioxidant Content of Butter Oil

The results in Table XLIX indicate that the tocopherol content of lard, edible tallow and some shortenings is very low. Due to the inherent instability of these fats, it is now common practice to add antioxidants such as propyl gallate, nordihydroguaiaretic acid (NDGA) and butylated hydroxyanisole (BHA) to these fats. If such stabilized fats are added to butter at a level of 5 to 15 percent, it is possible to detect these antioxidants in the butter oil by means of chemical tests. In Canada 0.003 percent propyl gallate and 0.01 percent of BHA are frequently added to lard and shortenings. Thus the addition of 10 percent of such a fat to butter would result in the presence of 0.0003 percent

of propyl gallate and 0.001 percent of BHA in the adulterated butter. The detection of so little propyl gallate is unlikely because it is easily oxidized in aqueous-fat systems. BHA, on the other hand, is not soluble in water if fat is present and thus remains in the fat phase. Therefore, a qualitative test for BHA should be made on all suspicious butter samples, especially if the tocopherol content is approximately normal for genuine butter oil. Employing this procedure a number of commercial butter samples have been found to be adulterated by virtue of their BHA content.

STUDIES OF THE LOSS OF ANTIOXIDANTS DURING THE STORAGE OF LARD AND OF PIE CRUST

A. Loss of Antioxidants in Oxidizing Lard

1. INTRODUCTION

Until comparatively recently little information has been recorded in the literature on the kinetics of the destruction of phenolic antioxidants in fats and oils. This lack of data has been attributed in part, at least, to the scarcity of satisfactory analytical methods for these phenolic compounds. However, with the development of improved analytical procedures a number of studies of the behaviour of individual antioxidants has been reported.

Filer et al. (41) have studied the oxidative destruction of gallic acid in commercially refined cottonseed oil, aerated at 110°C. These authors reported that the rate of loss of gallic acid was approximately constant and was virtually independent of the initial concentration. They concluded that the destruction of gallic acid exhibited characteristics of a zero order reaction.

Lundberg et al. (87) investigated the rates of destruction of four antioxidants added to lard held at 100°C under a stream of oxygen and simultaneously followed the change in peroxide value. These authors studied hydroquinone, catechol,

nordihydroguaiaretic acid (NDGA) and gallic acid, added separately to lard, at concentrations of 0.02, 0.10 and 0.50 percent by weight. These workers concluded that the deterioration of these phenolic antioxidants in oxidizing lard did not occur as a single low order reaction, but was complicated by the products formed from the fat and/or the antioxidants during the oxidative process. They observed that the use of the higher concentrations of these antioxidants, under their conditions, resulted in more rapid increase in peroxide value during the early stages of oxidation of lard.

In 1949 Kraybill et al. (74) reported the development of a new antioxidant formulation designated as "AMIF-72". This preparation consisted of 20 percent commercial butylated hydroxyanisole (BHA), 6 percent propyl gallate and 4 percent anhydrous citric acid with 70 percent propylene glycol as solvent. At that time no satisfactory methods were available for the determination of mixtures of these antioxidants. The "AMIF-72" antioxidant preparation is now widely used for the stabilization of edible fats. However, a procedure which permits the determination of combinations of propyl gallate, BHA, NDGA and tocopherols in lard and shortening was reported by Mahon and Chapman (88) in 1951 and has been described in more detail in preceding sections of this thesis. These workers have also developed a method for the estimation of

the 2-tertiary-butyl-4-hydroxyanisole (2-BHA) and 3-tertiary-butyl-4-hydroxyanisole (3-BHA) isomers in lard and shortening (89). Since there were no data in the literature on the destruction of propyl gallate and BHA when combined in a fat or oil, it was considered of interest to apply the foregoing procedures to the study of the relative rates of destruction of these phenolic antioxidants in oxidizing lard.

2. ANALYTICAL METHODS

The antioxidants employed were analyzed by the methods previously described in Sections "A", "B" and "D" of the analytical methods. Peroxides in the fat were estimated by Lea's "hot-method" (81).

3. EXPERIMENTAL CONDITIONS

Samples of fresh steam-rendered lard were obtained from two different commercial producers. Three 600 g. portions of each lard were warmed to 50°C and the required amount of the "AMIF-72 type" antioxidant preparation was added. The molten lard was stirred for ten minutes to ensure uniformity and 25 g. portions were poured into 50 ml. Pyrex beakers. The twenty-four beakers from each lard sample at each antioxidant level were divided into two groups of twelve beakers each and stored at 41°C and 61°C respectively, in constant temperature ovens. At suitable intervals, 12 g. portions of the lards were removed and propyl gallate, total BHA, 2-BHA,

3-BHA and peroxide value determined. Control lard samples to which no antioxidants had been added were also stored at 41° and 61°C and the peroxide value determined. In the case of the lard containing antioxidants, held at 61°C, analyses were conducted at intervals until all the propyl gallate and BHA had been destroyed. The destruction of the antioxidants in the lards stored at 41°C was relatively slow and this experiment was discontinued after thirty weeks and before the complete destruction of the antioxidants in this series of samples.

4. RESULTS AND DISCUSSION

a. Loss of Propyl Gallate and Butylated Hydroxyanisole in Lard Stored at 41 and 61°C

In the initial experiments the antioxidants were added at three levels. The calculated amounts of propyl gallate, BHA and citric acid incorporated in the lard are shown in Table LI. The concentrations actually found by analysis differ slightly from these values because there is frequently a small loss immediately after the addition of these antioxidants to the lard.

The analytical data obtained for the two different lots of lard containing the "AMIF-72 type" antioxidant preparation were identical within the limits of experimental error. Therefore, the results for only one lot are presented and

Table LI

Approximate Amounts of Antioxidant Added to Lard Samples A, B and C

Sample	Propyl Gallate Added %	Butylated Hydroxyanisole Added %	Citric Acid Added %
"Control"	0.000	0.000	0.000
A	0.012	0.040	0.008
B	0.006	0.020	0.004
C	0.003	0.010	0.002

discussed in this thesis. The results obtained for propyl gallate, BHA and peroxide values on lard samples "A", "B" and "C", stored at 61°C, are shown in Figures 13, 14 and 15.

Inspection of these graphs reveals that the rate of loss of propyl gallate was approximately constant with respect to time, for each concentration employed and, therefore, appeared to follow a zero order reaction. This conclusion is in agreement with those previously reported by Filer et al. (41) for gallic acid. Lundberg and co-workers (87) also followed the destruction of gallic acid in lard at 100°C in an atmosphere of oxygen, employing concentrations of 0.02, 0.10 and 0.50 percent. Their results indicated that at concentrations of 0.02 and 0.10 percent the rate of destruction of gallic acid was approximately constant with respect to time, suggesting a zero order reaction. The

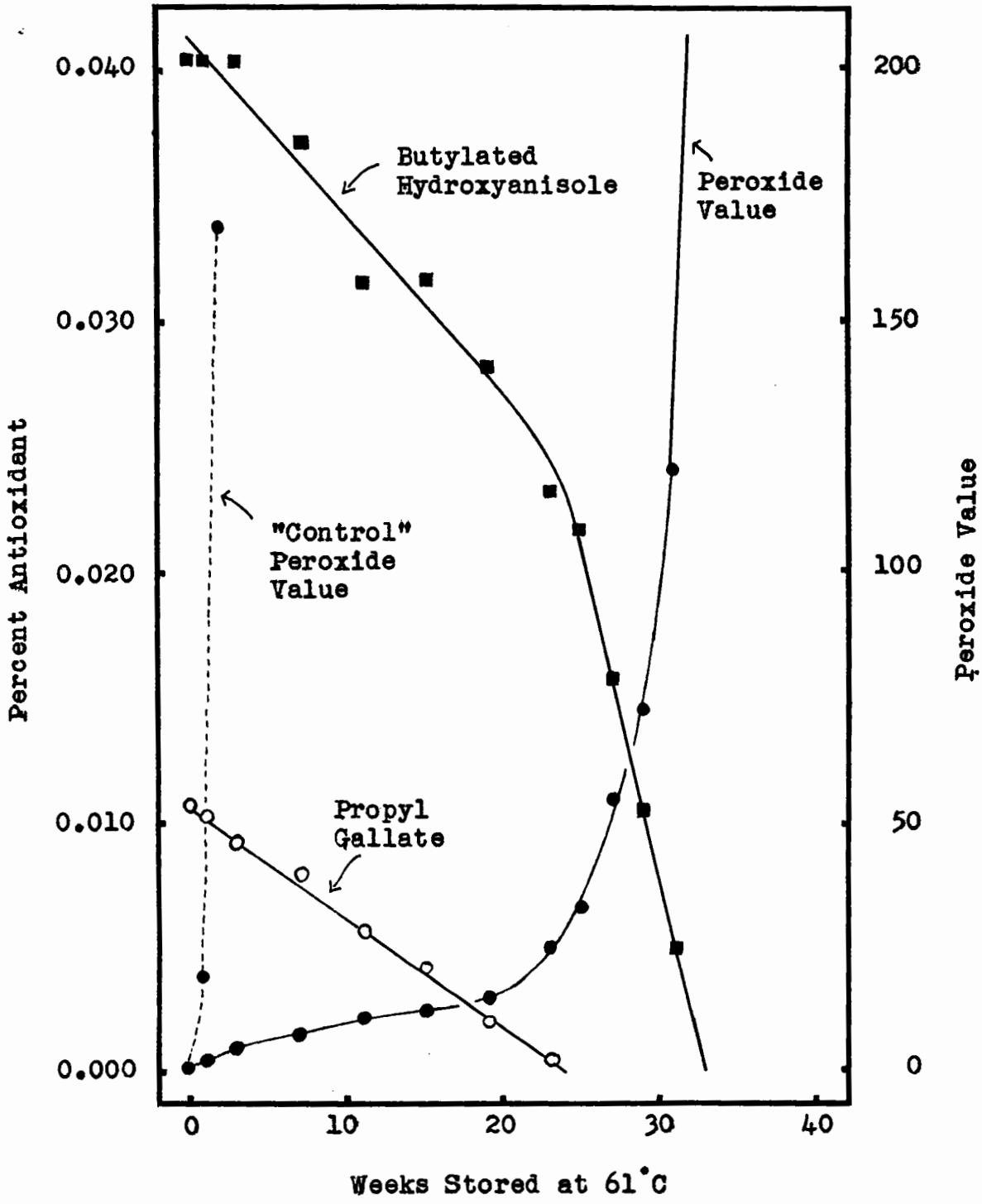


Figure 13 Peroxide Value and Concentration of Propyl Gallate and Butylated Hydroxyanisole in Lard Sample "A" Stored at 61°C

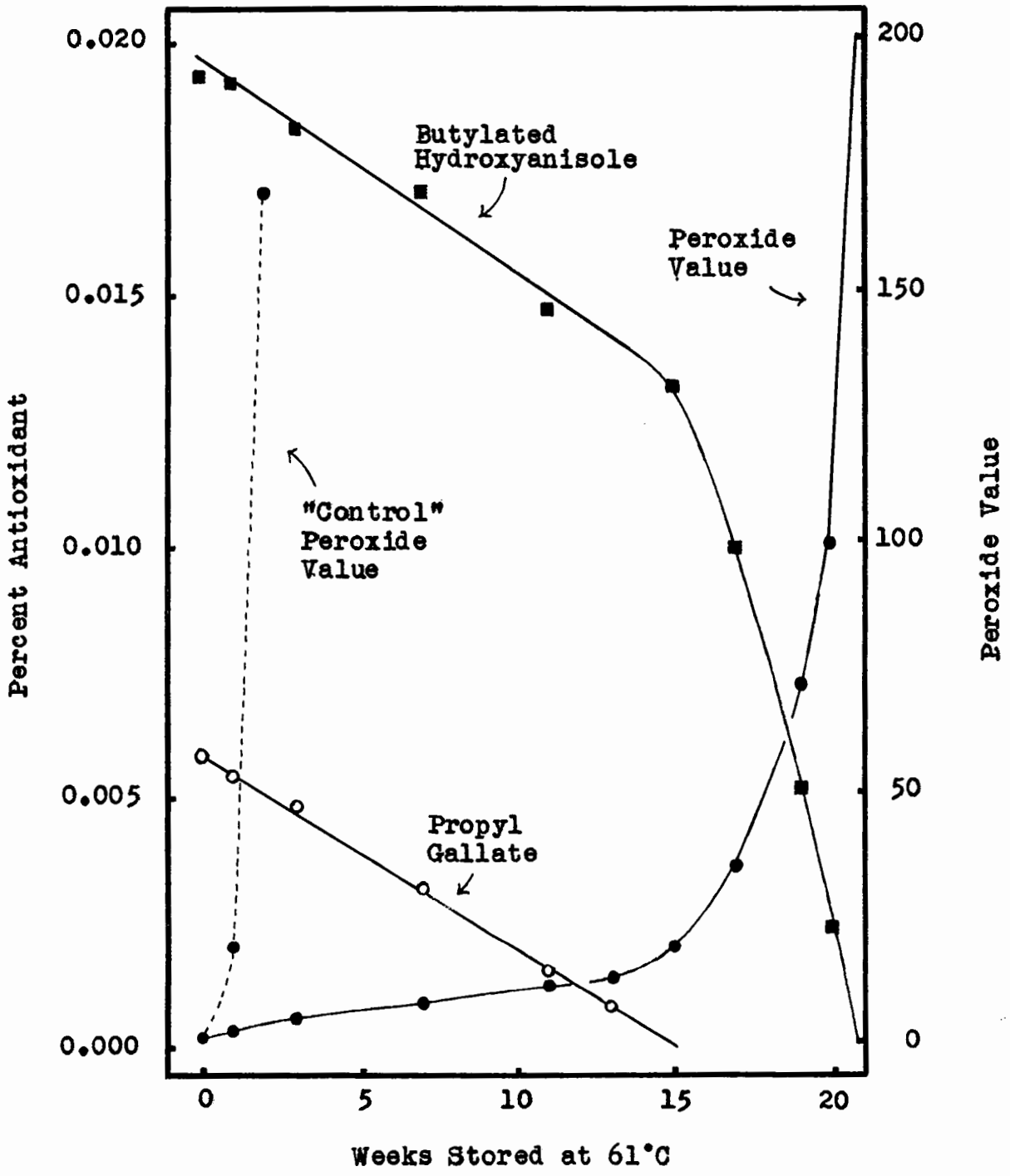


Figure 14 Peroxide Value and Concentration of Propyl Gallate and Butylated Hydroxyanisole in Lard Sample "B" Stored at 61°C

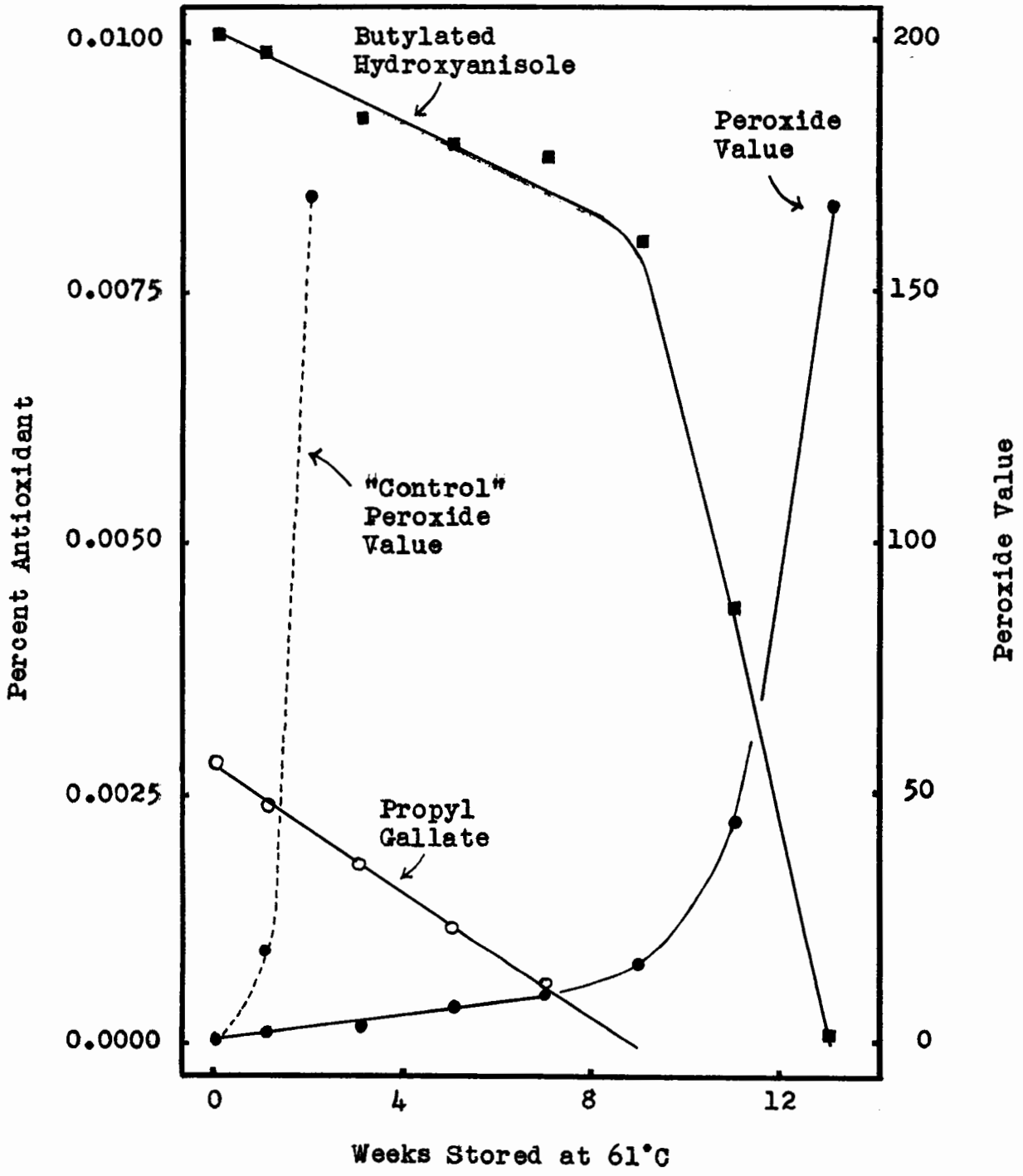


Figure 15 Peroxide Value and Concentration of Propyl Gallate and Butylated Hydroxyanisole in Lard Sample "C" Stored at 61°C

highly irregular results obtained by these workers (87) when employing 0.50 percent gallic acid was probably caused by the positive catalytic effect of this high antioxidant concentration upon the formation of fat peroxides.

The data in Figures 13, 14 and 15 indicate that the loss of BHA occurred in two stages. Initially there was a slow decrease in the BHA concentration which accelerated rapidly at approximately the point at which the propyl gallate could no longer be detected in the lard samples. The initial slow loss of BHA proceeded at an approximately constant rate for each concentration employed and was attributed at least in part to the vapourization of small amounts of BHA from the surface of the lard. This assumption was confirmed by placing 160 g. of lard containing 0.02 percent of BHA in a shallow bottle. A slow stream of nitrogen was passed over the surface of the lard, an area of approximately 150 square cm., and then through a series of four water-cooled traps containing absolute ethyl alcohol. This apparatus was placed in a constant temperature oven at 61°C for fifty hours, and the BHA trapped in the alcohol was determined. A total of 37.5 micrograms of BHA was found, corresponding to a loss of 4×10^{-3} micrograms of BHA per square cm. per hour. However, the losses of BHA during the initial stages of the storage of samples "A", "B" and "C" amounted to 3.0×10^{-2}, 2.9×10^{-2} and 1.1×10^{-2} micrograms

per square cm. per hour, respectively. These values range from three to seven times the amount actually recovered in the alcohol traps. It is possible that some BHA was destroyed during the process of vaporization from the lard or that during this initial period the BHA was also slowly reacting with the oxidizing fat. The second stage in the destruction of the BHA began about the same time as the complete disappearance of the propyl gallate and rapid increase in the peroxide value. The rate of destruction of the BHA during this latter period was much greater than during the initial stage. This acceleration in the rate of destruction is presumably attributable to an increase in the reactivity of BHA with the oxidizing fat.

The concentrations of the 2-BHA and 3-BHA isomers were also determined during the storage of lard samples "A", "B" and "C", held at 61°C. These values are shown in Figure 16. These results indicate that the destruction of the 2-BHA and 3-BHA isomers also occurred in two stages. During the initial stage the rates of loss of these isomers were approximately equal and remained constant with respect to time for each of the concentrations employed. As previously shown, evaporation of BHA is at least partially responsible for this slow initial loss. By reference to Figure 16 it can be seen that in all cases the rate of destruction of 3-BHA increased abruptly just prior to the complete destruction of the propyl

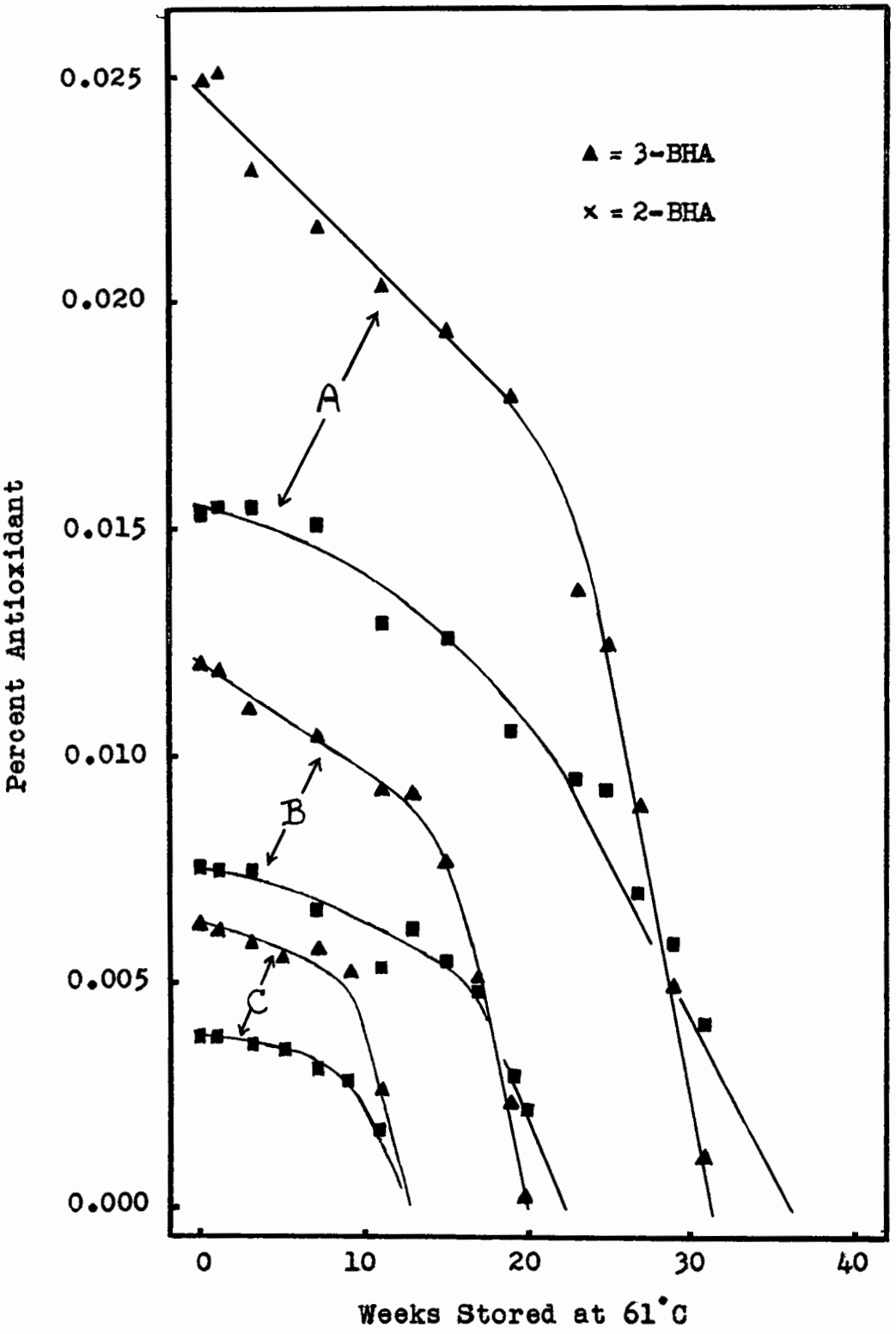


Figure 16 The Concentration of 2-Tertiary-butyl-4-hydroxyanisole and 3-Tertiary-butyl-4-hydroxyanisole in Lard Samples "A", "B", and "C" Stored at 61°C

gallate. It was concluded, therefore, that the combination of propyl gallate and BHA maintained a comparatively low peroxide value in the lard, but that BHA alone was relatively ineffective in preventing further peroxide formation after the destruction of the propyl gallate.

The results of the experiments on lard samples "A", "B" and "C" stored at 41°C, which was terminated at the end of thirty weeks, are given in Table LII.

Table LII

Propyl Gallate and BHA Content and Peroxide Values of Lard Samples "A", "B" and "C" Stored at 41°C for Thirty Weeks

Sample	Propyl Gallate at Start %	Propyl Gallate After 30 Weeks %	BHA at Start %	BHA after 30 Weeks %	Peroxide Value After 30 Weeks m.e./kg.
A	0.0115	0.0080	0.0380	0.0346	7.7
B	0.0057	0.0036	0.0195	0.0172	9.8
C	0.0028	0.0011	0.0101	0.0092	20.4

These results reveal a trend similar to the results obtained with the same samples stored at 61°C. The loss in propyl gallate ranged from 30 percent in sample "A" to 61 percent in sample "C". The loss in BHA, however, was relatively constant at approximately 10 percent for all samples.

b. Loss of Butylated Hydroxyanisole in Lard Stored at 61°C

In order to confirm the observation that BHA alone is relatively ineffective in maintaining a low peroxide value, it was decided to add BHA to a sample of fresh steam-rendered lard in the amounts shown in Table LIII.

Table LIII

Approximate Amount of Antioxidants Added to Lard Samples "L", "M" and "N"

Sample	BHA Added %	Citric Acid Added %
"Control"	0.00	0.000
L	0.04	0.008
M	0.02	0.004
N	0.01	0.002

The lard samples were stored in open beakers held at 61°C as previously described. The BHA content and the peroxide values were determined periodically with the results shown in Figure 17. These results indicate that the BHA was lost at a relatively rapid rate and that the peroxides accumulated with little or no induction period. Therefore, it was concluded that BHA alone was relatively ineffective in preventing peroxide formation in lard stored at 61°C as compared with a combination of propyl gallate and BHA (Figures 13, 14 and 15). This conslusion is in agreement with the statement of Kraybill and co-workers (73), who reported that BHA when

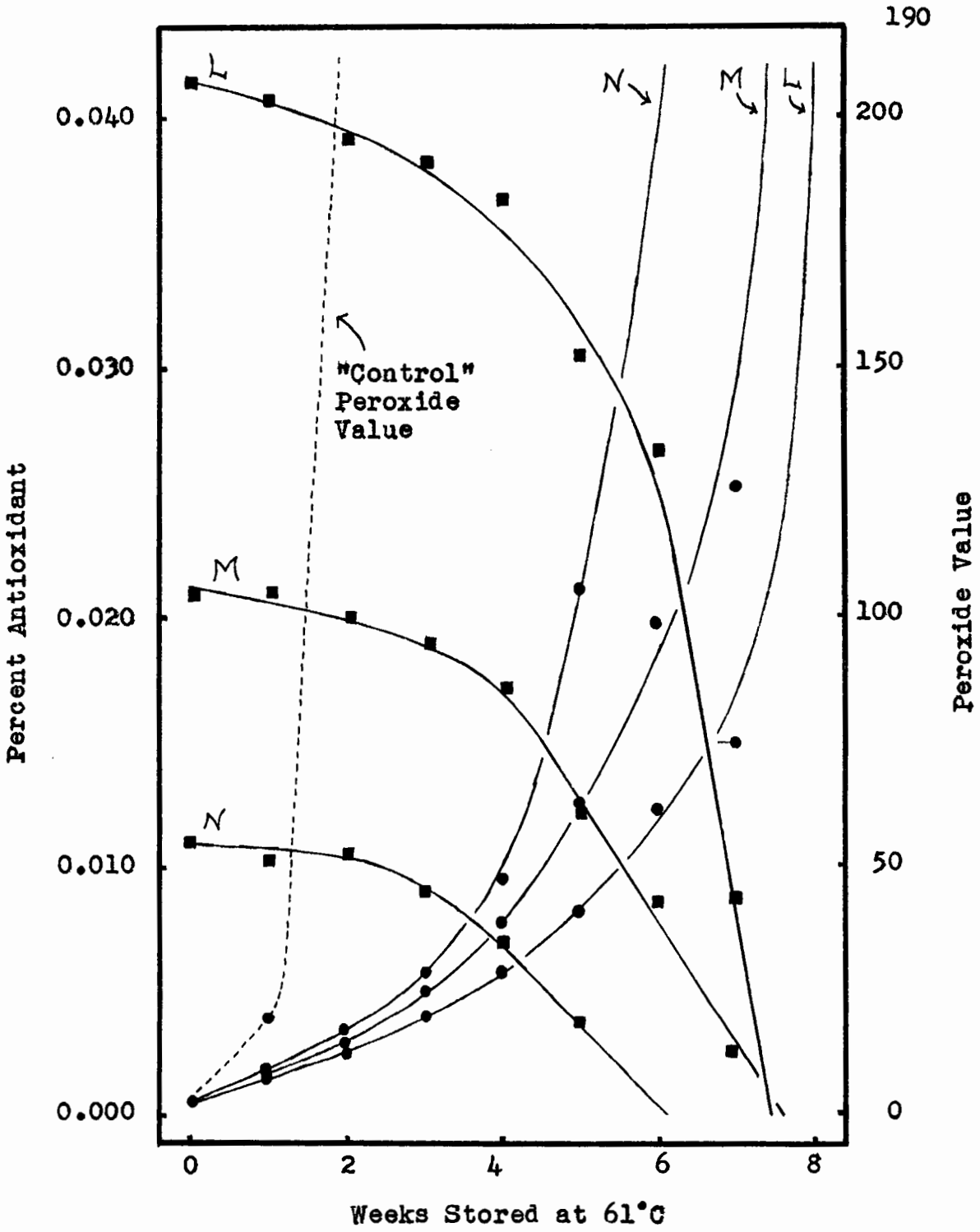


Figure 17 Peroxide Value and the Concentration of Butylated Hydroxyanisole in Lard Samples "L", "M", and "N" Stored at 61°C

used alone was not unusually effective in increasing the stability of lard as measured by the active oxygen method. Moore and Bickford (103) in a recent study of the effectiveness of a number of antioxidants also found propyl gallate more effective than BHA in stabilizing cottonseed oil, the same oil hydrogenated to shortening consistency and lard.

The results obtained by following the loss of the 2-BHA and 3-BHA isomers in samples "L", "M" and "N" are shown in Figure 18. These samples contain no propyl gallate and, therefore, it might be assumed that the data for the loss of BHA alone would resemble that portion of the curves in Figure 16 subsequent to the complete destruction of propyl gallate. Comparison of the data in Figures 16 and 18 indicate that this assumption is correct. It was noted from the data in Figure 18 that the loss of 3-BHA is initiated at an early stage in the storage period, whereas 2-BHA requires an induction period of approximately twenty-four days in the case of lard samples "L" and "M". It was also observed that with these samples the 3-BHA began to react at relatively low peroxide values, while a peroxide value of 25 to 35 milliequivalents per kg. was required before the rate of loss of 2-BHA became appreciable. No definite conclusions can be drawn from the results for sample "N", owing to the very low concentration of approximately 0.005 percent of each of the BHA isomers.

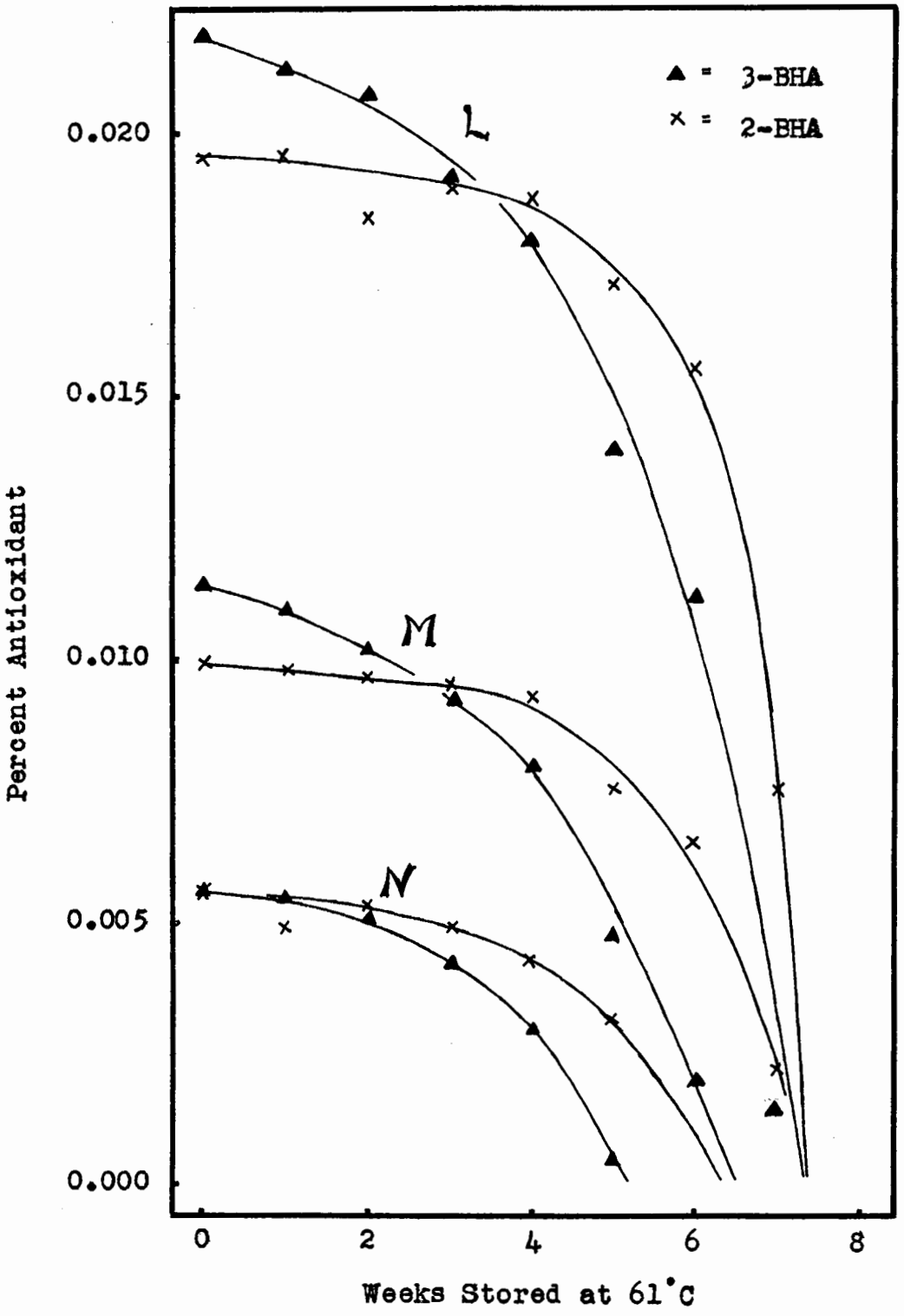


Figure 18 Concentration of 2-Tertiary-butyl-4-hydroxyanisole and 3-Tertiary-butyl-4-hydroxyanisole in Lard Samples "L", "M", and "N" Stored at 61°C

c. Relative Effectiveness of the 2-Tertiary-butyl-4-hydroxyanisole and 3-Tertiary-butyl-4-hydroxyanisole Isomers in Retarding the Rancidification of Lard Stored at 61°C

Since 3-BHA began to react at a lower peroxide value than 2-BHA, it could be argued that 3-BHA would inhibit peroxide formation to a greater extent and, therefore, should be a more effective antioxidant. In order to confirm this observation, amounts of 2-BHA, 3-BHA and citric acid as shown in Table LIV were added to fresh steam-rendered lard which was then stored at 61°C and analyzed at intervals for 2-BHA, 3-BHA and peroxide value.

Table LIV

Approximate Amount of Antioxidants Added to Lard Samples "X", "Y" and "Z"

Sample	2-BHA Added %	3-BHA Added %	Citric Acid Added %
"Control"	0.000	0.000	0.004
X	0.020	0.000	0.004
Y	0.000	0.020	0.004
Z	0.010	0.010	0.004

The results of the experiments with lard samples "X", "Y" and "Z" to which varying proportions of the BHA isomers plus citric acid were added and stored at 61°C, are presented in Figure 19. These data confirm the fact that 3-BHA alone is a more effective antioxidant than 2-BHA alone. Lard containing no added BHA and stored at 61°C attained a

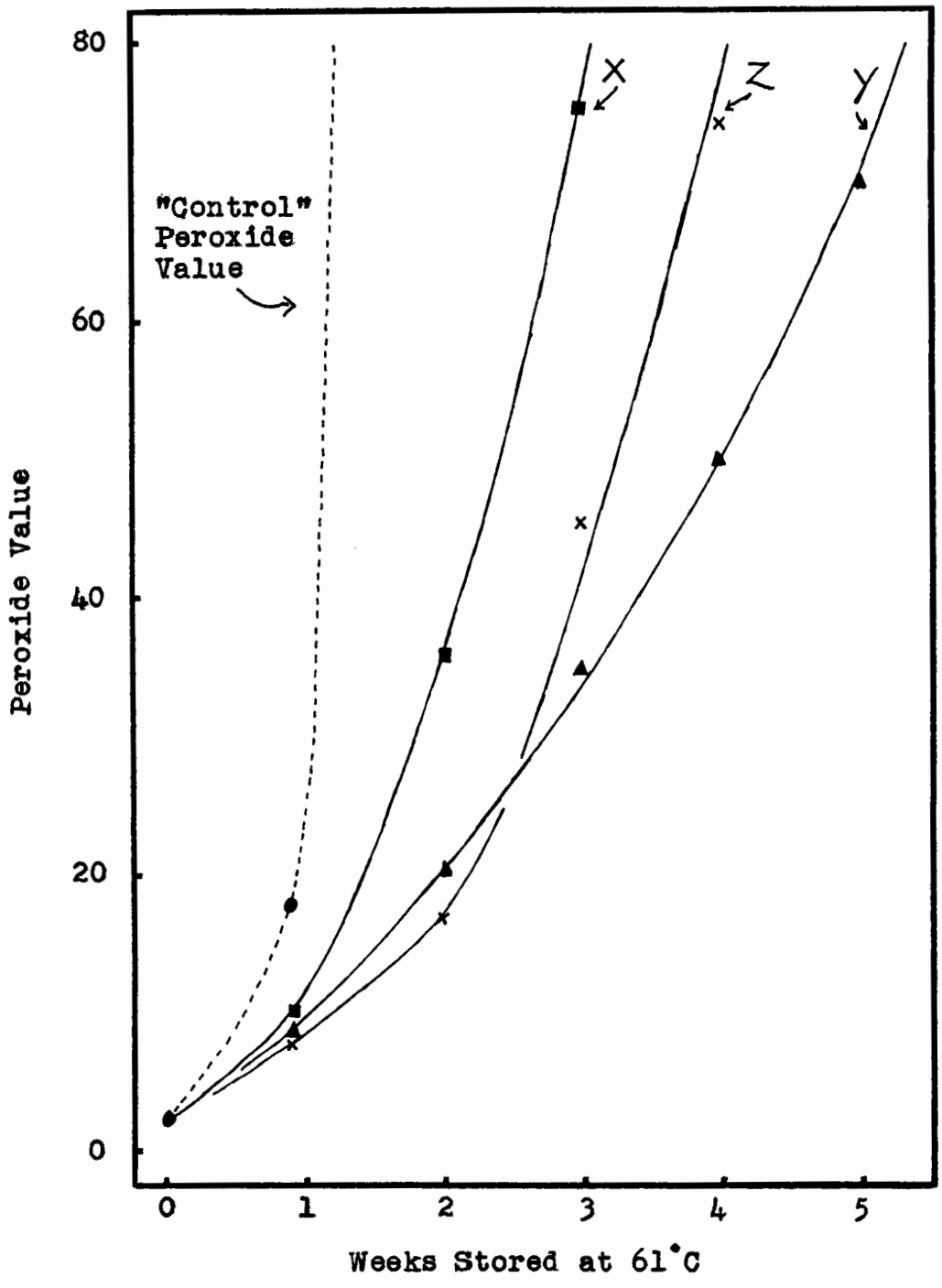


Figure 19 Peroxide Value in Lard Samples "X", "Y", and "Z" Stored at 61°C

peroxide value of 20 milliequivalents per kg. after only 6.2 days. On the other hand, lards containing 0.02 percent of 2-BHA and 0.02 percent of 3-BHA required 9.7 and 13.8 days, respectively, to attain a peroxide value of 20. The combination of equal amounts of 3-BHA and 2-BHA was almost as effective as 3-BHA in stabilizing the lard in the initial stages of the oxidation, but at higher peroxide values this curve was approximately midway between the curves for the two pure BHA isomers. These limited results indicated that the addition of 0.02 percent of the 2-BHA and 3-BHA isomers increased the stability of lard stored at 61°C by factors of 1.6 and 2.2, respectively, as compared to 1.0 for lard alone. At a concentration of 0.02 percent, 3-BHA exhibited an inhibition ratio of approximately 1.4 relative to 2-BHA when comparison was made at a peroxide value of 20. Moore and Bickford (103) have recently stated that a more realistic evaluation of the antioxidants in lard is obtained when comparisons are made at a peroxide value of 100 milliequivalents per kg. When the data in Figure 19 are compared at a peroxide value of 80, the maximum value for which figures were available, the inhibitor ratio of 3-BHA was approximately 1.5 as compared to unity for 2-BHA.

These results are in general agreement with those reported in the literature. Dugan et al. (31) investigated the relative effectiveness of 2-BHA and 3-BHA alone and as

mixtures of known composition on stabilization of eleven lards and concluded that 3-BHA exhibited an inhibitor ratio of 1.5 relative to 2-BHA when 0.01 percent of the isomers was employed and the active oxygen method used to evaluate the relative stability of the lards. Rosenwald and Chenicek (125) have reported that 3-BHA was 2.8 times as effective as 2-BHA in inhibiting the oxidation of lard when a concentration of 0.02 percent was employed.

The BHA employed in these investigations contained from 54 to 62 percent of 3-BHA. These samples were chosen intentionally in order that sufficient 2-BHA would be present to permit accurate analysis. Commercial BHA preparations containing higher proportions of 3-BHA have been encountered (see Table XIX) (89), and these might be expected to be slightly more efficient antioxidants.

5. VALIDITY OF THE ANALYTICAL VALUES FOR 2-TERTIARY-BUTYL-4-HYDROXYANISOLE AND 3-TERTIARY-BUTYL-4-HYDROXYANISOLE IN OXIDIZING LARD

The determination of the BHA isomers is based on the colour intensity obtained with the 2,6-dichloroquinonechloroimide - borax reagent relative to that obtained with the ferric chloride - α,α'-bipyridine reagents. If either of these reagents reacted with decomposition products formed during the oxidation of the fat, large errors in the determination of the isomers would result. In order to check the

validity of the determination of the 2-BHA and 3-BHA in oxidizing fat, the following experiment was conducted. To one sample of lard was added 0.02 percent of 3-BHA and to a second sample an equal amount of 2-BHA. These samples were held in open beakers at 61°C, and the proportion of the isomers in each sample was determined at intervals until the BHA was completely destroyed. The results of these experiments are given in Table LV.

Table LV

The Proportion of BHA Isomers as Determined During the Oxidation of Lard at 61°C

Storage Period at 61°C Weeks	2-BHA		3-BHA	
	Ratio of Absorbancies at 620/515 mμ	Found %	Ratio of Absorbancies at 620/515 mμ	Found %
0	0.138	100.0	0.787	100.0
1	0.137	100.2	0.766	96.8
2	0.128	101.6	0.764	96.5
3	0.132	101.0	0.784	99.6
4	0.125	102.0	0.788	100.1
5	-	-	0.763	96.3
6	-	-	0.767	96.9

The ratios given in Table LV represent the absorbancy obtained with the 2,6-dichloroquinonechloroimide - borax reagent divided by the absorbancy obtained with the ferric chloride - α,α'-bipyridine reagent. The average values of

101.2 percent for 2-BHA and 97.7 percent for 3-BHA are both within the experimental error of the method. If any substances produced during the oxidation of the fat had interfered with the determination, the percentage of the isomers would not have remained close to 100 percent until the complete destruction of the BHA. Therefore, it was concluded that the method for the determination of the isomers was satisfactory in the presence of oxidation products.

B. Loss of Antioxidants During the Preparation and Storage of Pie Crust

1. INTRODUCTION

The foregoing study of the loss of propyl gallate and butylated hydroxyanisole (BHA) in lard stored at 61°C was extended to include a study of these and other antioxidants in pie crust. All previous work in this field has been conducted by means of static experiments, that is, experiments in which the criterion has been the time under given conditions for the baked material to acquire a rancid smell or for the fat fraction to attain a given peroxide value. This study involved the addition of a number of antioxidant combinations to lard employed in the preparation of pie crust. The keeping quality of the pie crust and the disappearance of added antioxidants was estimated by periodically extracting the fat from a portion of the pie crust and analyzing it for added antioxidants and peroxide value.

2. EXTRACTION OF FAT FROM DOUGH OR PIE CRUST

a. Efficiency of Different Solvents in Extracting Fat from Dough

In order to follow the loss of antioxidants added to lard used to prepare pie crust, it was necessary to have a simple procedure for recovering the majority of the fat present in dough or pie crust. In a preliminary experiment 50 g. portions of dough were agitated in a Waring Blendor

for three minutes with 100 ml. portions of a number of fat solvents. The resulting dough suspensions were centrifuged at 1000 R.P.M. for five minutes, the solvent phase filtered, the solvent evaporated and the fat weighed. The efficiency of various solvents in extracting the fat from dough under these conditions is illustrated in Table LVI.

Table LVI

Efficiency of Various Solvents to Extract Fat from Dough

Solvent Employed	Percent Fat Recovered from Dough
Acetone	8
Benzene	96
Carbon tetrachloride	86
Chloroform	-
Ethyl ether	91
Methyl Cellosolve	-
Light Petroleum - 30 to 40° fraction	91
Light Petroleum - 30 to 50° fraction	89
Light Petroleum - 30 to 60° fraction	92
Light Petroleum - 34 to 38° fraction	91
Light Petroleum - 40 to 50° fraction	91
Light Petroleum - 50 to 60° fraction	90
Light Petroleum - 60 to 100° fraction	94

Most of the solvents tested in Table LVI were found unsatisfactory for one or more reasons. Acetone and methyl cellosolve, being miscible with water, took up the water present in the dough. The resulting aqueous - solvent mixtures were poor fat extractants. Chloroform, and carbon tetrachloride presented some difficulty in centrifuging down

the flour solids due to the high specific gravity of these solvents. Benzene, carbon tetrachloride, chloroform, methyl cellosolve and the 60 to 100°C light petroleum fraction were rejected because of their high boiling points which required undesirably high temperatures in order to remove all of the solvent. Since propyl gallate and other antioxidants are known to be destroyed by high temperatures, the use of such solvents are not feasible. Of the remaining solvests investigated, light petroleum with a 34 to 38°C boiling range was considered the most satisfactory. A single extraction with this solvent recovered approximately ninety-one percent of the fat in dough, employing the foregoing procedure. The lower boiling range (34°C) of this fraction avoided the possibility of this solvent boiling during all but the hottest summer weather. In addition, its low upper boiling range (38°C) greatly facilitated the complete removal of this solvent from the extracted fat. Light petroleum was employed in preference to ethyl ether because of the tendency of ethyl ether to form peroxides which might interfere with the accurate estimation of the peroxide value of the extracted fat.

b. Effect of Blending Time Upon the Recovery of Fat from Dough

Employing the 34 to 38°C light petroleum fraction and blending the sample for three minutes, the solvent boiled

vigorously because of the friction in the blendor. Therefore, experiments were conducted to determine whether the time of blending could be reduced. The effect of the time of agitation in a Waring Blendor upon the extraction of fat from dough is illustrated in Table LVII.

Table LVII

Effect of Blending Time on the Recovery of Fat from Dough, Employing 100 ml. of 34 - 38°C Light Petroleum Per 50 g. Dough

Time of Blending Minutes	Fat Recovery from Dough Percent
0.25	93
0.5	92
1.0	93
2.0	90
3.0	91
5.0	91

The results show that increasing the time of extraction above thirty seconds did not increase the efficiency of extraction. Therefore, thirty seconds blending was adopted for all subsequent experiments since the loss of solvent during blending was reduced.

c. Effect of the Volume of Solvent Upon the Recovery of Fat from Dough

The effect of the volume of 34 to 38°C boiling range light petroleum employing thirty seconds agitation in a

Waring Blendor upon the recovery of fat from dough was investigated. Volumes of light petroleum ranging from 50 to 175 ml. were employed to extract the fat from 60-g. portions of dough. The results of these experiments are given in Table LVIII.

Table LVIII

Effect of Light Petroleum Volume Upon the Recovery of Fat from Dough Employing a Blending Time of Thirty Seconds

Volume of 34-38°C Light Petroleum ml.	Fat Extracted from Dough Percent
50	67
75	80
100	87
125	91
150	92
175	94

Results in Table LVIII indicate that 150 ml. of 34 to 38°C light petroleum per 60 g. of dough recovered approximately 92 percent of the fat. Since it was intended to employ only 55 g. of dough or 45 g. of pie crust, 150 ml. of 34 - 38°C light petroleum was adopted to extract the fat. Subsequent extractions indicated that approximately 92 percent of the fat in dough and approximately 85 percent of the fat in pie crust was recovered with a single extraction.

3. PREPARATION OF PIE CRUST

a. Mixing of Dough

The dough was made from 100 parts flour, 60 parts lard and 50 parts water. Commercial pastry flour (not enriched), fresh steam rendered lard and distilled water were used. No sodium chloride was added, because this material may function as a pro-oxidant because of the traces of other metals it contains (59).

The antioxidant and/or acidic synergists were dissolved in a minimal volume of propylene glycol and thoroughly mixed into the lard at 45°C. The lard was cooled rapidly to 18°C and employed to make dough.

The flour and lard were cooled to approximately 18°C, and the lard creamed with half of the flour. The remainder of the flour was then mixed with the creamed portion, followed by the water, also at 18°C, which was mixed in lightly.

b. Baking of Pie Crust

The dough was spread to a depth of one-quarter of an inch in rectangular aluminium trays (11" x 11" x 3/8") and baked in an oven at 205°C, until lightly browned. To avoid scorching near the edge of the trays, the turned-up edge was only 3/8" high, because this edge served as an additional surface for heat absorption. The layer of dough was made slightly thicker near the edge of the tray in order to

utilize this additional heat input. Uniformity of baking of the pie crusts is of the utmost importance in order to obtain reliable keeping times.

c. Storage of Pie Crust

The pie crust was stored in an oven at 61°C. Periodically, the fat from a portion of pie crust was extracted and analyzed for added antioxidants and peroxide values.

4. ANALYTICAL METHODS

a. Reagent

Light Petroleum - (34 - 38°C) - Shake Skellysolve A (28 - 38°C) with one-tenth of its volume of concentrated sulphuric acid for two to five minutes. Run off the acid layer and wash the light petroleum with water and dilute alkali until free of acid. Distil the light petroleum in an all-glass fractionating column and collect the fraction boiling between 34 to 38°C.

b. Extraction of Fat from Dough and/or Pie Crust

Place 55 g. of dough or 45 g. of pie crust into a Waring Blendor. Add 150 ml. of 34 to 38°C light petroleum, cover and blend for thirty seconds. Transfer the contents of the blendor to a 250 ml. centrifuge bottle, stopper and centrifuge at 1000 R.P.M. for five minutes. Decant the slightly turbid light petroleum solution into a double 15 cm. Whatman

No. 54 filter and collect the filtrate in a 250 ml. flask. Remove the light petroleum under reduced pressure employing a water pump while warming the flask in a 40°C water-bath. Remove the last traces of light petroleum with a Hyvac pump. Such an extraction will recover approximately 92 percent of the fat in dough and 85 percent of that in pie crust.

c. Analysis for Phenolic Antioxidants and Peroxide Value

The extracted fat was analyzed for added phenolic antioxidants employing the procedures previously outlined. The peroxide value of the fat was determined employing Lea's "hot method" (81).

5. RECOVERY OF ANTIOXIDANTS FROM DOUGH AND PIE CRUST

a. Propyl Gallate

It was found impossible to recover any of the propyl gallate added to dough. However, lard containing 0.006 percent of propyl gallate upon being blended with light petroleum, centrifuged, filtered and the light petroleum evaporated under reduced pressure (as for the extraction of fat from dough) yielded as much as 98 percent of the added propyl gallate. Therefore, the procedure for extracting the fat from the sample occasioned no serious loss of propyl gallate.

The use of autoclaved flour also resulted in zero

recoveries of propyl gallate from dough, hence enzymatic activity was not responsible for the destruction of propyl gallate. In addition, flour not subjected to any oxidizing "improvers" such as chlorine dioxide also resulted in the complete loss of added propyl gallate. Therefore, the use of such oxidizing agents was not responsible for the destruction of propyl gallate.

Since propyl gallate reacts with metallic ions such as iron, it was suspected that this antioxidant was being oxidized or complexed by the metallic ions present in flour. In an attempt to prove this point, flour was fractionated into three crude fractions as follows:-

Flour was moistened with distilled water to form a dough which was allowed to stand at room temperature for thirty minutes. This dough was rubbed up by hand in a large volume of distilled water to separate crude starch and protein fractions. The starch suspension was filtered through cheese cloth, centrifuged, resuspended in dilute alkali and again centrifuged. This procedure was repeated until the supernatant solution was essentially free of protein. The resulting starch was washed with water to remove any alkali, centrifuged and air dried. The crude protein fraction was dissolved in dilute alkali and centrifuged to separate any starch present. The protein was precipitated by the addition of ethyl alcohol at an acid pH. This protein precipitate

was suspended in distilled water and adjusted to approximately pH7 and then dried in vacuo. All remaining solutions were collected, adjusted to pH 7, evaporated to dryness and are referred to as the "residue fraction".

The original flour and each of the three flour fractions was made into dough employing lard containing 0.006 percent of added propyl gallate. The partial analyses of the flour fractions and the recovery of propyl gallate from the resulting doughs are given in Table LIX.

Table LIX

Recovery of Propyl Gallate Added to Dough Made with Flour or Four Flour Fractions

Type of Flour Fraction	Starch %	Protein %	Ash %	Propyl Gallate Recovered %
Starchy Fraction 1	93.5	1.7	0.1	94
Starchy Fraction 2	93.0	4.5	0.2	80
Original Flour	72.0	10.8	0.4	0
Residue Fraction	27.3	42.5	16.6	0
Proteinacious Fraction	2.8	92.7	2.0	0

Data in Table LIX indicate that high recoveries of propyl gallate were possible when predominately starchy flour fractions were employed to make dough. However, as the protein and ash in the flour fractions increased, the recovery of propyl gallate rapidly decreased to zero. These results

indicated that propyl gallate was destroyed or complexed by some component of flour. In this connection Sair and Hall (129) observed that some factor in flour plays an important role in the carry-through of antioxidants. These authors considered that the metallic content of flour might be responsible but were unable to confirm this assumption, since the addition of metallic deactivators to dough did not increase the keeping quality of pie crust.

In the case of pie crust made from dough containing added citric acid or disodium dihydrogen ethylenediaminetetraacetate ("Versene"), followed by the addition of propyl gallate as a spray in a lard solution, resulted in recoveries of three to ten percent of the added propyl gallate. However, after a few hours at room temperature no propyl gallate could be recovered from the pie crust. These results are in agreement with the views of Sair and Hall (129). Nevertheless, the role of metallic ions in flour should not be underrated; in most cases the metallic deactivators employed are inadequate to complex all the metallic ions in flour and in addition the small amount of propyl gallated added (approximately 0.003 percent of the flour) could be complexed by a small fraction of the metallic content of flour.

b. Lauryl Gallate

Lauryl gallate recoveries as high as thirty percent

from dough and twenty percent from freshly baked pie crust have been obtained. The recovery of lauryl gallate sprayed onto pie crust containing "Versene" was sixty-four percent after a few hours at room temperature. In this case "Versene" was employed at 0.28 percent of the flour by weight. These results indicated that the loss of added antioxidants could be reduced and the keeping quality of the pie crust increased if a sufficiently large amount of a powerful metal deactivator was employed. (See Table LX).

The greater stability of lauryl gallate in dough as compared to propyl gallate employed at the same molecular concentration was explained on the basis that lauryl gallate is insoluble in water while being soluble in fat. These properties should restrict the lauryl gallate to the lipid portion of dough, thus avoiding such direct contact with the metallic ions of flour which should be concentrated in the aqueous phase. Propyl gallate, on the other hand, is slightly water-soluble and would diffuse into the aqueous phase and be complexed by the metallic ions.

c. Nordihydroguaiaretic Acid (NDGA)

The apparent recovery of NDGA from dough was of the order of two to six percent of that added while no NDGA was recovered from pie crust. The loss of NDGA added to dough was attributed to the same factors responsible for the loss of propyl gallate.

d. Butylated Hydroxyanisole (BHA)

Recoveries of BHA from dough have been as high as ninety-eight percent, though in most instances recoveries of eighty to ninety-two percent were obtained. In the case of pie crust, the time of baking normally employed resulted in a further loss of fifteen to twenty percent of the BHA present.

i. Effect of Time of Baking Upon the Recovery of Butylated Hydroxyanisole from Pie Crust

The effect of baking time in a 205°C oven upon the recovery of BHA from pie crust was investigated, with the results given in Figure 20.

These results indicate that the loss of BHA proceeds at an ever-increasing rate during the baking process. The loss of BHA was due, at least in part, to the steam-distillation of BHA from the dough.

ii. Recovery of Butylated Hydroxyanisole from Oven Vapours

A series of three experiments were conducted with the object of recovering the BHA vapourized during the baking of pie crust. Air was drawn through an oven kept at 205°C and containing 700 g. of dough, made with lard containing 0.02 percent BHA, at a rate of approximately 620 ml. per second. On leaving the oven the air and oven-vapours were passed through a series of two straight condensors and one spiral-condensor, cooled to 4°C by a rapid stream of water.

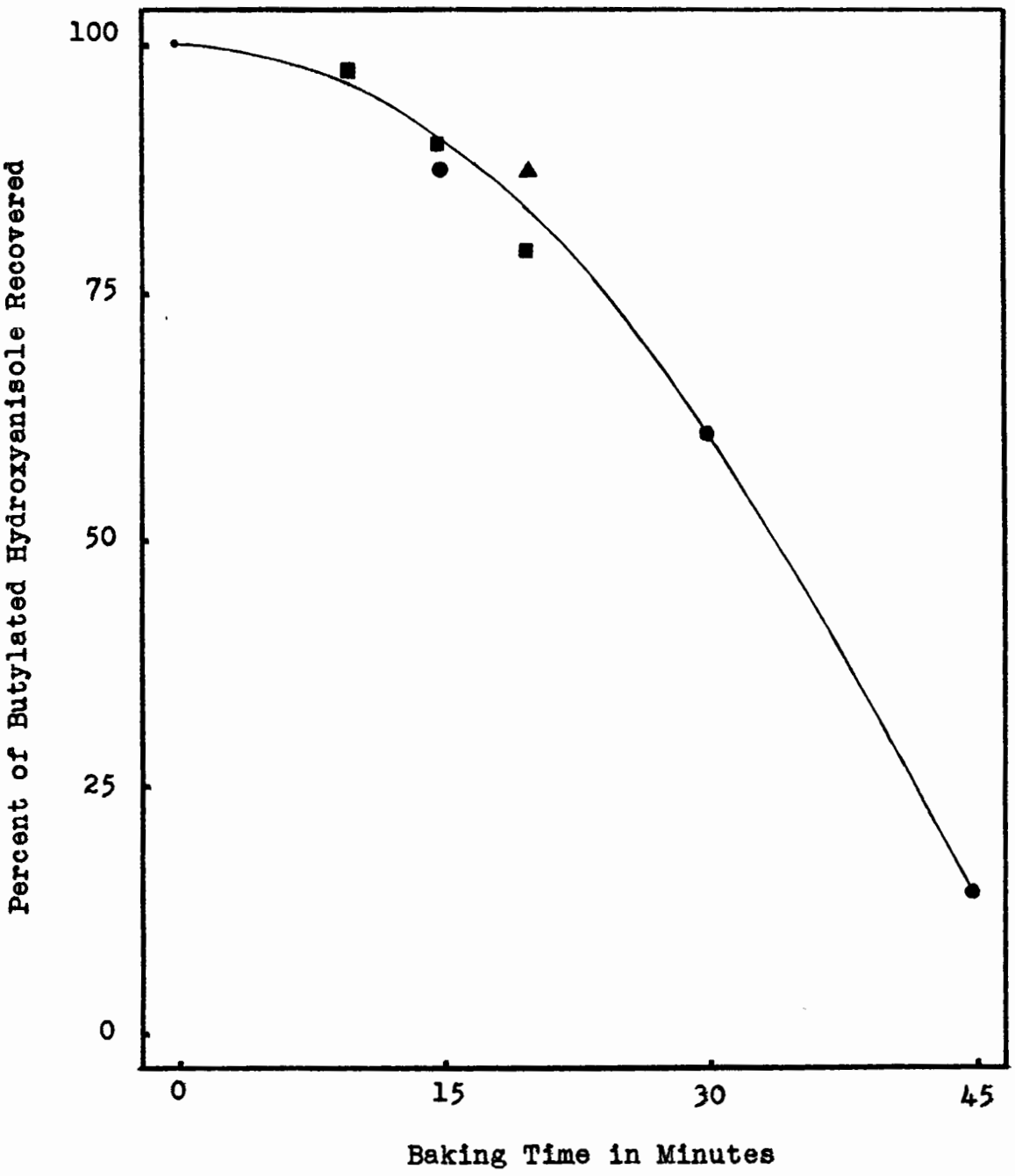


Figure 20 Effect of Baking Time at 205°C on Recovery of Butylated Hydroxyanisole from Pie Crust

The condensate amounted to eighty to eighty-six percent of the weight loss during the baking of the dough. Fifty-five to 80 percent of the BHA lost during the baking, was recovered in the condensate. If the amount of BHA actually present in the condensate is calculated on the basis of the total weight lost during the baking of the pie crust, then from sixty-four to eighty-five percent of the BHA lost during baking should have been in the oven vapours. It is clear, therefore, that the greater portion of BHA lost during the baking of the pie crust is due to vapourization.

iii. Recovery of Butylated Hydroxyanisole Vapourized from Pie Crust Stored at 61°C

Lard containing 0.02 percent BHA was used to make pie crust, 640 g. of this pie crust being placed in a large jar held at 61°C. A slow stream of nitrogen (10 ml. per minute) was passed through this jar and then through a series of six traps filled with absolute ethyl alcohol. After a period of ninety-one hours, the remaining alcohol in the traps was analyzed employing the 2,6-dichloroquinone-chloroimide - borax reagents and found to contain 0.7 mg. of BHA. Since this reagent couples with BHA to produce a blue complex, errors due to other reducing substances are avoided. Due to the large volume of nitrogen employed and the partial evaporization of the alcohol in the traps, it is considered significant that so much BHA was recovered from the traps.

It is important to note that whereas the loss of BHA during the baking of pie crust is normal and unavoidable, the loss of BHA by vapourization from pie crust stored at 61°C would not be encountered under normal storage conditions. It is unlikely that any appreciable amount of BHA would be vapourized from pie crust held at room temperature or under refrigeration.

6. PIE CRUST STORAGE EXPERIMENTS

Doughs were prepared, employing lard to which one or more of the following materials had been added, propyl gallate, lauryl gallate, NDGA, BHA, citric acid, phosphoric acid, α-amino acetic acid (glycine) and disodium dihydrogen ethylenediaminetetraacetate ("Versene"). When phenolic antioxidants were added, the fat in a portion of the dough was extracted and analyzed for the added phenolic antioxidants, prior to the baking process. The dough was then baked as previously described, the fat from a portion of the fresh pie crust was extracted and analyzed for antioxidant content and peroxide value. The remainder of the pie crust was stored at 61°C for further analyses. Samples containing acidic synergists but no phenolic antioxidants were analyzed for peroxide value only.

7. RESULTS AND DISCUSSION OF PIE CRUST STORAGE TESTS

For the purpose of comparison the results for peroxide

values were summarized and expressed in terms of the time required at 61°C for the fat in the pie crust to attain a peroxide value of 60 milliequivalents per kg. of fat. These results are given in Table LX.

Table LX

Effect of Different Antioxidant and Synergistic Combinations Upon the Time Required for the Fat of Pie Crust Held at 61°C to Attain a Peroxide Value of 60

Material Added to Lard Employed to Make Pie Crust		Time at 61°C to Attain a Peroxide Value of 60 Days
Blank		4
CA	Acidic Synergists	5
H_3PO_4 + Glycine	Acidic Synergists	5
Versene	Acidic Synergists	7
High CA	Acidic Synergists	7
NDGA + CA		7
PG + CA		7
CA (Baked) + PG (Sprayed on Pie Crust)		7
High CA (Baked) + PG (Sprayed on Pie Crust)		7
LG + CA		13
Versene (Baked) + PG and CA (Sprayed on Pie Crust)		15
Versene (Baked) + LG and CA (Sprayed on Pie Crust)		18
BHA + NDGA + CA		21
BHA + High CA		22
BHA + PG + CA		23
BHA + LG + CA		24
BHA + CA		25

Key to Table LX

Additive Abreviations	Additives Expressed as Percentage of the Lard Employed
CA	0.004% Anhydrous Citric Acid
High CA	0.460% Anhydrous Citric Acid
H_3PO_4	0.040% Phosphoric Acid
Glycine	0.040% ∝-Amino Acetic Acid
Versene	0.460% Disodium Dihydrogen ethylenediamine-tetraacetate
PG	0.006% Propyl Gallate
LG	0.0095% Lauryl Gallate
NDGA	0.010% Nordihydroguaiaretic Acid
BHA	0.008% 2-Tertiary-butyl-4-hydroxyanisole 0.012% 3-Tertiary-butyl-4-hydroxyanisole

The results shown in Table LX indicate that the presence of acidic synergists alone resulted in little improvement in the keeping quality of pie crust stored at 61°C. The addition of propyl gallate or NDGA in the presence of citric acid exerted no additional effect, thereby indicating that these antioxidants were rendered chemically inactive upon addition to the dough. Lauryl gallate, on the other hand, produced a significant improvement in the keeping time of pie crust. This is attributed to the fact that lauryl gallate is insoluble in water while being fat-soluble. These properties should tend to keep lauryl gallate in the fat phase, thereby avoiding direct contact with the metallic ions which are presumably located in the aqueous phase. For these reasons lauryl gallate might avoid oxidation or complexing by the

metallic ions. Dough baked with added "Versene" together with subsequent spraying of the pie crust with propyl gallate and citric acid or with lauryl gallate and citric acid resulted in a significant improvement in the stability of the pie crust. This fact indicates that the relatively large amount of "Versene" employed was able to complex a large portion of the metallic ions present, thus avoiding their destructive action on the propyl gallate or lauryl gallate added subsequently.

The most stable pie crusts were those containing BHA and citric acid. In all cases the BHA employed was a mixture of 60 percent pure 3-BHA and 40 percent of pure 2-BHA. The use of propyl gallate plus BHA plus citric acid or NDGA plus BHA plus citric acid resulted in no further increase in stability. The use of additional citric acid in the presence of BHA produced no additional stability. It is surprising, however, that the use of lauryl gallate plus BHA plus citric acid did not produce greater stability than BHA plus citric acid. Because the use of lauryl gallate plus citric acid produced a significant increase in stability, hence the use of lauryl gallate plus BHA plus citric acid would be expected to produce greater stability than that of BHA plus citric acid. Nevertheless, these results did not confirm this supposition.

The literature contains numerous references to the

relative effectiveness of antioxidants incorperated into pie crust. Of these, the data of Sair and Hall (129) are considered representative. These authors assigned the following values to the stability of pie crusts employing fat containing 0.01 percent of added antioxidant together with 0.003 percent of citric acid:- Control 2 to 3 days; propyl gallate 3 to 4 days; NDGA 3 to 4 days; lauryl gallate 4 to 5 days; and BHA 16 days at 61°C. These data are in agreement with the results cited in Table LX. The lower keeping times obtained by Sair and Hall (129) might be due, at least in part, to the pro-oxidant effects of the sodium chloride which was included in their dough mixture.

The disappearance curves for BHA in the presence of citric acid and other antioxidants are given in Figures 21, 22,23,24,25,26 and 27. In all cases there was approximately fifteen percent loss of BHA during the baking process. Thereafter, the loss of total BHA, 2-BHA and 3-BHA approximated straight-line functions of time. In the case of doughs containing propyl gallate plus BHA plus citric acid or NDGA plus BHA plus citric acid, no propyl gallate or NDGA was found in the pie crust and, therefore, no disappearance curves for these antioxidants are given in Figures 23 and 24. It is important to note that in all cases the peroxide value began to increase rapidly while a significant amount of the added BHA still remained in the pie crust. Peroxide values

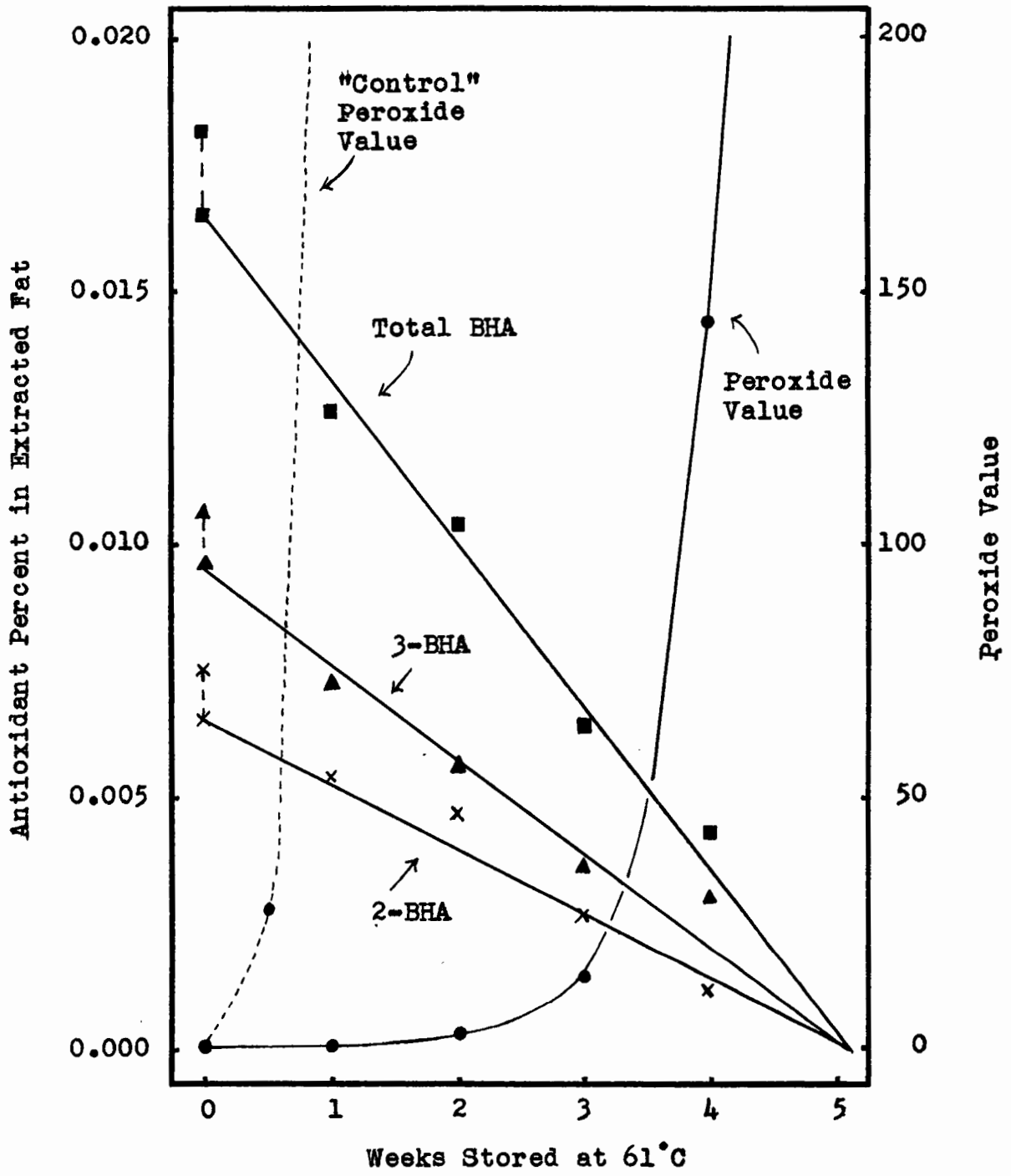


Figure 21 Loss of Butylated Hydroxyanisole from Pie Crust Containing Butylated Hydroxyanisole and Citric Acid

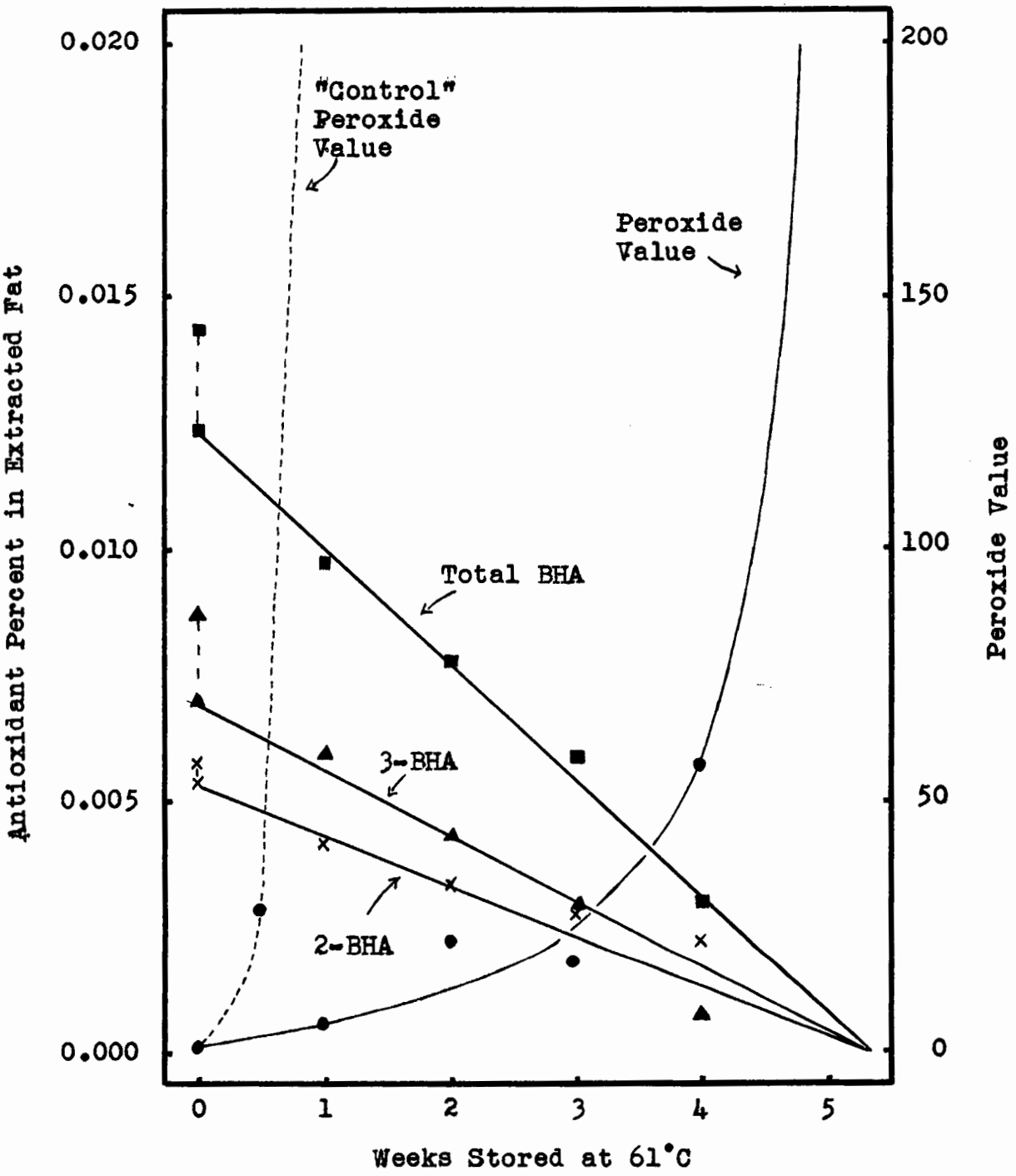


Figure 22 Loss of Butylated Hydroxyanisole from Pie Crust Containing Butylated Hydroxyanisole and High Citric Acid

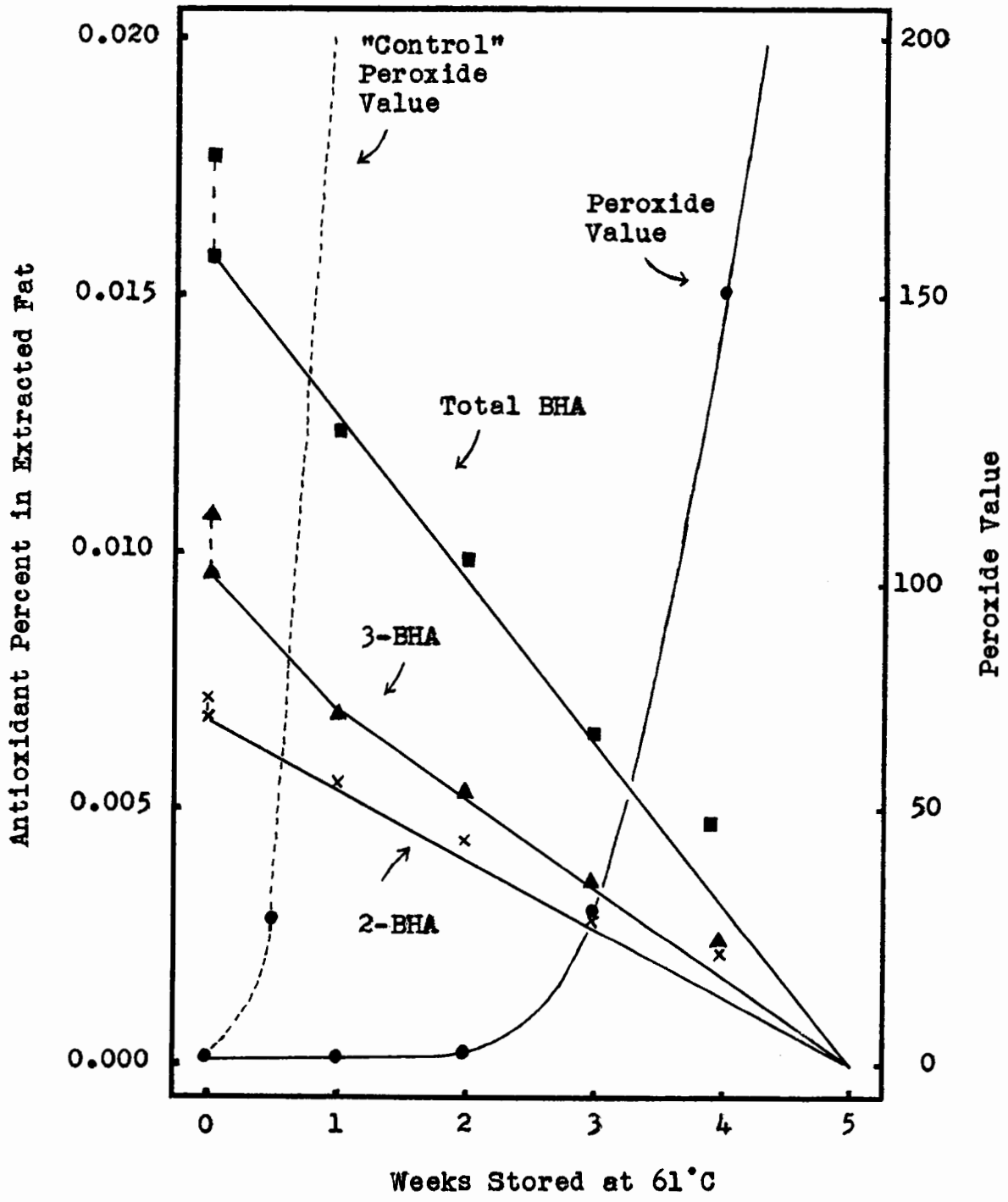


Figure 23 Loss of Butylated Hydroxyanisole from Pie Crust Containing Propyl Gallate, Butylated Hydroxyanisole and Citric Acid

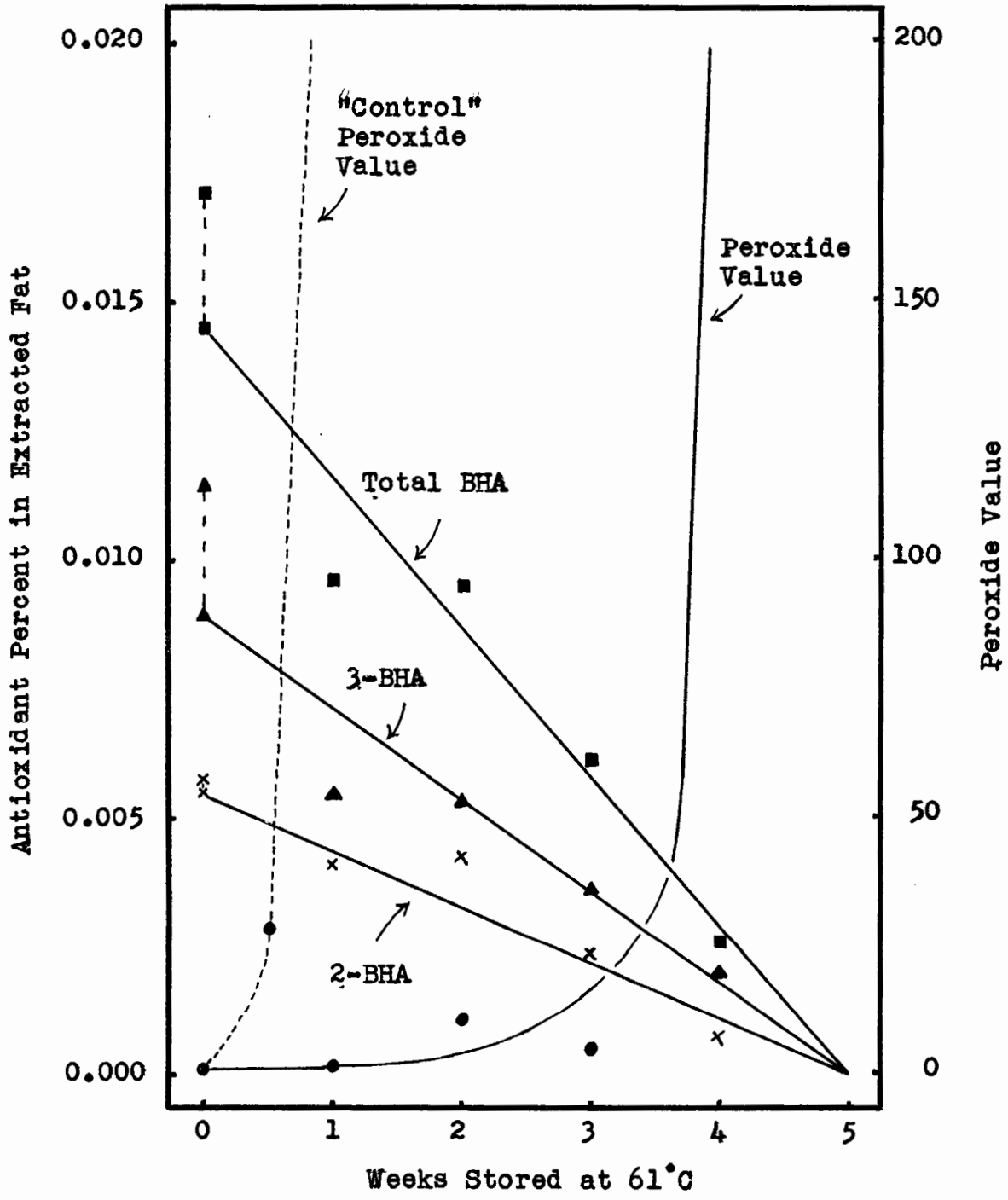


Figure 24 Loss of Butylated Hydroxyanisole from Pie Crust Containing Nordihydroguaiaretic Acid, Butylated Hydroxyanisole and Citric Acid

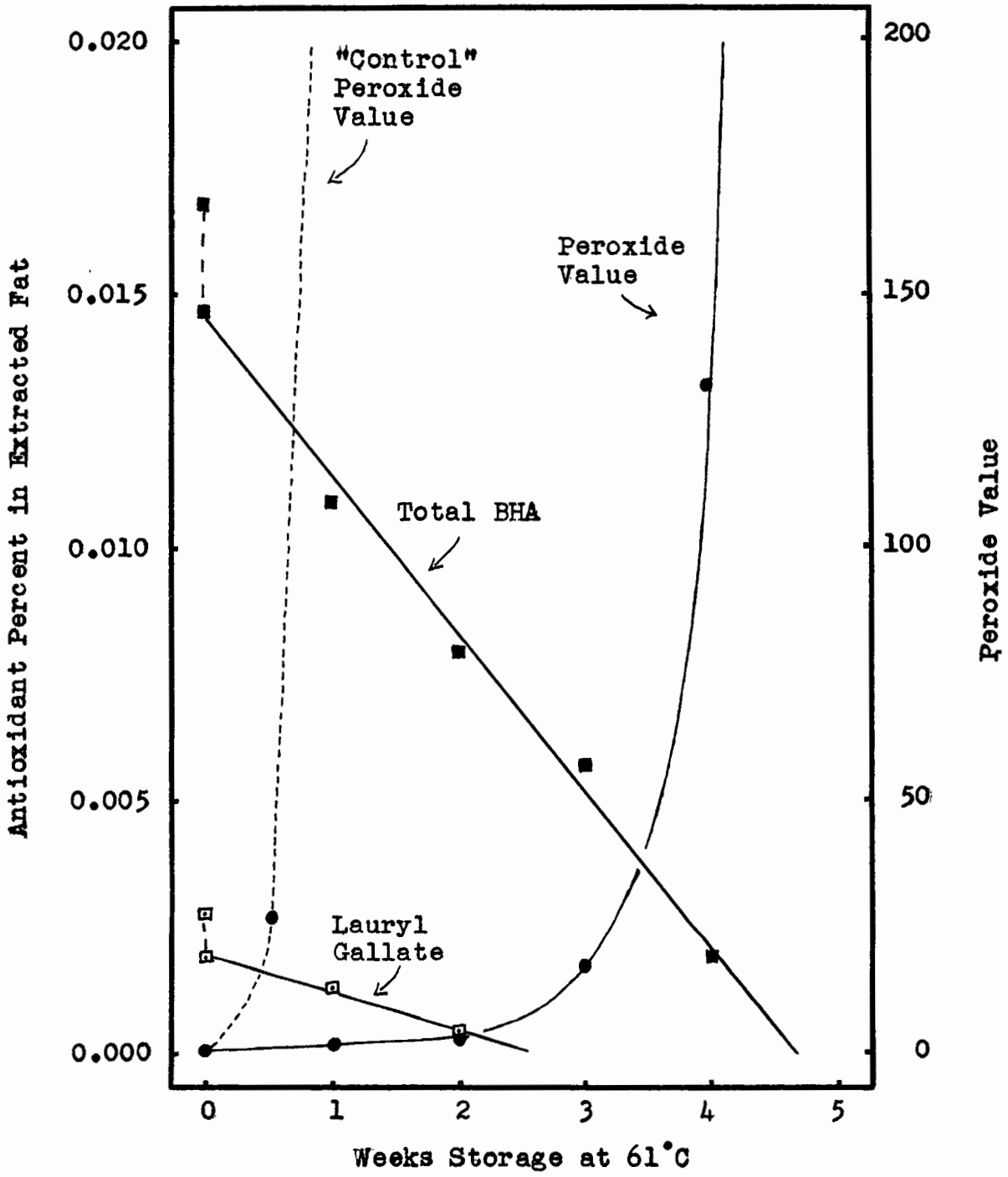


Figure 25 Loss of Butylated Hydroxyanisole and Lauryl Gallate from Pie Crust Containing Lauryl Gallate, Butylated Hydroxyanisole and Citric Acid

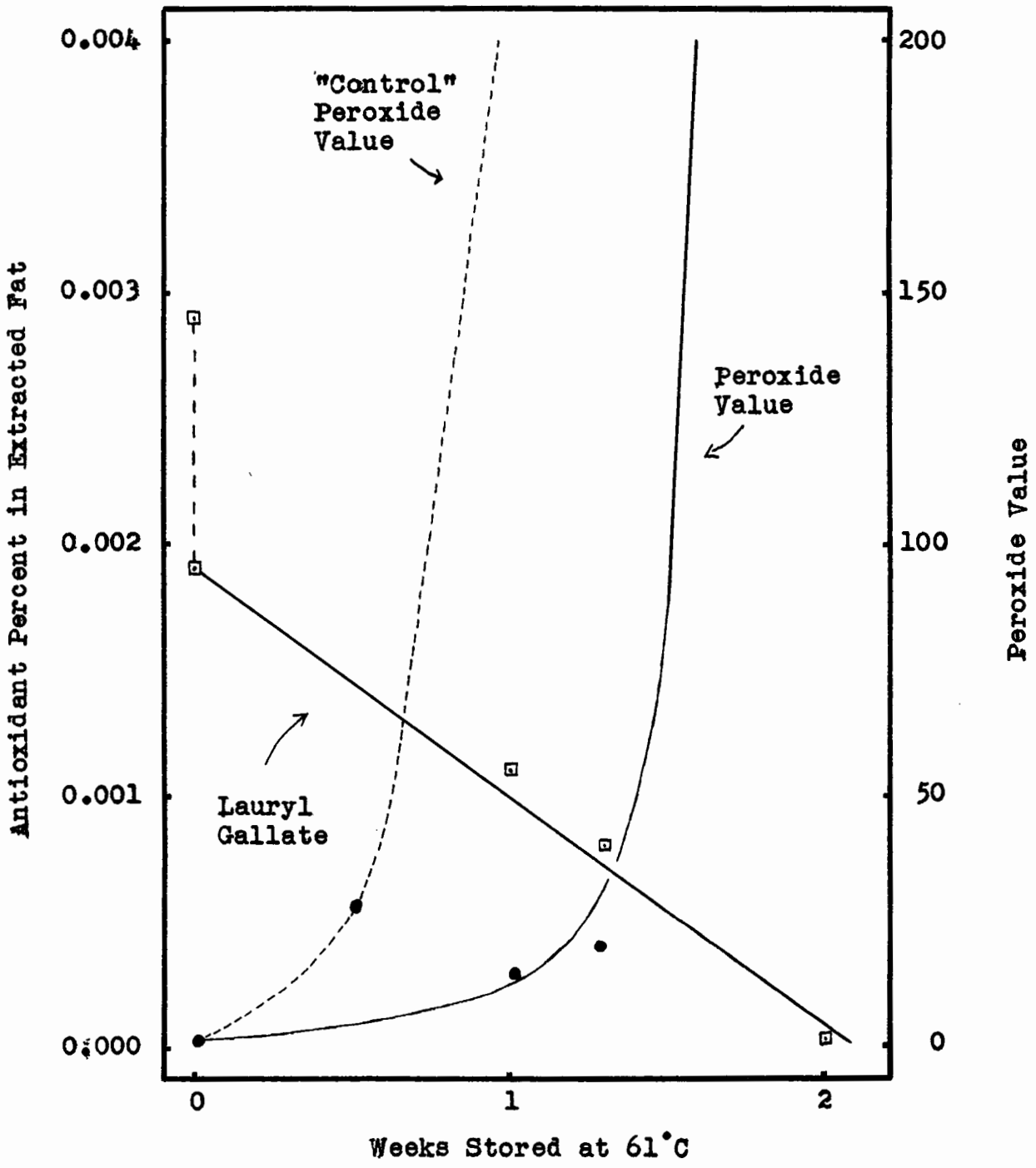


Figure 26 Loss of Lauryl Gallate from Pie Crust Containing Lauryl Gallate and Citric Acid

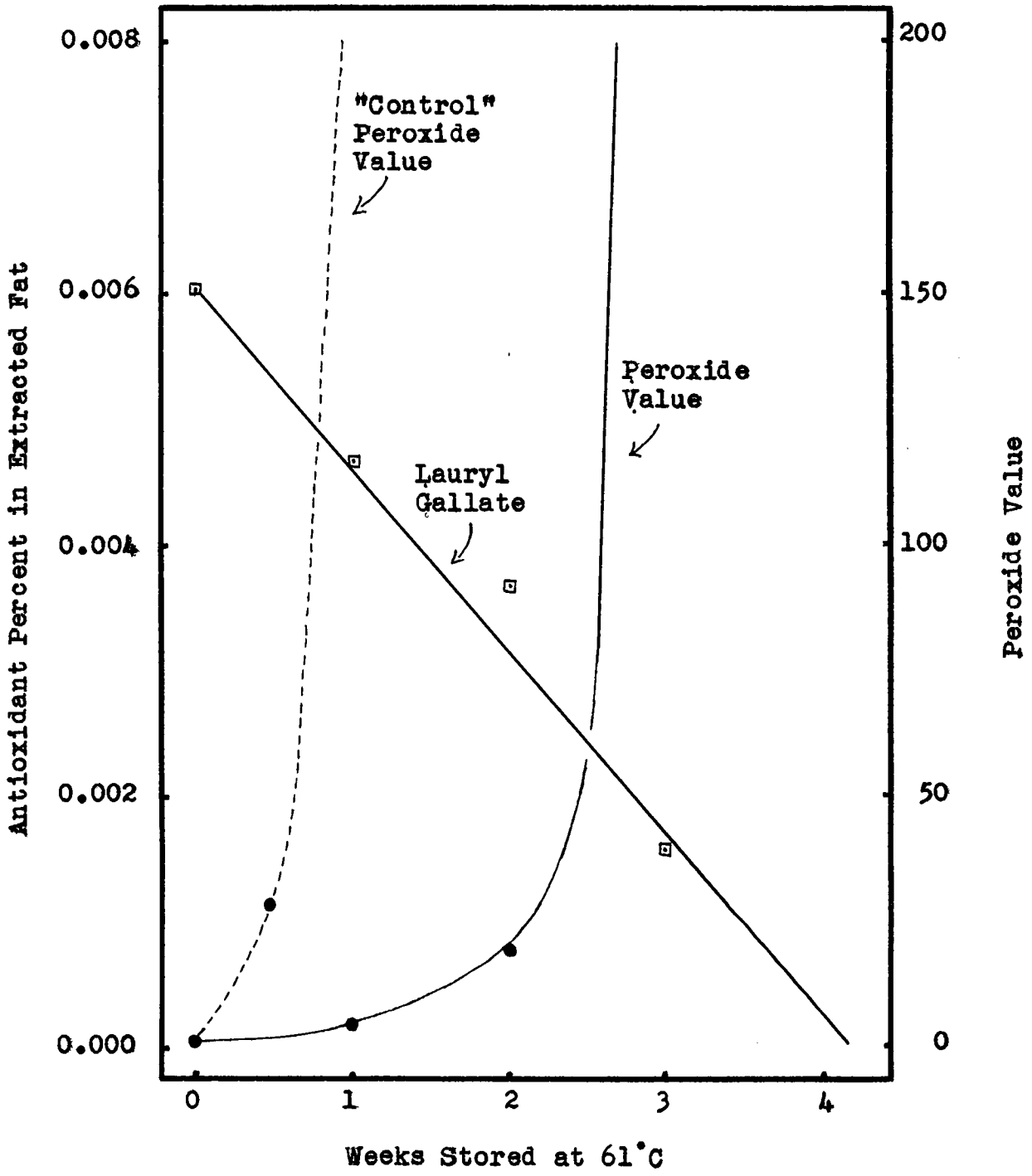


Figure 27 Loss of Lauryl Gallate from Pie Crust. Pie Crust Baked with Added Versene and then Sprayed with Lauryl Gallate and Citric Acid

as high as 280 milliequivalents per kg. of fat were recorded in the presence of analyzable quantities of BHA.

The disappearance curves for lauryl gallate plus BHA in the presence of citric acid are given in Figure 25. The loss of both lauryl gallate and BHA followed straight-line functions of time. In this case, it was impossible to estimate the 2-BHA and 3-BHA isomers present, because the analytical method is not applicable in the presence of lauryl gallate. Here again appreciable peroxide values were obtained before all the BHA disappeared.

The disappearance curve for lauryl gallate in the presence of citric acid is given in Figure 26 and is approximately a straight-line function of time. A high peroxide value was attained before all the lauryl gallate was lost.

In the case of pie crust made from dough containing "Versene" and then sprayed with lauryl gallate plus citric acid, the loss of lauryl gallate approximated a straight-line function of time, Figure 27.

Pie crust made from dough containing "Versene" and then sprayed with propyl gallate plus citric acid contained 10 percent of the added propyl gallate after a short interval. However, after seven days storage at 61°C no propyl gallate was found, therefore, no disappearance curve for propyl gallate was obtained.

8. CONCLUSIONS

Acidic synergists such as citric acid, phosphoric acid, α-amino acetic acid (glycine) or disodium dihydrogen ethylenediaminetetraacetate ("Versene") produced no significant improvement in the keeping quality of pie crust. Propyl gallate plus citric acid, or NDGA plus citric acid produced no significant effect on the keeping quality of pie crust owing to the fact that propyl gallate and NDGA are completely destroyed in dough made with flour. Lauryl gallate plus citric acid produced a significant improvement in the keeping quality of pie crust. The addition of "Versene" to dough, followed by spraying the pie crust with a solution of lauryl gallate plus citric acid or propyl gallate plus citric acid resulted in an increase in stability. The addition of BHA plus citric acid to dough resulted in the greatest stability of the pie crust. However, the addition of propyl gallate, lauryl gallate or NDGA to this dough produced no further increase in the stability of the pie crust. BHA is partly vapourized from dough during the baking process and also during the storage of pie crust at 61°C.

SUMMARY

1. A procedure has been developed for the quantitative separation of propyl gallate, butylated hydroxyanisole and tocopherol when present in the same fat sample. Propyl gallate is extracted from the fat in light petroleum solution by means of an aqueous solution of ammonium acetate; butylated hydroxyanisole is subsequently extracted with 72 percent ethyl alcohol, while tocopherol remains in the light petroleum solution of the fat.
2. Sensitive qualitative tests are described for the detection of gallic acid, propyl gallate, lauryl gallate, butylated hydroxyanisole and gum guaiac in fats.
3. Colorimetric methods are presented for the quantitative determination of propyl gallate, butylated hydroxyanisole, nordihydroguaiaretic acid and tocopherol in lard and shortening. These procedures permit the quantitative analysis of all combinations of these antioxidants except when propyl gallate and nordihydroguaiaretic acid are present in the same fat sample.
4. A rapid colorimetric procedure has been developed for the determination of butylated hydroxyanisole in lard and shortening. This procedure, employing a 2,6-dichloroquinonechloroimide - borax reagent, is recommended for monitoring the addition of butylated hydroxyanisole to

commercial lard or shortening. The presence of propyl gallate or nordihydroguaiaretic acid do not introduce analytical errors, since the antioxidant preparation employed is used as a reference standard.

5. A method has been developed for estimating the proportion of 2-tertiary-butyl-4-hydroxyanisole and 3-tertiary-butyl-4-hydroxyanisole in commercial butylated hydroxyanisole. This procedure is applicable to fat samples containing butylated hydroxyanisole in the presence of propyl gallate or tocopherol
6. A colorimetric method is described for the quantitative estimation of lauryl gallate in lard and shortening.
7. A rapid quantitative procedure is described for determining tocopherol in butter oil. This procedure avoids interferences due to carotene, vitamin A alcohol, the synthetic colours, Oil Yellow AB and Oil Yellow OB and is suitable for detecting the adulteration of butter oil with vegetable oils.
8. Analytical values are given for the amounts of propyl gallate, total butylated hydroxyanisole, 2-tertiary-butyl-4-hydroxyanisole, 3-tertiary-butyl-4-hydroxyanisole and tocopherol found in a number of commercial lards and shortenings.
9. Analytical values are presented for the tocopherol content of butter, vegetable and animal fats and oils.

10. Experiments were conducted to ascertain the effectiveness of different concentrations of propyl gallate plus butylated hydroxyanisole plus citric acid for the prevention of rancidification of lards stored at 41° and 61°C.

11. The effectiveness of different concentrations of butylated hydroxyanisole plus citric acid in preventing the rancidification of lard stored at 61°C has been investigated. The results indicate that butylated hydroxyanisole in the absence of propyl gallate is relatively ineffective as an antioxidant for lard.

12. The relative antioxidant activity of 2-tertiary-butyl-4-hydroxyanisole and 3-tertiary-butyl-4-hydroxyanisole was investigated in lard stored at 61°C. The 3-tertiary-butyl-4-hydroxyanisole isomer was found to be the more effective antioxidant.

13. The rates of loss for propyl gallate, total butylated hydroxyanisole, 2-tertiary-butyl-4-hydroxyanisole and 3-tertiary-butyl-4-hydroxyanisole were determined in lard stored at 61°C.

    a. In the presence of butylated hydroxyanisole, the loss of propyl gallate approximated a straight-line function of time.

    b. In the presence of propyl gallate, the loss of butylated hydroxyanisole was slow; after the propyl

gallate had been destroyed, the rate of loss of butylated hydroxyanisole increased rapidly.

c. In the absence of propyl gallate the loss of 3-tertiary-butyl-4-hydroxyanisole proceeded at a more rapid rate than for 2-tertiary-butyl-4-hydroxyanisole.

d. When propyl gallate and butylated hydroxyanisole were present in the same lard sample, the peroxide value increased very slowly until all the propyl gallate had been destroyed: thereafter the peroxide value increased rapidly. In lard containing only butylated hydroxyanisole, the peroxide value increased rapidly after a short induction period. High peroxide values were encountered in the presence of detectable quantities of this antioxidant.

14. Storage tests were conducted to determine the relative effectiveness of different combinations of propyl gallate, lauryl gallate, butylated hydroxyanisole, nordihydroguaiaretic acid, citric acid, ortho-phosphoric acid, glycine and disodium dihydrogen ethylenediamine-tetraacetate ("Versene") upon the stability of pie crust held at 61°C.

a. Acidic synergists such as citric acid, phosphoric acid, α-amino acetic acid (glycine) or disodium dihydrogen ethylenediamimetetraacetate ("Versene") produced no significant improvement in the keeping quality of pie crust.

b. Propyl gallate plus citric acid, or nordihydroguaiaretic acid plus citric acid produced no significant effect upon the keeping quality of pie crust owing to the fact that the activity of these antioxidants was completely destroyed in dough made with flour.

c. Lauryl gallate plus citric acid produced a significant improvement in the keeping quality of pie crust.

d. The addition of "Versene" to dough, and spraying the pie crust with a solution of lauryl gallate plus citric acid or propyl gallate plus citric acid resulted in a significant increase in stability.

e. The addition of butylated hydroxyanisole plus citric acid to dough resulted in the greatest stability of the pie crust. However, the addition of propyl gallate, lauryl gallate or nordihydroguaiaretic acid to this dough produced no further increase in the stability of the pie crust.

f. Butylated hydroxyanisole is partly vapourized from dough during the baking process and also during the storage of pie crust at 61°C.

15. The rates of loss of propyl gallate, lauryl gallate, total butylated hydroxyanisole, 2-tertiary-butyl-4-hydroxyanisole, 3-tertiary-butyl-4-hydroxyanisole and nordihydroguaiaretic acid were determined in doughs, freshly baked pie crusts and during the subsequent storage of pie crust at 61°C.

a. Propyl gallate and nordihydroguaiaretic acid were completely lost upon addition to dough.

b. A large proportion of the lauryl gallate was lost in the dough and during the baking of pie crust. The loss of the remaining lauryl gallate from pie crust approximated a straight-line function of time.

c. Eight to 15 percent of the butylated hydroxyanisole added to dough could not be recovered. Of the remaining butylated hydroxyanisole, a further 15 to 20 percent was lost during the baking of the pie crust. In pie crust, stored at 61°C, the losses of total butylated hydroxyanisole, 2-tertiary-butyl-4-hydroxyanisole and 3-tertiary-butyl-4-hydroxyanisole approximated straight-line functions of time.

d. In pie crust stored at 61°C, the rate of peroxide formation was much more rapid than in lard stored at 61°C. Large peroxide values were found in pie crust, even in the presence of detectable quantities of added antioxidants such as lauryl gallate or butylated hydroxyanisole.

e. The presence of relatively large amounts of "Versene" in dough, followed by the addition of lauryl gallate or propyl gallate as a surface spray, greatly increased the recoveries of these antioxidants and also their antioxidant activity.

CLAIMS TO ORIGINAL RESEARCH

The writer claims to have made the following contributions to the analytical chemistry of antioxidants used in foods and to the study of the stability of food fats:-

1. The development of a procedure for the quantitative extraction of propyl gallate from fat in light petroleum solution. This procedure is based upon the extraction of propyl gallate with 1.67 percent aqueous ammonium acetate, followed by the extraction of butylated hydroxyanisole employing 72 percent ethyl alcohol, while leaving the tocopherol and fat in the light petroleum phase.
2. The discovery that propyl gallate, lauryl gallate and possibly other gallate esters produce an intense rose colour with sodium carbonate and other alkalis, provided acetone is present.
3. An application of Mitchell's ferrous tartrate reagent to the quantitative analysis of propyl gallate.
4. The development of a method for the quantitative analysis of lauryl gallate in fats based in part on the discovery mentioned in paragraph 2, and employing a sodium carbonate reagent in the presence of acetone.
5. The development of a procedure for the quantitative determination of both butylated hydroxyanisole and nordihydroguaiaretic acid when present in the same fat sample.

The procedure is based on the different rates of reaction of these two substances with a reagent containing ferric chloride and α,α'-bipyridine.

6. The application of the Gibbs' 2,6-dichloroquinonechloroimide - borax reagent to the qualitative and quantitative determination of butylated hydroxyanisole.

7. The development of an analytical procedure for determining the proportion of 2-tertiary-butyl-4-hydroxyanisole and 3-tertiary-butyl-4-hydroxyanisole isomers in commercial butylated hydroxyanisole added to fats.

8. An adaption of the Emmerie and Engel ferric chloride plus α,α'-bipyridine reagents to the quantitative determination of tocopherol in lard and shortening.

9. The development of a rapid analytical procedure for the determination of tocopherol in fats and oils which eliminates interferences from Oil Yellow AB, Oil Yellow OB, vitamin A alcohol and carotene. This method is suitable for the routine examination of commercial butter oils for the detection of adulteration with vegetable oils other than coconut oil. Such adulteration is indicated by the presence of excessively high tocopherol concentrations.

10. The use of a qualitative test for butylated hydroxyanisole in butter oil, as a means of detecting the adulteration of butter with fats containing added butylated hydroxyanisole.

11. The relative rates of destruction of propyl gallate, total butylated hydroxyanisole, 2-tertiary-butyl-4-hydroxyanisole and 3-tertiary-butyl-4-hydroxyanisole when added together at three different concentrations to lard stored at 61°C have been studied for the first time.

12. The relative rates of destruction of 2-tertiary-butyl-4-hydroxyanisole and 3-tertiary-butyl-4-hydroxyanisole alone or together in lard stored at 61°C have been studied for the first time.

13. The losses of various combinations of propyl gallate, butylated hydroxyanisole, nordihydroguaiaretic acid and lauryl gallate in dough, during the baking and subsequent storage of the pie crust at 61°C have been studied. Tentative conclusions have been drawn as to the relative importance of the parts played by vapourization during baking, protective actions of sequestering agents, and effects of storage at 61°C.

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