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DEVELOPMENT OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY PROCEDURE FOR EVALUATING ANTIOXIDANT ACTIVITY

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By

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ABSTRACT

DEVELOPMENT OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY PROCEDURE FOR EVALUATING ANTIOXIDANT ACTIVITY

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Antioxidant activity of 3-dehydroshikimic acid (DHS) in a bulk oil and an oil-in-water emulsion was evaluated using two techniques. DHS was found to be an excellent antioxidant in lard when evaluated using the peroxide value method. The compound was also evaluated in a 10% corn oil-in-water emulsion using the measurement of conjugated diene hydroperoxides to gauge the extent of oxidation. This was accomplished using a high performance liquid chromatography method to eliminate the effects of other compounds which may absorb in the 230-235 nm range. The accelerated procedure is very simple and avoids many of the pitfalls of established methods, including interference by other compounds and the use of excessive heat. In the emulsion, DHS was either inactive or acted as a prooxidant depending on the techniques used to accelerate peroxidation in the system.

Interfacial phenomena may explain why DHS was effective in the bulk oil and not in the emulsion. This hydrophilic compound afforded excellent protection in the bulk oil system by most likely being oriented in the air-oil interface. In the emulsion, most of the DHS was likely dissolved in the water phase and was thus ineffective in protecting the oil against peroxidation.

To my parents, my brother Andrew, Jennifer, and Molly

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LIST OF ABBREVIATIONS

AOM	Active Oxygen Method
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
DHS	3-Dehydroshikimic Acid
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic Acid
FFA	Free Fatty Acids
HPLC	High Performance Liquid Chromatography
MDA	Malondialdehyde
MRP	Maillard Reaction Product
TBA	2-Thiobarbituric Acid
TBARS	2-Thiobarbituric Acid Reactive Substances
TBHQ	*tert*-Butylhydroquinone
UV	Ultraviolet

INTRODUCTION

The peroxidation of food lipids can result in the formation of unpleasant tastes and odors and is generally recognized as one of the primary factors limiting the shelf-life of certain food products. The most commonly used method of retarding lipid peroxidation is by the addition of antioxidants. Although many of these compounds are highly effective, most are synthetic. Use of synthetic compounds has been connected to certain health risks and a general public concern persists regarding their safety. These concerns have prompted a search for alternative natural sources of antioxidants.

In the past, the use of natural antioxidants has been limited due to color and flavor problems and high costs. Thus, there is a need for low cost, versatile and effective natural antioxidants. One inexpensive experimental antioxidant is 3-dehydroshikimic acid (DHS). This compound can be produced easily and inexpensively from D-glucose by suitably engineered microbes.

The methodology to evaluate natural antioxidants must be carefully interpreted as an antioxidant may display very different levels of effectiveness depending on which substrate, acceleration techniques, and testing methods are used for its analysis. Therefore, the conclusions reached in many studies may not be correct due to the use of inappropriate methods to follow peroxidation.

The first objective of this study was to evaluate the antioxidant activity of DHS relative to other established antioxidants in bulk oil. Lard was chosen as the substrate and the extent of peroxidation was monitored by measuring the peroxide value at designated time intervals. This method, although empirical, is widely accepted as a means to follow peroxidation.

The second objective of this study was to evaluate the antioxidant activity of DHS in a 10% corn oil-in-water emulsion using a procedure involving the quantification of conjugated diene hydroperoxides. The measurement of conjugated dienes by ultraviolet spectroscopy is commonly criticized because changes in absorbance in the range of 230-235 nm cannot be uniquely attributed to the hydroperoxides. To minimize this problem, a high performance liquid chromatographic (HPLC) method was developed to separate the conjugated diene hydroperoxides from other interfering compounds. Peaks were confirmed to be hydroperoxides via a chemiluminescence reaction and a simple reduction technique.

The final aim of this research was to compare the performance of DHS in the bulk oil and the emulsion and speculate as to why any differences in antioxidant activity may have occurred.

LITERATURE REVIEW

I. Lipid Peroxidation

The peroxidation of food lipids, which eventually leads to rancidity is a major concern to food scientists and many others who work in the food processing industry. The oxidative degradation of mono and polyunsaturated lipids can result in the formation of unpleasant tastes and odors and may alter the color, viscosity or solubility of certain substrates (Gutteridge and Haliwell, 1990). Therefore, it is desirable to slow down the rate of oxidation since it is often the decisive factor in determining the storage life of many food products (Coupland and McClements, 1996).

The role of lipid peroxidation in health and disease has also received much attention. Peroxidation has been implicated in a variety of pathological processes including atherosclerosis, retinal degeneration and acceleration of the aging process (Bidlack and Tappel, 1973; Csallany and Isayaz, 1976; Jain and Hochstein, 1980). There is also evidence that free radicals produced via the oxidation pathway may directly and/ or indirectly affect the initiation and progress of cancer (Choe and Yu, 1995).

II. Chemical Mechanism of Lipid Peroxidation

Lipid peroxidation is a classic free radical chain reaction that occurs in four phases: initiation, propagation, branching, and termination (Kanner *et al.*, 1987):

Initation	LH	+	initiator	→	$L^{\cdot}$
Propagation	$L^{\cdot}$	+	O_2	→	$LOO^{\cdot}$
	$LOO^{\cdot}$	+	LH	→	$LOOH + L^{\cdot}$
Branching	LOOH			→	$LO^{\cdot} + HO^{\cdot}$
	2LOOH			→	$LOO^{\cdot} + LO^{\cdot} + H_2O$
Termination	$LO^{\cdot}$	+	$LO^{\cdot}$	→	non radical polymers
	$LOO^{\cdot}$	+	$LOO^{\cdot}$	→	non radical polymers

Where: LH = unsaturated fatty acid

$HO^{\cdot}$ = hydroxyl radical

$L^{\cdot}$ = allyl radical

$LO^{\cdot}$ = alkoxyl radical

$LOO^{\cdot}$ = peroxyl radical

LOOH = hydroperoxide

During *initiation*, a carbon-centered lipid radical ($L^{\cdot}$) is formed by the abstraction of a hydrogen atom from an unsaturated fatty acid (LH) in the presence of an initiator. *Propagation* involves further reactions of the carbon-centered lipid radical with oxygen and lipid to yield peroxyl radicals ($LOO^{\cdot}$) and lipid hydroperoxides (LOOH). During *branching*, these hydroperoxides break down

into alkoxyl ($LO^{\cdot}$) and hydroxyl ($HO^{\cdot}$) radicals which ultimately results in the formation of more hydroperoxides (LOOH). *Termination* occurs upon the formation of a non-radical final product from two radicals (Kanner *et al.*, 1987).

III. Role of Transition Metals

The ground state of a lipid molecule is of singlet multiplicity whereas that of oxygen is of triplet multiplicity (Miller *et al.*, 1990). Therefore, the direct reaction of unsaturated fatty acids (LH) with oxygen is spin-forbidden and precluded by other thermodynamic constraints (Aust *et al.*, 1985). Thus, lipid peroxidation must be preceded by alteration of the electronic structure of oxygen or the unsaturated fatty acid.

Transition metals such as copper and iron possess a labile d-electron system and are thus well suited to catalyze redox reactions. These metals can achieve a range of oxidation states which enables them to easily transfer electrons. Stable paramagnetic states, resulting from the presence of unpaired electrons, are common for transition metals and facilitate their reaction with radical substrates. Thus, they are able to remove the spin restriction between oxygen and unsaturated fatty acids and thereby promote lipid peroxidation (Kanner *et al.*, 1987).

Among the transition metals, iron makes the most significant contribution to the acceleration of lipid peroxidation. Iron may catalyze this reaction via two basic mechanisms:

MECHANISM I: LOOH-Dependent Lipid Peroxidation (Minotti and Aust, 1987)

$$Fe(II) + LOOH \rightarrow Fe(III) + OH^- + LO^\cdot$$

$$LO^\cdot + LH \rightarrow L^\cdot + LOH$$

Transition metals can substantially enhance the propagation of lipid peroxidation (Minotti and Aust, 1987). Here, Fe(II) catalyzes the decomposition of trace amounts of preformed LOOHs to form alkoxyl radicals ($LO^\cdot$) which abstract hydrogen from a neighboring allylic bond. This reaction is very important because food products almost always contain at least small amounts of preformed LOOH molecules (Minotti, 1992).

MECHANISM II: LOOH-Independent Lipid Peroxidation

A $Fe(II) + O_2 \rightarrow Fe(III) + O_2^{\cdot -}$

B $2\,O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$

C $Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + {}^\cdot OH$

D $O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH^- + {}^\cdot OH$

E ${}^\cdot OH + LH \rightarrow H_2O + L^\cdot$

Here, Fe(II) reacts with oxygen to yield a superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) (equations *A* and *B*). Then, Fe(II) and hydrogen peroxide may react to form hydroxyl radicals (${}^\cdot OH$) via the Fenton reaction (equation *C*). This can also occur via the iron-catalyzed Haber-Weiss reaction (equation *D*). Although this reaction is exothermic, a transition metal (e.g. Fe or Cu) is needed for catalysis (Hsieh and Kinsella, 1989). Neither hydrogen peroxide nor

superoxide is thought to cause lipid peroxidation directly (Hsieh and Kinsella, 1989). However, hydroxyl radicals are very reactive and have the ability to abstract hydrogen from unsaturated fatty acids (LH) (equation *E*) (Minotti, 1992). This can occur in the absence of preformed LOOH molecules, hence the name "LOOH-Independent Lipid Peroxidation."

IV. Antioxidants

The unavoidable presence of transition metal catalysts and oxygen in food products poses a serious challenge to the food technologist to minimize the undesirable peroxidation of the lipid constituents (Graf, 1994). Although there are many methods available to minimize this reaction, the most commonly used method of retarding lipid oxidation in foods is by the addition of antioxidants. An antioxidant is defined as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell, 1990).

Antioxidants can be divided into three major categories based on mode of action: Type I, Type II, and Type III (Labuza, 1971). Type I antioxidants are primarily phenolic compounds which donate a proton to a free radical. These are also known as "free radical chain stoppers" and include compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylated hydroquinone (TBHQ), and the tocopherol family.

Type II antioxidants include metal chelators such as citric acid, ethylenediaminetetraacetic acid (EDTA) and some forms of ascorbic acid. These

substances work by chelating metal ion catalysts, thereby suppressing the generation of free radicals and reducing the overall rate of lipid peroxidation. Inhibition of hydroxyl radical generation by binding metal ions can occur by two mechanisms. First, binding of the metal ions may alter their redox potential and/or accessibility such that they can no longer participate in the formation of hydroxyl radicals. A second possibility is that binding of a catalytic transition metal ion to an antioxidant does not prevent the redox reactions, but instead these reactions are directed onto the antioxidant thereby sparing other more important targets (Halliwell *et al.*,1995).

Type III antioxidants are very broad in scope and include alteration of various environmental factors (e.g., storage conditions). Reduction of the oxygen partial pressure in a package falls under this general description. This can be accomplished through de-aeration, headspace control, gas or vacuum packaging, and gas-tight sealing. Lowering storage temperature via refrigeration or freezing will also slow down lipid peroxidation. Finally, preventing contamination by catalytic pro-oxidative metals can also greatly reduce the rate of this reaction.

Other type III antioxidants that can be applied to fats and oils include refining, deodorization, and partial hydrogenation. This makes them less susceptible to peroxidation and thereby less likely to become rancid as quickly as they would in their original state.

IV.1. Mechanism of Free Radical Scavenging

Chain breaking antioxidants (AH) interfere with the free radical chain mechanism of peroxidation by donating an easily abstractable hydrogen atom to a lipid radical. This acts to compete with the chain propagating reactions of lipid peroxidation (equations *A* and *B*). The antioxidant derived radical, $A^{\cdot}$, may react further with another peroxyl radical, dimerize to A_2, or may even get converted back to AH by reaction with another molecule (equations *C-E*) (Gutteridge and Halliwell, 1990).

A $AH + LOO^{\cdot} \rightarrow LOOH + A^{\cdot}$

B $LO^{\cdot} + AH \rightarrow LOH + A^{\cdot}$

C $LOO^{\cdot} + A^{\cdot} \rightarrow LOOA$

D $LO^{\cdot} + A^{\cdot} \rightarrow LOA$

E $A^{\cdot} + A^{\cdot} \rightarrow A_2$

Many food antioxidants such as propyl gallate and TBHQ act by scavenging free radicals. They reduce the primary radical to a non-radical chemical species and subsequently are transformed into oxidized antioxidant radicals. There are two basic conditions that a chemical must satisfy in order to be classified as a chain-breaking antioxidant. First, when present in low concentrations relative to the substrate to be oxidized, it must be able to delay or prevent the free-radical mediated oxidation. Second, the oxidized antioxidant

radical formed after scavenging must be of such low reactivity that no further reactions with lipids can occur (Halliwell, 1990).

Most of these chemicals are generally phenolic compounds with one or more hindered phenolic hydroxyl groups. These compounds can readily donate hydrogen atoms and electrons and they are able to form stable resonance hybrids.

V. Search for Novel Antioxidants of Natural Origin

The addition of synthetic chain-breaking antioxidants such as BHA and TBHQ can control lipid oxidation in foods (Hettiarachchy, 1996). However, use of these synthetic compounds has been connected to certain health risks. For example, BHA can induce cancer of the rat forestomach when ingested in large doses (Schildermann *et al.*, 1995). Thus, a general public concern persists regarding the safety of synthetic food additives. As a result, these concerns have prompted a search for alternative sources of antioxidants. Natural antioxidant substances are presumed to be safe by the general public since they occur in plant foods and are seen as more desirable than their synthetic counterparts such as BHA, BHT and TBHQ (Frankel, 1993).

One commonly used antioxidant is alpha-tocopherol. This substance is a naturally occurring fat-soluble molecule that can act as a chain breaking (type I) antioxidant and its presence within lipoprotein particles has been shown to confer resistance to oxidation on constituent unsaturated fatty acids (Esterbauer *et al.*, 1992). However, it has been reported that in the absence of co-antioxidants such

as ascorbic acid, alpha- tocopherol may act as a prooxidant, propagating as opposed to breaking chains of peroxidation (Stocker, 1994).

Another large group of natural antioxidants come from spice and herb extracts. Several efficient antioxidant compounds have been extracted from rosemary (Frankel *et al.*, 1996; Houlihan *et al.*, 1985), oregano (Kikuzaki and Nakatani, 1989), thyme (Miura and Nakatani, 1989), and ginger (Kikuzaki and Nakatani, 1993). Other sources include fenugreek seeds, a low cost food ingredient traditionally consumed in the far east without reported ill effects (Hettiarachchy *et al.*, 1996), and potatoes (Al-Saikhan *et al.*, 1995).

Antioxidant compounds may also be produced during certain chemical reactions. Maillard reaction products (MRPs) comprise another group of what may be considered natural antioxidants because the reactions occur normally in foods (Smith and Alfawaz, 1995). MRPs prepared from histidine and glucose or enzymatic hemoglobin hydrolyzate and glucose have been shown to improve the oxidative stability of sausage during frozen storage (Lingert and Lundgren, 1980). Also, Smith and Alfawaz (1995) found that MRPs prepared from egg albumin acid hydrolyzate and glucose significantly slowed the development of oxidative rancidity and off-flavor development in cooked ground beef during storage at 4^0 C.

In the past, the use of natural antioxidants has been limited due to their high cost of isolation and purification and color and flavor problems (Hettiarachchy *et al.*, 1996). Therefore, further investigations directed towards finding, safe, low cost, versatile and effective antioxidants are needed. One such compound is 3-dehydroshikimic acid (DHS). Its antioxidant efficacy has been

evaluated recently in a model system containing liposomes impregnated with a fluorophore (3-(*p*-(6-phenyl)-1,3,5-hexatrienyl) phenylpropionic acid). In this system, DHS displayed more potent antioxidant activity than did alpha tocopherol and weaker antioxidant activity than did propyl gallate, gallic acid, and TBHQ (Richman *et al.*, 1996). This substance was also tested for its ability to inhibit the formation of hydroperoxides in lard incubated at 60^0 C. Here, the antioxidant activity of DHS was equal to or superior to the antioxidant activities of propyl gallate, gallic acid, and TBHQ (Richman *et al.*, 1996).

In addition to showing significant antioxidant properties, DHS can also be produced easily and inexpensively. DHS can be synthesized from D-glucose by suitably engineered microbes (Figure 1).

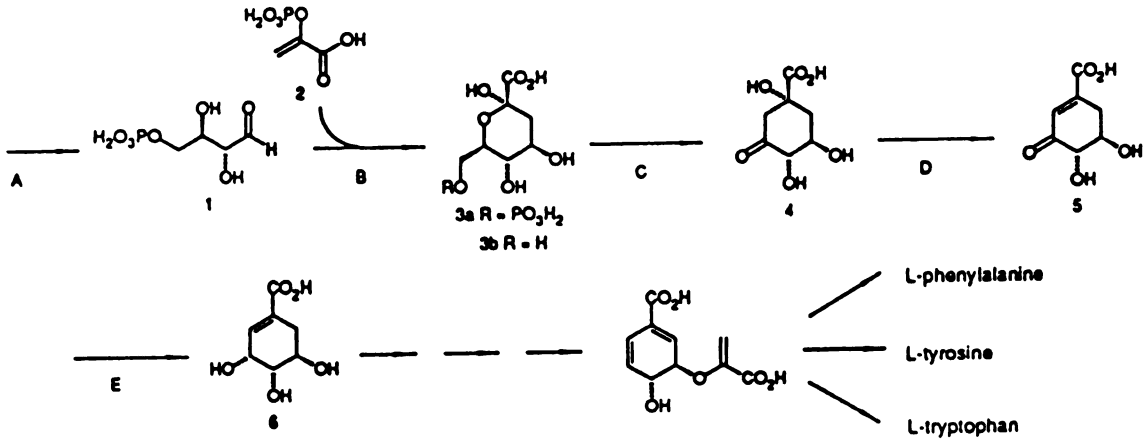

Figure 1. Common pathway of aromatic amino acid biosynthesis in *E. coli aro*E. Enzyme E is missing from this strain, thus DHS (compound 5) accumulates (Draths and Frost, 1990).

D-glucose is derived from inexpensive plant starch and is in abundant supply. This natural biogenesis may allow DHS to be used in nearly all foods in the future. However, as with all potential food additives, this compound must be extensively evaluated with regards to its safety, including a complete toxicological profile in mammals.

VI. Measurement of Lipid Peroxidation

Measurement of lipid peroxidation can be divided into two main categories: measurement of primary changes and measurement of secondary changes. Methods that measure primary changes are those that quantify the loss of reactants (e.g., oxygen or unsaturated fatty acids) or the formation of primary products of peroxidation (hydroperoxides) (Gray and Monahan, 1992). Methods that measure secondary changes are used when peroxidation occurs at an accelerated rate because hydroperoxides rapidly decompose to stable secondary products (e.g. hexanal) in this type of environment (Gray and Monahan, 1992). Five of the most frequently used methods to monitor the extent of lipid peroxidation will be discussed.

VI.1. Measurement of Primary Peroxidation Products

One of the most commonly used methods to measure the formation of hydroperoxides in foods is the determination of the peroxide value. This is accomplished via an iodometric technique (AOAC, 1973) in which the peroxide

value is reported as milliequivalents of iodine per kilogram of fat. This method is applicable to all normal fats and oils, is simple, and useful for bulk lipids.

However, the iodometric determination of the peroxide value suffers from many limitations. The technique is highly empirical, so even slight variation in procedure could mean large variation in results. Also, the official method fails to adequately measure low peroxide values because of difficulties with determination of the endpoint. This could prove to be a hindrance when trying to evaluate the efficacy of many highly effective antioxidant compounds.

Another technique commonly used to measure hydroperoxides involves measurement of conjugated diene structures which absorb ultraviolet (UV) light in the range of 230-235 nm. Peroxidation of unsaturated fatty acids is accompanied by the formation of such structures (Figure 2). Measurement of this absorbance is extremely useful in studies with pure lipids and it is excellent for measuring the early stages of the peroxidation process (Halliwell and Chirico, 1993).

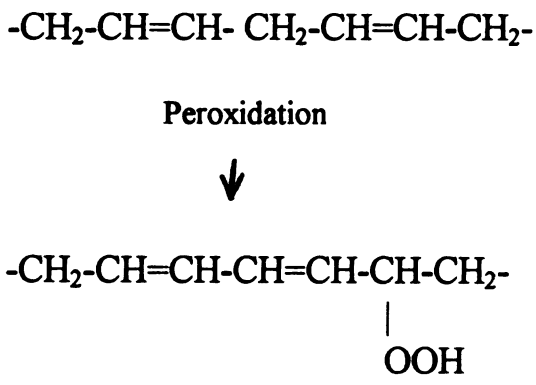

Figure 2. Formation of a conjugated double bond system from the peroxidation of linoleic acid (Halliwell and Chirico, 1993).

The measurement of conjugated dienes, like the peroxide value, also has certain limitations. In this procedure, the lipid sample is dissolved in an appropriate organic solvent (e.g., isooctane) and diluted to 0.01 milligram of sample per milliliter of solvent. Absorbance is then measured by spectrophotometry (AOAC, 1973). However, application of this simple technique to complex biological and food systems can lead to misinterpretation of data because changes in absorbance in the 230-235 nm range cannot be uniquely attributed to conjugated diene hydroperoxides. For example, Dormandy and Wickens (1987) have shown that human body fluids contain octodeca-9-*cis*-11-*trans*-dienoic acid. This substance is a non-oxygen-containing isomer of linoleic acid that absorbs UV radiation in the 230-235 nm range. Also, conjugated diene products may be present in the diets of animals and can cause problems if this technique is used to assess the extent of peroxidation in animal tissues (Holley and Slater, 1991). These problems can often be eliminated with the use of high performance liquid chromatography (HPLC).

In addition, in order for this method to work, unsaturated fatty acids with more than one double bond must be present. Therefore, if oleic acid represents a large portion of the fatty acids in the food being analyzed, this technique may not be a true measure of the extent of lipid peroxidation.

Another method that involves measurement of primary oxidation products is also one of the newest and most promising techniques. This method is based on detection of chemiluminescence generated during the oxidation of luminol by hydroperoxides (Yang, 1992). Chemiluminescence is the emission of light

resulting from chemical reactions that produce a reactive product in an excited state. It is the radiative decay of this product that emits light (Yang, 1992). In a solution containing hydroperoxides, addition of luminol and a catalyst (e.g., cytochrome c) results in chemiluminescence. This emission of light is most likely based on the action of superoxide, which is formed through interaction of the catalyst with hydroperoxides (Yang, 1992). This superoxide oxidizes luminol to the semiquinone radical which produces light.

This assay is commonly used with postcolumn HPLC detection. Therefore, interference from antioxidants is minimized due to their separation from the hydroperoxides by the column prior to chemiluminescence detection (Yamamoto *et al.*, 1988). The use of HPLC also makes it easier to identify and characterize the nature of the lipid hydroperoxides based on retention times and comparison to those of standard lipid hydroperoxides.

Another positive aspect of this method is its high level of sensitivity. Even though the chemiluminescent species degrade quickly (in less than one minute), a postcolumn detector coupled with proper tubing lengths results in retained separation of resolved peaks and high sensitivity (Yang, 1992). Drawbacks of this method include a need for skilled operators and expensive equipment.

VI.2. Measurement of Secondary Peroxidation Products

The most popular method used to assess peroxidative damage to lipids is the 2-thiobarbituric acid (TBA) test. The TBA test monitors the formation of malonaldehyde (Nielsen, 1994). Malonaldehyde (MDA) is a secondary product of

lipid peroxidation and may arise by a number of mechanisms in an oxidizing lipid system (Figure 3).

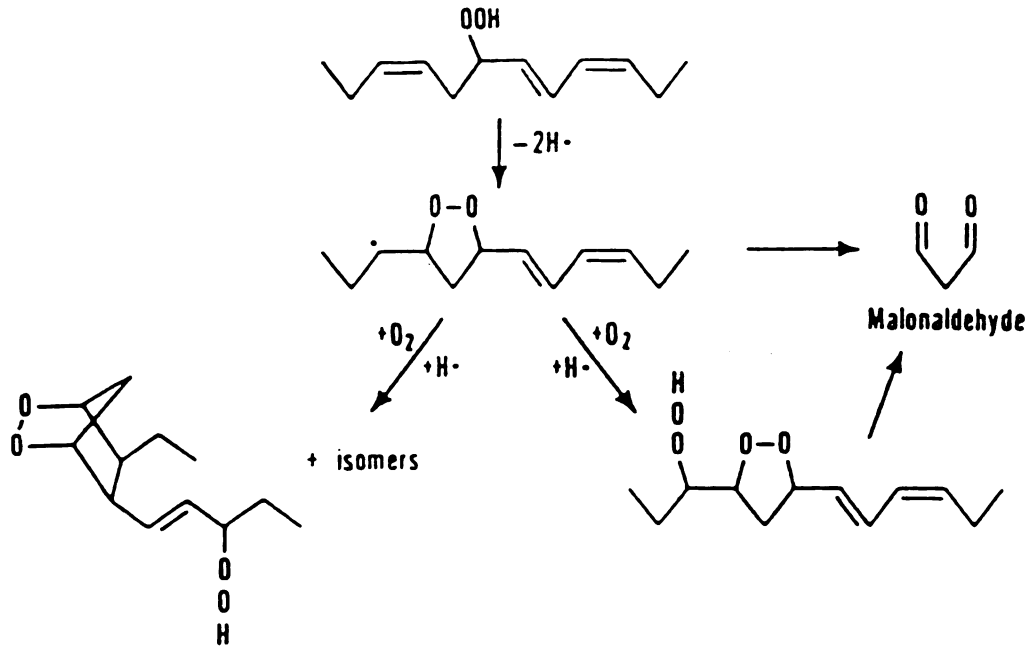

Figure 3. Mechanism of 1,3-cyclization of 12- and 13-hydroperoxides of linolenate and formation of malonaldehyde (Frankel, 1984).

The TBA test is a spectrophotometric assay that measures the amount of pink chromagen formed by reaction of two molecules of TBA with one molecule of MDA. This pink crystalline pigment has an absorbance maxima of 532-535 nm and a secondary maxima at 245-305 nm (Halliwell and Chirico, 1993). As lipids are oxidized, the amount of TBA-reactive substances (TBARS) increases, which results in the formation of larger amounts of pigment. Extent of oxidation can be determined by absorbance levels.

This assay is simple to use, very versatile, and provides a useful index of lipid peroxidation (Gray and Monahan, 1992). For these reasons, it is the most widely used single assay for measuring lipid peroxidation in biological systems (Gutteridge and Halliwell, 1990). However, the TBA test has many limitations. First, MDA or MDA-like substances can be produced during the assay itself from precursors formed during the peroxidation process. Among these are 2-alkenals, 2,4-alkadienals, and MDA bound to proteins (Esterbauer *et al.*, 1991). Also, aldehydes other than MDA can form chromagens with the target absorbance of 532 nm (Halliwell and Chirico, 1993).

The TBA test is most effective when analyzing products that are composed mainly of fatty acids with three or more double bonds. Therefore, this assay should not be used on substances containing mostly oleic and/ or linoleic acids (Frankel, 1993). Lastly, the formation of MDA occurs very far downstream from the initial production of primary oxidation products and thus it is difficult to use this assay to accurately evaluate antioxidant activity of test compounds (Arora, 1997).

In recent years, specific volatile products arising from lipid peroxidation have been used as indices of oxidation. Hexanal is one of the major secondary products formed during the oxidation of linoleic acid. Frankel *et al.* (1989) developed a rapid gas chromatographic method to monitor the formation of hexanal. This test is simple, can be used as a good measure of lipid peroxidation, and is becoming increasingly popular (Frankel, 1993).

Aldehydes such as hexanal are related to development of off-flavors in foods (Buttery and Teranishi, 1963; Rayner *et al.*, 1978; Warner *et al.*, 1978). Thus, analysis of these volatiles by gas chromatography may in some cases be closely related to results of sensory panel flavor tests and can be used as marker compounds to follow the development of rancid flavors in meats. This method is also helpful in determining the origin of flavor and odor volatiles and their precursors (Frankel, 1993).

The chemical composition of the end products of lipid peroxidation will depend on many factors. Two of the most important are the fatty acid composition of the lipid substrate used and which metal ions are present in the system (Gutteridge and Halliwell, 1990). For example, copper and iron ions may give different end-product distributions when lipid peroxidation is monitored via the TBA test. Also, copper salts decompose peroxides which can lead to low amounts of measurable peroxides but higher amounts of certain carbonyl compounds which may interfere with some assays (e.g. fluorescence assays). It follows that selection of only one test to monitor lipid peroxidation can give misleading results (Gutteridge and Halliwell, 1990). Thus, multiple assays, different lipid substrates, and different acceleration techniques should be employed when evaluating the efficacy of potential antioxidant compounds.

VII. Accelerated Tests

The previously described assays can be used to measure the oxidation status and/or stability of lipids. Accelerated stability tests are commonly used in tandem with these methods to more quickly assess the efficacy of antioxidants. In these tests, lipid peroxidation is hastened by subjecting the sample to heat, oxygen, light, metal catalysts, or enzymes. Heating is most commonly used to accelerate oxidation because the rate reaction increases exponentially with the absolute temperature (Ragnarrson and Labuza, 1977). Table 1 lists several standard accelerated stability tests.

Table 1. Standard Accelerated Stability Tests (Frankel, 1993)

TEST	CONDITIONS	CHARACTERISTICS
Ambient storage	Room temperature, atmospheric pressure	Too slow
Light	Room temperature, atmospheric pressure	Different mechanism
Metal catalysis	Room temperature, atmospheric pressure	More decomposition
Weight-gain method	30-80^0 C, atmospheric pressure	Endpoint questionable
Schaal oven	60-70^0 C, atmospheric pressure	Fewest problems
Oxygen uptake	80-100^0 C, atmospheric pressure	Different mechanism
Oxygen bomb	99^0 C, 65-115 psi O_2	Different mechanism
Active oxygen	98^0 C, air bubbling	Different mechanism
Rancimat	100-140^0 C	Endpoint questionable

Even though ambient conditions most closely resemble real storage conditions of foods, this procedure is too slow to be of practical value to the food industry in determining the efficacy of possible antioxidants. In addition,

reproducibility of results is compromised by many variables that are difficult to control over long periods of time (Frankel, 1993).

Elevated temperature tests such as oxygen uptake, oxygen bomb (American Society for Testing Materials), the active oxygen method (AOM), and Rancimat (Metrohm Ltd., CH-9100, Herisau, Switzerland) are all performed at temperatures ranging from 80^0 C to over 100^0 C. These tests are not representative of real-life storage conditions since the mechanism of lipid peroxidation changes significantly under these harsh conditions (Frankel, 1993).

There are many other limitations associated with testing at such high temperatures. Volatile antioxidants such as BHA and BHT may undergo significant degradation at elevated temperatures. These conditions may also decompose phenolic antioxidants found in plant extracts (Frankel, 1993). Finally, Maillard reaction products that possess proven antioxidant activities may be formed during testing (Ragnarsson and Labuza, 1977).

The Schaal oven test involves storage of the oxidizable substrate in an oven at 60-70^0 C (Frankel, 1993). As noted in Table 1, this acceleration technique has the fewest problems. First, the endpoint represents a lower degree of oxidation compared to the other methods (e.g. AOM, Rancimat) and results of the Schaal oven test correlate well with actual shelf life determination (Pomeranz and Meloan, 1987). Also, testing methods which measure primary changes (e.g., the peroxide value) can be used with this acceleration technique because the primary hydroperoxides formed during lipid peroxidation do not decompose under the conditions of the procedure. Furthermore, side reactions are minimized at 60^0 C

whereas at temperatures near 100^0 C and above, these reactions can give false results (Ragnarsson and Labuza, 1977).

VIII. Choice of Medium for Evaluation of Antioxidant Activity

Lipid peroxidation has been studied extensively in bulk fats and oils and there is a good understanding of the factors that effect peroxidation in these types of systems (Coupland and McClements, 1996). However, the mechanics of lipid peroxidation and the action of antioxidants is not very well understood in systems in which the fat is dispersed as emulsion droplets (Coupland and McClements, 1996).

Food emulsions are a very large and important category of products. Some examples of emulsions include dressings, sauces, various beverages, soups, and baby foods (Larsson and Friberg, 1990). Therefore, it is important to evaluate the effectiveness of any new antioxidant in emulsion systems.

The peroxidation of lipids in an emulsion is different from that of bulk lipids. This is mainly due to the presence of the droplet membrane, the interactions between the ingredients and the partitioning of the ingredients between the aqueous, oil, and interfacial region. Thus, it should be noted that an antioxidant that is very effective in a bulk oil system may have no effect or even be a prooxidant in an emulsion system (Coupland and McClements).

MATERIALS AND METHODS

I. INITIAL SCREENING OF PHENOLIC ANTIOXIDANTS

The ability of DHS, TBHQ, propyl gallate, and gallic acid to suppress peroxidation in lard was evaluated using the peroxide value method coupled with Schaal oven acceleration.

I.1. Materials

Prime steam lard (produced from a continuous industrial hot rendering process, titre=38; free fatty acids (FFA)=0.5 max.), was obtained from Monfort, Inc. (Greeley, CO). TBHQ was purchased from Eastman Chemical (Kingsport, TN). DHS was donated by Dr. John Frost, Department of Chemistry, Michigan State University (East Lansing, MI). Propyl gallate and gallic acid were obtained from Sigma Chemicals (St. Louis, MO). Acetic Acid (99.7% purity) and chloroform (99.8% purity) were purchased from E.M. Science (Gibbstown, NJ). Potassium iodide was purchased from J.T. Baker (Phillipsburg, NJ). Starch indicator solution (1%) was obtained from Sigma Chemicals (St. Louis, MO). Sodium thiosulfate (0.1011 N) was from Aldrich Chemicals (Milwaukee, WI). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Fair Lawn, NJ). All glassware used in the study was acid-washed.

I.2. Determination of Antioxidant Activity

Lard was melted in a 50^0 C water bath. The melted lard (60 g) was placed in each of thirty nine 100 ml glass beakers. DMSO (500µl) was added to the lard in each of 3 control systems (containing no antioxidant).

Treated systems (containing antioxidants) were prepared as follows:

Three concentrations (0.02%; 0.01%; 0.002%) of four antioxidants (gallic acid; TBHQ; DHS; propyl gallate) were tested in triplicate. Antioxidants were added on a weight/weight basis. The antioxidants were dissolved in 500 µl DMSO and mixed into the melted lard thoroughly with a glass stirring rod.

The antioxidant activity of the compounds was determined by measuring their ability to inhibit peroxide formation in the lard samples. The samples were kept in an oven at 60 ± 5^0 C in the dark over a 9-week period. Peroxide values were determined at 7 day intervals by the official method (Cd 8-53) of the American Oil Chemists Society (AOAC, 1973).

Briefly, aliquots (5 g) of each of the lard samples were collected for peroxide value measurements every seven days. Saturated potassium iodide solution (500 µl) was added to a solution containing 30 ml glacial acetic acid-chloroform (3:2 v/v) and 5 g lard sample. The solution was then allowed to stand (with occasional shaking) for approximately 1 minute. Distilled water (30 ml) and 1% starch indicator solution (1 ml) were then added. The resulting lard solution was titrated with vigorous shaking against 0.1 N or 0.01 N sodium thiosulfate

solution. The end point was determined by the disappearance of blue color from the chloroform layer. All measurements were done in triplicate.

II. SCREENING OF PHENOLIC ANTIOXIDANTS IN A CORN OIL-IN-WATER EMULSION

The ability of DHS, TBHQ, propyl gallate, and gallic acid to suppress peroxidation in a 10% corn oil-in-water emulsion was evaluated by an HPLC method which monitored the formation of conjugated dienes. Peroxidation was accelerated in both studies by incubating the samples in a 55^0 C water bath. In addition, one study included the addition of ferrous ions to the system to further hasten peroxidation.

II.1. Materials

Corn oil, stripped of tocopherols, was obtained from Acros Organics (NJ). Polyoxyethylene sorbitan monolaurate (Tween 20) was obtained from Sigma Chemicals (St. Louis, MO). $FeCl_2 \cdot 4H_2O$ was from Mallinckrodt (Paris, KY). Antioxidants (propyl gallate, gallic acid, TBHQ, and DHS) were obtained from the same sources mentioned earlier. Acetonitrile (HPLC grade), chloroform (99.8% purity), and hexane (96.8% purity) were obtained from E.M. Science (Gibbstown, NJ). Methanol (100% purity) was from J.T. Baker (Phillipsburg, NJ). All glassware used in the study was acid-washed.

II.2. Preparation of Emulsions

Ten percent stripped corn oil-in-water emulsions were prepared according to Frankel *et al.* (1994) with slight modifications. The oil (2.5 g) was placed in a 50 ml Erlenmeyer flask. To the control systems (i.e., those containing no antioxidant), 250 µl methanol were added. Antioxidants were added to the emulsion system at concentrations of 0.02% (weight/weight). The antioxidant was dissolved in 250 µl methanol and added directly to the oil. The flask was then swirled for approximately one minute to achieve mixing of the antioxidant with the oil. Emulsions were then brought up to volume (25 ml) with NANOpure water (filtered by an apparatus from Barnstead (Dubuque, IA)). Tween 20 (0.25 g) was then added to each flask. Emulsions were prepared using a Polytron mixing device from Kinematica (Switzerland). The speed was set at 5 and thorough mixing of the emulsion ingredients was achieved in 20 seconds. These emulsions were physically stable during oxidation at 55^0 C. Sonication in an ice bath did not produce emulsions with similar stability as claimed by Frankel *et al.* (1994).

The experiment was set up as follows: One concentration (0.02%) of each antioxidant (gallic acid; TBHQ; DHS; propyl gallate) was tested in triplicate in the system with ferrous ions and the system without ferrous ions. Antioxidants were added on a weight/weight basis. The antioxidants were weighed out, dissolved in 250 µl of methanol and mixed into the oil by swirling. Emulsions were then prepared from this oil as described earlier.

II.3. Acceleration of Peroxidation

Samples were incubated in 55^0 C water in a Gyrorotary shaker bath (New Brunswick Scientific Co., Edison, NJ) to minimize separation of the oil and water phases. The Erlenmeyer flasks were wrapped in aluminum foil to minimize exposure to light.

In the study involving iron, 0.015 g $FeCl_2 \cdot 4H_2O$ was mixed (by vortexing for 15 s) in 250 µl water. This solution was added to the emulsion immediately after mixing with the Polytron. Metal solutions were prepared daily in nitrogen-sparged water immediately prior to use.

II.4. Extraction of lipid hydroperoxides from the corn oil-in-water emulsion

Aliquots (50 µl) of the corn oil-in-water emulsions were taken from the reaction system at pre-determined time intervals. Further reactions were minimized by the immediate addition of 1 ml cold methanol and the aliquots were extracted with 1 ml cold hexane (the methanol and hexane were stored in an ice bath during the experiments). The mixture of lipid, methanol, and hexane was then vortexed for 15 seconds and centrifuged at 2500 RPM for 30 seconds at 0^0 C using a Sorvall Superspeed RC2B automatic refrigerated centrifuge (Ivan Sorvall Inc. Newton, CT). The hexane layer was then collected. The methanol layer was washed twice with 1 ml aliquots of cold hexane, repeating the same procedure as in the first step. The combined hexane extracts were evaporated to dryness under nitrogen. After the tube was completely dry, its sides were washed down with 500 µl chloroform which was subsequently evaporated to dryness.

The test tubes were then stored at -80^0 C in the dark until the sample extracts were analyzed by HPLC (which occurred no later than 36 hours after extraction).

II.5. HPLC Method of Analysis of Conjugated Diene Hydroperoxides

The dried extracts were re-dissolved in 35 µl chloroform, of which 30 µl was recovered and injected onto a reverse phase C-8 column (3 µm d., 150 x 4.6 mm) (Supelco, Bellefonte, PA). The mobile phase consisted of 100% acetonitrile at a flow rate of 1.4 ml/ minute. A Waters 486 tunable absorbance detector (Milford, MA) set at a wavelength of 234 nm was used to detect the presence of conjugated diene structures.

III. PEAK CONFIRMATION BY HPLC-CHEMILUMINESCENCE

As previously noted, structures other than hydroperoxides may absorb in the 234 nm range. Therefore, it was necessary to confirm that the target peaks were indeed conjugated diene hydroperoxides. This was achieved via HPLC-chemiluminescence.

III.1. Materials

Potassium tetraborate, 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) and cytochrome c from horse heart (prepared without TCA) were purchased from Sigma Chemicals (St. Louis, MO). All other reagents used were as described earlier.

III.2. HPLC-Chemiluminescence Assay

A similar set-up as that previously described in section II.5 was used. However, the eluate passing through the absorbance detector was mixed with a chemiluminescence cocktail at a post-column mixing tee. The reagent was prepared as described by Miyazawa *et al.* (1987). It consisted of cytochrome c (10 μg/ml) and luminol (1μg/ml) dissolved in 50mM nitrogen-sparged borate buffer adjusted to a pH of 10.0. This solution also consisted of 2% acetonitrile to facilitate mixing with the mobile phase. The cocktail was prepared immediately before use, degassed, continuously sparged with helium, and stored in a container with coating to block out UV light. The flow rate was 1.4 ml/minute. The chemiluminescence generated by the conjugated diene hydroperoxides was detected by a Waters 474 fluorescence detector. This assay confirmed that the target peaks were indeed conjugated diene hydroperoxides, most likely from linoleic acid.

IV. PEAK CONFIRMATION BY SODIUM BOROHYDRIDE REDUCTION

Further confirmation that the target peaks were hydroperoxides was achieved via a simple reduction. During this reaction, the hydroperoxides were reduced and thus, total peak area was lessened.

IV.1. Materials

Sodium borohydride was purchased from Fisher Scientific Co. (Fair Lawn, NJ). All other reagents used were as described earlier.

IV.2. Reduction Reaction

Six emulsion mixtures containing no added ferrous ions or antioxidants were prepared as described in section II.2. These samples were then incubated for 12 hours as described in section II.3. The extraction procedure described in section II.4 was followed. Three of these dried extracts were re-dissolved and injected as described in section II.5. The three remaining extracts were reduced with a mixture of 500 µl methanol and 0.005 g sodium borohydride. The reaction was allowed to proceed to completion. This mixture was then evaporated to dryness, immediately re-dissolved in 35 µl chloroform and injected onto the column as in section II.5.

RESULTS AND DISCUSSION

I. Initial Screening of Phenolic Antioxidants

Determination of the peroxide value is one of the most commonly used methods to evaluate the efficacy of antioxidants. This procedure is widely used in the food industry because it is applicable to all normal fats and oils and is useful for bulk lipids. Also, the simplicity of this assay allows for the evaluation of many antioxidant compounds easily and relatively quickly when coupled with Schaal oven acceleration.

Prime steam lard was chosen as the test substrate because of its fatty acid composition and the fact that it does not contain any naturally occurring antioxidants. The most abundant unsaturated fatty acids in lard are oleic (approx. 44%) and linoleic acids (approx. 11%). The remaining 45% is composed mainly of saturated fatty acids (Christie, 1987). Therefore, lard is susceptible to peroxidation. Furthermore, this substance has been used in past studies to successfully measure the efficacy of antioxidants (Gordon and Weng, 1992; Economou *et al.*, 1991; Banias *et al.*, 1992).

Of the assays discussed in the literature review, the peroxide value determination is best suited for an initial screening process because chemiluminescence is a very involved process. Also, measurement of conjugated diene hydroperoxides at 234 nm would not be as effective because these structures are not formed during peroxidation of oleic acid, the most abundant fatty acid in lard.

Measuring the induction period before the appearance of oxidation products and the onset of rancidity can give an indication of the effectiveness of antioxidant compounds in slowing lipid peroxidation. The induction period is defined as the length of time before detectable rancidity or rapid acceleration of lipid peroxidation. This can be determined by calculating the maximum of the second derivative with respect to time or by manually drawing tangents to lines on graphs such as Figures (4 - 7) (Nielsen, 1994).

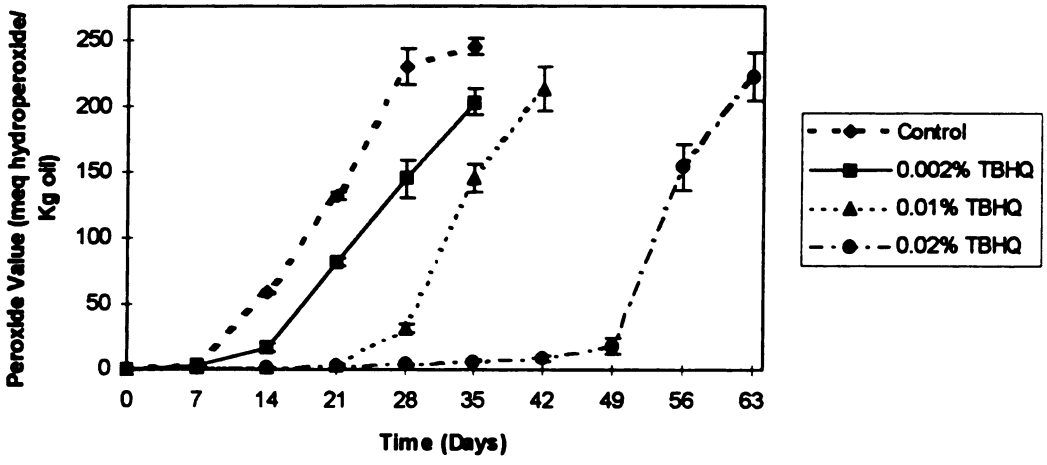

Figure 4. Effect of TBHQ on the peroxidation of lard at 60^0 C. The rate of peroxidation was analyzed by measurement of the peroxide value at seven day intervals. Values represent the mean $\pm$ standard deviation of triplicate measurements.

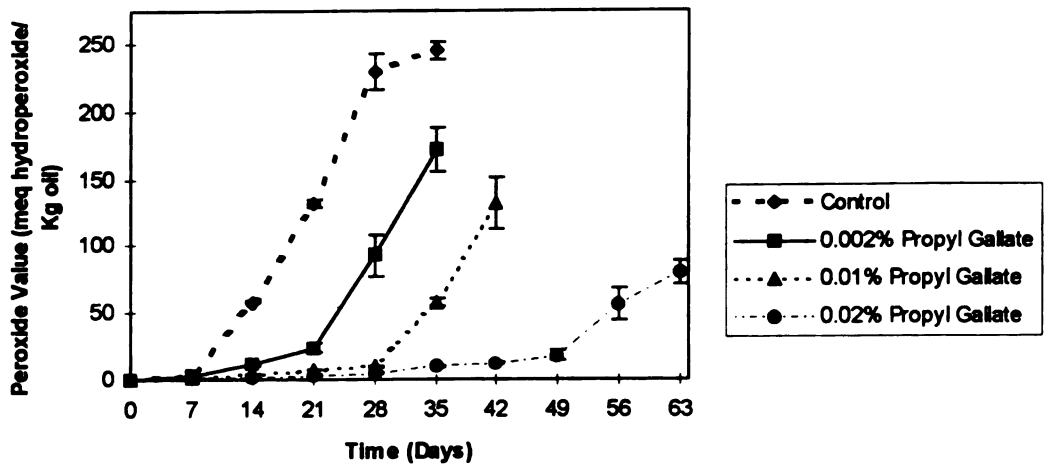

Figure 5. Effect of propyl gallate on the peroxidation of lard at 60^0 C. The rate of peroxidation was analyzed by measurement of the peroxide value at seven day intervals. Values represent the mean $\pm$ standard deviation of triplicate measurements.

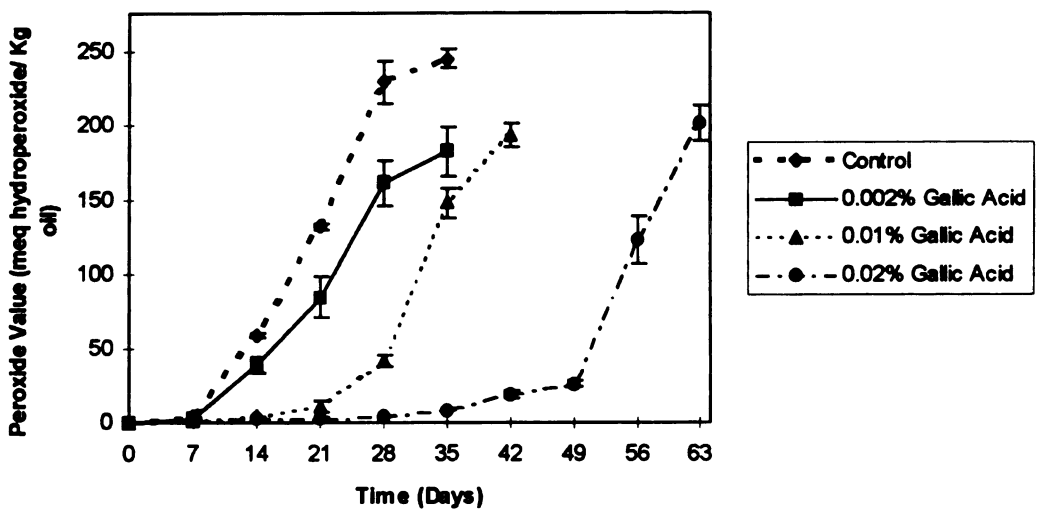

Figure 6. Effect of gallic acid on the peroxidation of lard at 60^0 C. The rate of peroxidation was analyzed by measurement of the peroxide value at seven day intervals. Values represent the mean $\pm$ standard deviation of triplicate measurements.

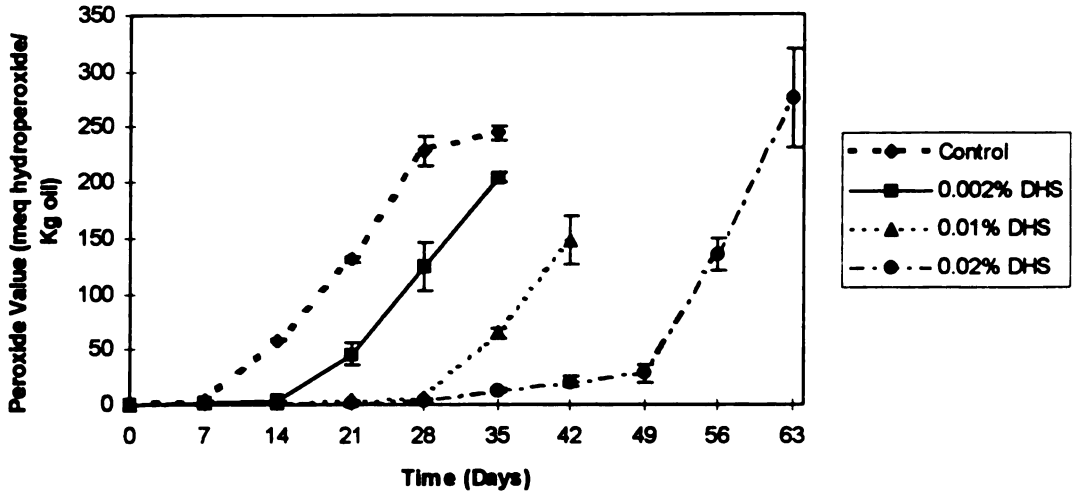

Figure 7. Effect of DHS on the peroxidation of lard at 60^0 C. The rate of peroxidation was analyzed by measurement of the peroxide value at seven day intervals. Values represent the mean $\pm$ standard deviation of triplicate measurements.

TBHQ, propyl gallate and gallic acid all provided protection against peroxidation at levels as low as 0.002%. At 0.01%, all antioxidants were even more effective (Figures 4-7). At 0.02%, which is the maximum allowable concentration by the United States Food and Drug Administration, induction periods of 48, 49, and 47 days were obtained for TBHQ, propyl gallate, and gallic acid respectively (Table 2).

Table 2. Induction periods and protection factors for selected phenolic antioxidants in lard at various concentrations (wt/wt).

COMPOUND	CONC. (%)	INDUCTION PERIOD (DAYS)	PROTECTION FACTOR**
Control	0	8	1.00
TBHQ	0.02	48	6.00
TBHQ	0.01	26	3.25
TBHQ	0.002	14	1.75
Propyl gallate	0.02	49	6.13
Propyl gallate	0.01	29	3.63
Propyl gallate	0.002	20	2.50
Gallic acid	0.02	47	5.89
Gallic acid	0.01	23	2.89
Gallic acid	0.002	8	1.00
DHS	0.02	48	6.00
DHS	0.01	29	3.63
DHS	0.002	16	2.00

**Protection factors were calculated as (Induction Period/ 8). The higher the protection factor, the more efficient the antioxidant.

The corresponding protection factors were 6.00 for TBHQ, 6.13 for propyl gallate, and 5.89 for gallic acid. These antioxidants were chosen due to their structural similarities to DHS (Figure 8), which performed just as well with an induction period of 48 days and protection factor of 6.00. DHS also performed just as well as established antioxidant compounds at concentrations of 0.01 and 0.002%. Thus, this compound possesses excellent antioxidant capabilities in a lard system.

Although this peroxide assay is very commonly used, its limitations should be noted. This technique is highly empirical, so even slight variation in procedure could mean a large variation in results. Lighting in the hood in which the

experiments were performed coupled with operator error resulted in variation in some of the peroxide value measurements. However, with practice this variation was minimized. Therefore, DHS could be considered to be a very effective antioxidant compound in bulk oil based on the results of this study.

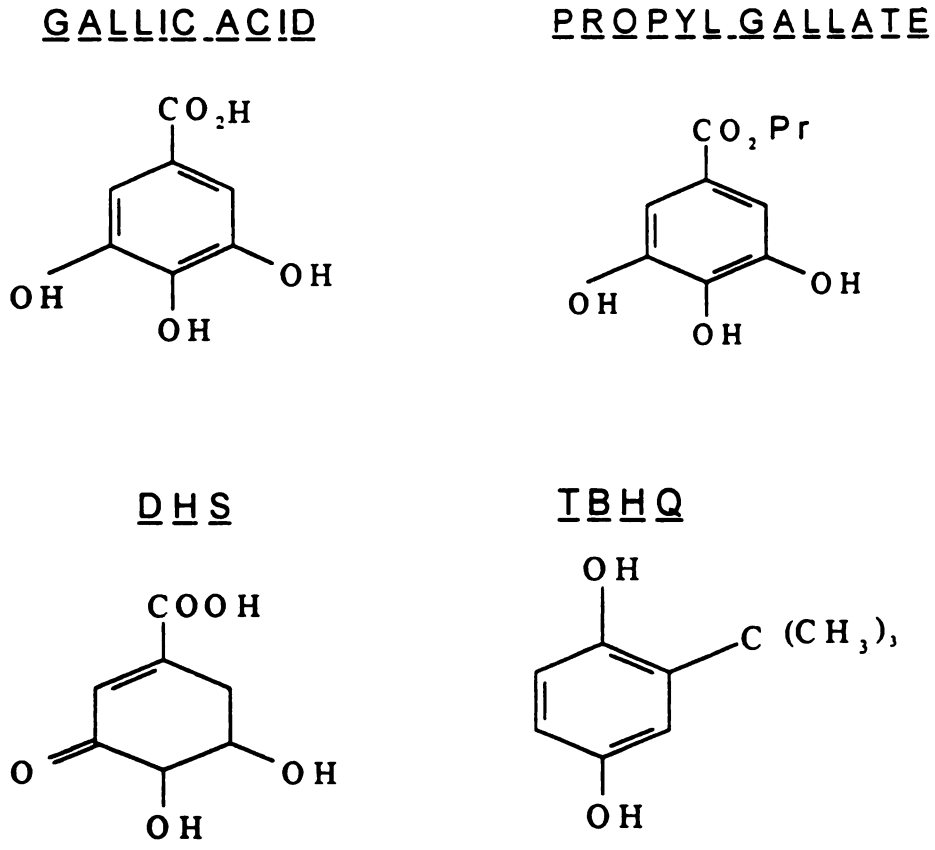

Figure 8. Structures of selected phenolic antioxidants and DHS

II. Efficacy of Phenolic Antioxidants in 10% Corn Oil-in-Water Emulsion

Food emulsions are a very large and important category of products. There are "semisolid" varieties such as margarine and butter as well as liquid ones such as dressings, sauces and various beverages (Larsson and Friberg, 1990). The 10% oil-in-water emulsion used in this study is representative of a typical low-fat salad dressing.

Stripped corn oil was chosen as the test substrate because of its fatty acid composition and due to the fact that successful studies have been performed previously with corn oil-in-water emulsions (Frankel *et al.*, 1994). The most abundant unsaturated fatty acids in corn oil are linoleic (approx. 52%) and oleic (approx. 34%). Saturated fatty acids make up the remaining 14% (Christie, 1987). Therefore, corn oil is even more susceptible to peroxidation than lard, provided its naturally occurring antioxidants are removed.

One of the main problems with emulsions is their instability. Several attempts were made to mix the emulsions with sonication in an ice bath as was done by Frankel *et al.* (1994). The emulsions produced with this technique were not stable throughout the duration of the experiments. Also, although a sonifier can produce very small globules, its energy consumption is very high and the globule size distribution obtained is wide (Larsson and Friberg, 1990). Therefore, emulsions were made up with a turbo mixer. This technique also gives a very wide spread in globule size (Larsson and Friberg, 1990). However, thorough mixing is achieved in a much shorter time and energy consumption is less. Furthermore, the

emulsions were placed in a shaker bath during oxidation to minimize separation of the oil and water phases.

A system which monitors the formation of conjugated diene structures was chosen to measure lipid peroxidation in the emulsions. Conjugated diene hydroperoxides are formed from polyunsaturated fatty acids such as linoleic acid. Therefore, this assay was chosen to determine the efficacy of propyl gallate, gallic acid, DHS, and TBHQ as antioxidants in the emulsions.

The measurement of conjugated dienes by UV absorbance to monitor lipid peroxidation has been criticized in the past. This is because changes in absorbance in the 230-235 nm range cannot always be uniquely attributed to conjugated diene hydroperoxides. Other conjugated diene structures that may interfere with this assay have been found in human body fluids (Dormandy and Wickens, 1987) and animal diets (Holley and Slater, 1991). However, HPLC was used to separate the hydroperoxides from other interfering substances prior to detection, thereby minimizing any such problems.

The HPLC conditions were based on past literature and trial and error. Previously, Miyashita *et al.* (1990) had used analytical reversed phase HPLC to monitor autoxidation of synthetic triacylglycerols containing linoleate and linolenate. Their solvent mixture consisted of acetonitrile/methylene chloride/methanol (85:15:1) at a flow rate of 1.5 ml/min. The column was a 5 micron C-18 (25 X 0.46 cm) and the effluent was monitored with a variable UV detector set at 235 nm. Although excellent peak separation and resolution were achieved with this system, run times were in excess of 35 minutes.

In these studies, a 3 micron C-8 column (15 X 0.46 cm) was employed. The mobile phase consisted of 100% acetonitrile at a flow rate of 1.4 ml/min. This system was very simple to set up and maintain, and preparation of the mobile phase required no mixing. Run times were only 25 minutes long and the column was allowed to flush for 5 minutes between each injection. This simple procedure was very effective in that peak resolution and separation were both excellent (Figure 9). The two peaks which became largest most rapidly were chosen to measure the extent of lipid peroxidation. These peaks were subsequently confirmed to be hydroperoxides by chemiluminescence and reduction procedures.

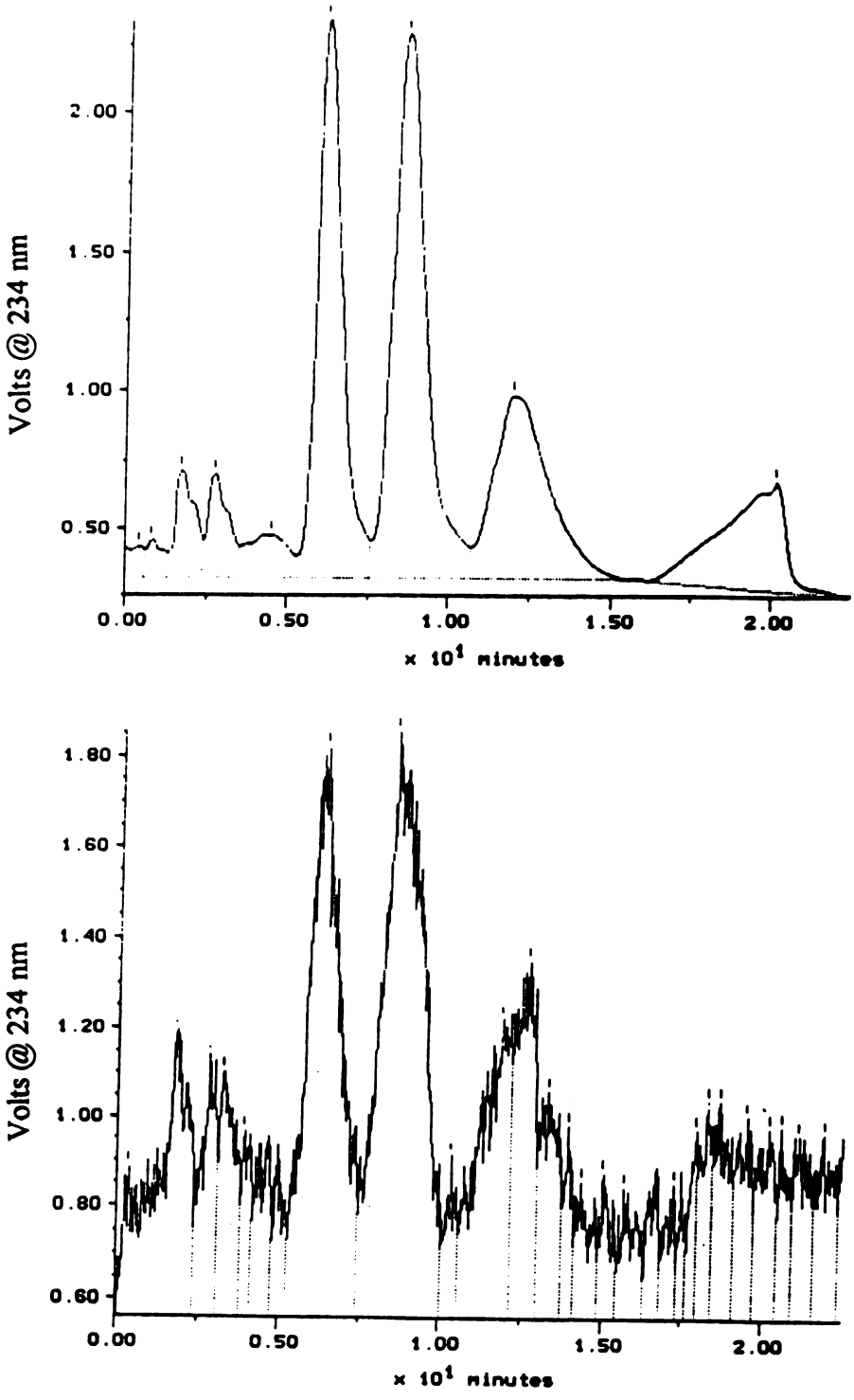

Figure 9. Representative chromatograms of conjugated diene hydroperoxide development in an oxidizing corn oil-in-water emulsion. The top chromatogram is representative of those used to construct figures 10 and 11. The two largest peaks (at 6.1 and 8.6 minutes) represent conjugated diene hydroperoxides as confirmed by chemiluminescence (bottom chromatogram). Peak areas were calculated by computer integration.

Further confirmation that the above peaks (Figure 9) were hydroperoxides was achieved via a reduction reaction. Prior to injection, three samples were reduced with sodium borohydride.

Table 3. Peak areas of extractions from reduced and control (non-reduced) samples.

	CONTROL (NON-REDUCED) SAMPLES*	REDUCED SAMPLES*
	4.32 e-7	2.34 e-7
	4.61 e-7	2.12 e-7
	4.39 e-7	2.87 e-7
Mean	**4.44 e-7**	**2.44 e-7**
Standard Deviation	0.15 e-7	0.39 e-7

*Peak area integration units

Table 3 shows that the mean peak area was reduced by approximately 45% for the three trials. Thus, the concentrations of hydroperoxides were lower in the reduced samples than in the control samples. This provided further evidence that the target peaks were indeed hydroperoxides. Therefore, this HPLC method could be used for evaluating the antioxidant activity of certain compounds in a corn oil-in-water emulsion.

TBHQ is one of the most commonly used synthetic antioxidants in food systems. This compound was shown to be a very effective inhibitor of peroxidation in the corn oil-in-water emulsion both in the presence and absence of ferrous ions (Figures 10 and 11). Calculated efficiency factors (Table 4) were 0.25 and 0.03 with and without Fe(II), respectively. These results were not only expected, but also validated the procedure as an effective method to monitor lipid peroxidation in the emulsion.

Table 4. Efficiency factors of selected phenolic antioxidants in an oxidizing corn oil-in-water emulsion.

COMPOUND	EFFICIENCY FACTOR WITH FE(II)**	EFFICIENCY FACTOR WITHOUT FE(II)**
Control	1.00	1.00
TBHQ	0.25	0.03
Propyl gallate	3.10	0.08
Gallic acid	2.33	0.13
DHS	0.97	1.20

**Efficiency factors were calculated as total peak area of treated emulsion injection/ total peak area of control emulsion injection at the furthest extent of oxidation (4 hours with Fe(II) and 16 hours without Fe(II)). Thus, the lower the efficiency factor, the more efficient the antioxidant.

Propyl gallate is also commonly used as a food antioxidant. Like TBHQ, this substance was very effective in suppressing peroxidation in lard (Figure 5). It was also an effective antioxidant in the emulsion when ferrous ions were not present (Figure 10). An efficiency factor of 0.08 was calculated for this antioxidant (Table 4). However, when ferrous ions were added to accelerate the

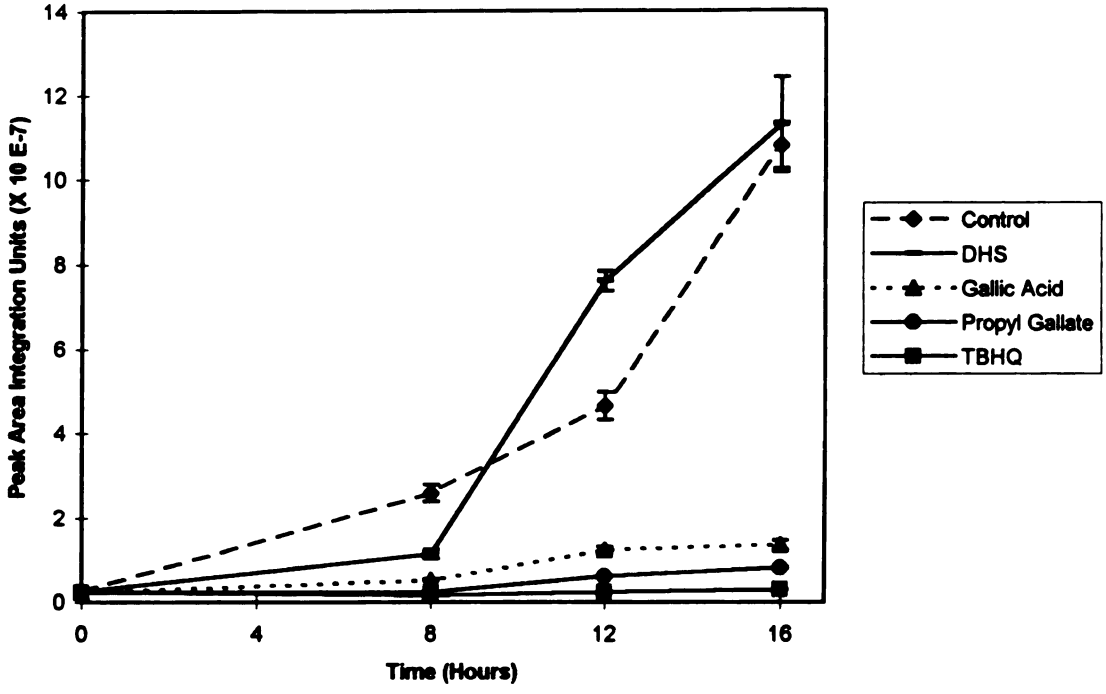

Figure 10. Peroxidation of 10% corn oil-in-water emulsion without Fe(II). Peroxidation was monitored by observing an increase in peak areas measured at 234 nm over a period of 16 hours (see figure 9 for example chromatograms). Values represent the mean $\pm$ standard deviation of triplicate measurements.

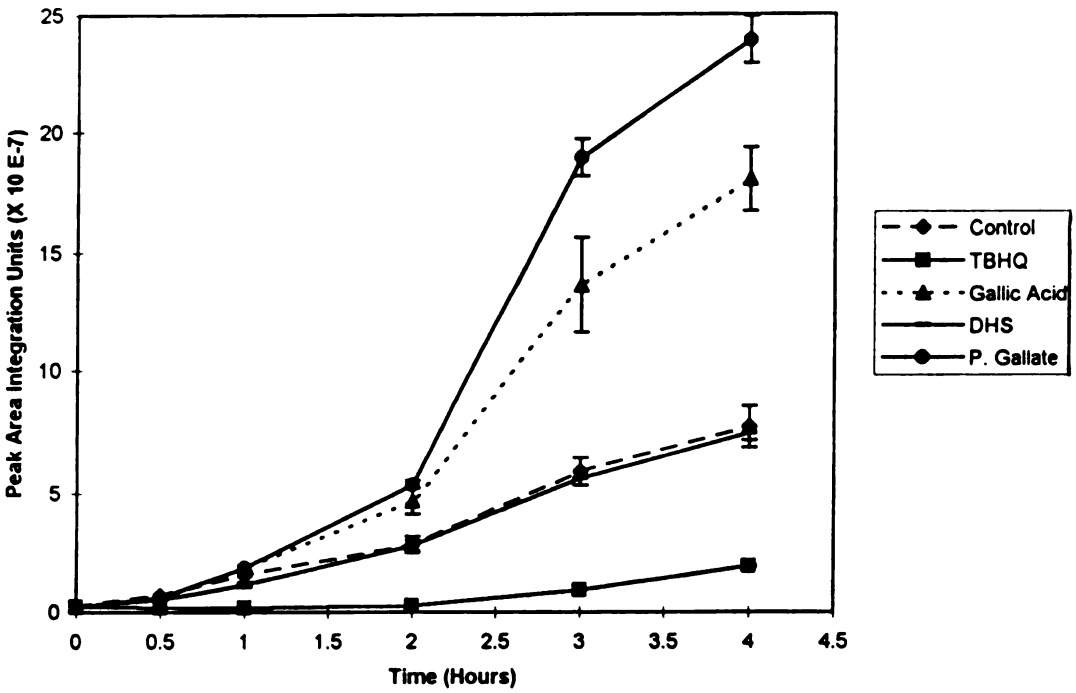

Figure 11. Peroxidation of 10% corn oil-in-water emulsion containing added Fe(II). Peroxidation was monitored by observing an increase in peak areas measured at 234 nm over a period of 16 hours (see figure 9 for example chromatograms). Values represent the mean $\pm$ standard deviation of triplicate measurements.

peroxidative progress (Figure 11), propyl gallate acted as a strong prooxidant (efficiency factor of over 3) and the emulsion turned a deep purple color during the experiment. Gallic acid exhibited the same type of characteristics. Its efficiency factor was 0.13 without Fe(II) and nearly 3 when ferrous ions were added. There are several reasons that may explain this observation.

Many lipid-soluble chain-breaking antioxidants can have prooxidant properties under certain circumstances. This is because they have the ability to bind Fe(III) ions and reduce them to Fe(II) ions. Propyl gallate has limited solubility in water, but it is soluble enough to allow it to accelerate peroxidation indirectly via the Fenton reaction or Haber-Weiss reaction (Aruoma *et al.*, 1990).

The following mechanisms illustrate how Fe(II) can act to accelerate peroxidation:

MECHANISM I: LOOH-Dependent Lipid Peroxidation (Minotti and Aust,1987)

$$Fe(II) + LOOH \rightarrow Fe(III) + OH^- + LO^\cdot$$

$$LO^\cdot + LH \rightarrow L^\cdot + LOH$$

Here, Fe(II) catalyzes the decomposition of trace amounts of preformed LOOHs to form alkoxyl radicals ($LO^\cdot$) which abstract hydrogen from a neighboring allylic bond (Minotti, 1992). This reaction is probably not very important to this study at the beginning because the corn oil likely did not contain large amounts of preformed hydroperoxides. However, as the experiments proceeded, more and more hydroperoxides accumulated and the Fe(II) acted to speed up oxidation.

MECHANISM II: LOOH-Independent Lipid Peroxidation

A $Fe(II) + O_2 \rightarrow Fe(III) + O_2^{\cdot-}$

B $2\,O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$

C $Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + {}^{\cdot}OH$

D $O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + OH^- + {}^{\cdot}OH$

E $^{\cdot}OH + LH \rightarrow H_2O + L^{\cdot}$

Here, Fe(II) reacts with oxygen to yield a superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (equations *A* and *B*). Then, Fe(II) and hydrogen peroxide may react to form hydroxyl radicals ($^{\cdot}OH$) via the Fenton reaction (equation *C*). This can also occur via the iron-catalyzed Haber-Weiss reaction (equation *D*). Although this reaction is exothermic, a transition metal (e.g., Fe) is needed for catalysis (Hsieh and Kinsella, 1989). It is the hydroxyl radicals which are very reactive and have the ability to abstract hydrogen from unsaturated fatty acids (LH) (equation *E*) (Minotti, 1992). This can occur in the absence of preformed LOOH molecules. Thus, LOOH-independent lipid peroxidation was most likely accelerated indirectly by the propy gallate and gallic acid which acted to keep the iron in the ferrous state. The deep purple color was most likely the result of the antioxidant—iron complexes.

It should be noted that when antioxidants such as propyl gallate are added to a lipid system without the addition of ferrous ions, their chain-breaking antioxidant activity most often outweighs their prooxidant action in reducing metal ions. Therefore peroxidation is inhibited (Halliwell *et al.*, 1995). This was seen not only in the lard, but also in the emulsions that did not contain added iron.

Like the other three compounds, DHS was also very effective in suppressing peroxidation in the lard. However, DHS had no effect on peroxidation in the emulsion where ferrous ions were added (efficiency factor=0.97) to accelerate the process (Figure 11). In the study where iron was omitted, this compound exhibited only slight antioxidant properties (Figure 10) up to 8 hours and then acted as a slight prooxidant (efficiency factor of 1.20 at 16 hours).

This can probably be best explained by the so-called polar paradox that oil-soluble antioxidants are better in emulsions than in oils and water-soluble antioxidants are more effective in oils than in emulsion systems (Porter *et al.*, 1989). Thus, in bulk oil systems where oil is the main phase, hydrophilic antioxidants are very effective. However, in oil-in-water emulsions where water is the main phase, hydrophilic antioxidants such as DHS remain in the water and are less effective antioxidants in the oil-water interface where peroxidation occurs (Frankel *et al.*, 1996). The results of this study are consistent with the notion in that DHS is slightly more polar and therefore more water soluble than TBHQ, propyl gallate, or gallic acid. Therefore, DHS exhibited only slight antioxidant properties in one of the emulsion studies. It is likely that there are many other factors involved and further research is needed to better explain these differences.

It has been shown that an antioxidant may display very different levels of effectiveness depending on which substrate, acceleration techniques, and testing methods are used for its analysis. Although TBHQ was effective in all systems, propyl gallate, gallic acid, and especially DHS exhibited very different levels of

effectiveness when tested in different substrates and under different acceleration conditions. Thus, the effectiveness of an antioxidant in an emulsion may be considerably different from that of the same antioxidant in a bulk oil. This demonstrates the need for careful consideration when selecting an appropriate antioxidant system for certain applications.

SUMMARY AND CONCLUSIONS

A general public concern persists regarding the safety of synthetic food additives. Therefore, it is important to diligently search for and evaluate natural antioxidant compounds. As shown in this study, one such compound is DHS. DHS can be produced easily and inexpensively from D-glucose by suitably engineered microbes.

With the discovery of so many potential antioxidant compounds each year, it is important to thoroughly screen each one as efficiently as possible. Two widely accepted methods were used to evaluate DHS in this study. First, this compound was found to be an excellent antioxidant in a bulk oil (lard) when the peroxide value method was employed to evaluate the extent of oxidation. However, when tested in an emulsion (10% corn oil-in-water) using conjugated diene absorbance to evaluate its efficiency, DHS displayed no antioxidant properties.

The measurement of conjugated dienes to assess the extent of oxidation has been criticized in the past. This is because changes in absorbance in the 230-235 nm range cannot be uniquely attributed to conjugated diene hydroperoxides. Therefore, a method employing HPLC was developed in order to separate the hydroperoxides from other interfering compounds. This method is more time consuming than a simple absorbance procedure. However, what it lacks

in speed, it makes up for in increased sensitivity and specificity. Also, the possibility of interference by other compounds in virtually eliminated.

The developed HPLC method proved to be a very effective tool for evaluating the antioxidant activity of a potential antioxidant compound in a corn oil-in-water emulsion. This accelerated test could be performed in just one day and factors such as interfering compounds and very high heat were virtually eliminated. Thus, this assay could prove to be of use in the industry with some further refinements. It is also likely that it could be used to not only evaluate antioxidant activity in emulsions, but also in bulk oils and other foodstuffs with only minor modifications to the incubation and extraction processes.

Also, this study illustrates the importance of using different acceleration techniques, substrates, and testing methods when evaluating potential antioxidant compounds. DHS was very effective in lard and not at all effective in the emulsion due to its hydrophilic nature.

Before DHS is used as a food additive it must be extensively evaluated with regards to its safety. Also, whether or not consumers will accept a compound that is produced by a genetically engineered microbe as "natural" remains to be seen.

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