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Measuring Antioxidant Effectiveness in Food

ERIC A. DECKER,*,† KATHLEEN WARNER,‡ MARK P. RICHARDS,§ AND FEREIDOON SHAHIDI#

Department of Food Science, University of Massachusetts, Amherst, Massachusetts 01003; National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604; Meat Science and Muscle Biology Laboratory, University of Wisconsin−Madison, 1805 Linden Drive West, Madison, Wisconsin 53706; and Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland A1B 3X9, Canada

Many new in vitro methods have been developed to evaluate antioxidant activity. Unfortunately, these in vitro methods often correlate poorly with the ability of compounds to inhibit oxidative deterioration of foods because the in vitro assays do not account for factors such as the physical location of the antioxidant, its interaction with other food components, and environmental conditions. To accurately evaluate the potential of antioxidants in foods, models must be developed that have the chemical, physical, and environmental conditions expected in food products. This paper outlines model systems of the evaluation of antioxidants in three types of foods: bulk oil, oil-in-water emulsions, and muscle foods. These model systems are not intended to be inclusive of all possible methods to measure lipid oxidation and antioxidant activity. However, use of these models would allow researchers to more easily compare research results from one paper to another.

KEYWORDS: Antioxidants; foods; oil; emulsions; muscle foods; meat; lipid oxidation; omega-3 fatty acids

INTRODUCTION

Many new in vitro methods have been developed to evaluate antioxidant activity. Unfortunately, these in vitro methods often do not correlate with the ability of compounds to inhibit oxidative deterioration of foods. This is because the activity of antioxidants in food systems depends not only on the chemical reactivity of the antioxidant (e.g., free radical scavenging and chelation) but also on factors such as physical location, interaction with other food components, and environmental conditions (e.g., pH). One of the major factors affecting the activity of antioxidants that scavenge free radicals in foods is their partitioning behavior in lipids and water. For example, hydrophilic antioxidants are often less effective in oil-in-water emulsions than lipophilic antioxidants, whereas lipophilic antioxidants are less effective in bulk oils than hydrophilic antioxidants (*1*, *2*). Differences in the effectiveness of the antioxidants in bulk oils and emulsions are due to their physical location in the two systems. Polar antioxidants are more effective in bulk oils presumably because they can accumulate at the air−oil interface or in reverse micelles within the oil, the locations where lipid oxidation reactions would be greatest due to high concentrations of oxygen and prooxidants. In contrast, predominantly nonpolar antioxidants are more effective in emulsions because they are retained in the oil droplets and/or may accumulate at the oil−water interface, the location where interactions between hydroperoxides at the droplet surface and pro-oxidants (e.g., transition metals) in the aqueous phase occur. In addition, in emulsions polar antioxidants would tend to partition into the aqueous phase where they would not be able to protect the lipid. The tendency for lipophilic antioxidants to work best in foods with high water content, whereas polar antioxidants are most effective in bulk oil, has been termed the "antioxidant paradox" (*1*, *2*).

In vitro assays that measure free radical scavenging activity assays such as the ferric reducing/antioxidant power (FRAP), Trolox equivalent antioxidant activity (TEAC), and oxygen radical absorbant capacity (ORAC) are performed in the absence of lipids. This means that the impact of antioxidant partitioning is not evaluated, thus leading to possible lack of correlation between the results of in vitro assays and antioxidant performance in foods. To accurately evaluate the potential of antioxidants in foods, models must be developed that have the chemical, physical, and environmental (e.g., pH and ionic strength) conditions expected in food products. Because these factors are not consistent throughout all food systems, individual models must be developed. In this paper we will outline models for three types of foods lipids: bulk oil, oil-in-water emulsions, and muscle foods. These models and recommendations are not

* Author to whom correspondence should be addressed [telephone (413) 545-1026; fax (413) 545-1262; e-mail edecker@foodsci.umass.edu].
† University of Massachusetts.
‡ U.S. Department of Agriculture.
§ University of Wisconsin−Madison.
# Memorial University of Newfoundland.

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intended to be inclusive of all possible methods to measure lipid oxidation and antioxidant activity. In some cases a researcher may want to add additional analytical techniques. However, if possible we recommend following these basic procedures so that results can be compared more easily from one paper to another.

GENERAL CONSIDERATION FOR EVALUATION OF FOOD ANTIOXIDANTS

Although major differences will exist in the behavior of antioxidants in different kinds of foods, there are some general considerations that should be observed for all food products:

1. Avoid high oxidation temperatures (>60 °C) during storage studies because the mechanisms and kinetics of oxidation are not the same as at lower temperatures. For example, high temperatures can cause rapid decomposition of hydroperoxides, decomposition or volatility of antioxidants, and depletion of oxygen (oxygen solubility decreases with increasing temperature).

2. Ensure that the starting lipid does not contain high levels of oxidation products. El-Magoli et al. (*3*) reported that oxidation products of lipids such as hexanal and 2,4-decadienal act as pro-oxidants. In addition, free fatty acids have shown a pro-oxidative effect (*4*). An AOCS collaborative study reported that corn, sunflower, canola, and soybean oils oxidized to peroxide values of 17.2−17.9 had significant levels of rancid and/or painty flavors (*5*). Oils with high peroxide values should not be used in oxidation studies because the oil is already rancid and thus would have little relevance to food quality. In addition, high levels of pre-existing lipid oxidation products could result in the rapid decomposition of antioxidants, thus altering conclusions about antioxidant effectiveness.

3. Effectively analyze the activity of antioxidants by measuring both primary (e.g., hydroperoxides, conjugated dienes) and secondary oxidation (carbonyls, volatile compounds) products. In some cases antioxidants (e.g., tocopherols) can increase primary lipid oxidation products by donating hydrogen to a peroxyl radical to form a lipid hydroperoxide while simultaneously decreasing formation of low molecular weight volatile secondary oxidation products. Other compounds can decrease lipid hydroperoxides (e.g., oxidizing agents such transition metals) while increasing the secondary lipid oxidation products that cause rancidity. Thus, if one were to only measure lipid hydroperoxides to evaluate an antioxidant, an extract of biological material high in iron could appear to be antioxidative because lipid hydroperoxides would never accumulate because they are being decomposed into the volatile oxidation products that cause rancidity. It is also possible that nucleophillic compounds can form complexes with aldehydes (*6*). Thus, if one was only measuring a single aldehyde as an index of lipid oxidation, certain compounds could appear to be antioxidative because they could complex that aldehyde while not affecting other volatile lipid oxidation components that can affect rancidity.

4. Types of fatty acid decomposition products formed during oxidation are related to the fatty acid composition of the oil. For example, hexanal is derived from the oxidation of omega-6 fatty acids such as linoleic acid, and propanal is derived from the oxidation of omega-3 fatty acids such as linolenic acid. For oleic acid rich oils, nonanal may be used as a marker of oxidation. Therefore, it is essential to know the fatty acid composition of the oil being oxidized when a method for measuring secondary oxidation products is chosen. In addition, it is essential to use methods that are sensitive enough to measure low levels of oxidation because at higher levels of oxidation the food product is already rancid and, thus, results do not pertain to food preservation. If possible, sensory evaluations should be conducted to confirm results from instrumental and chemical analyses.

5. Use either crude extracts of biological materials or pure compounds as the source of antioxidants. If possible, it is best to use pure compounds to allow a better evaluation and understanding of how the compound functions alone and in combination with other food components. If phenolic compounds are expected to be the major antioxidants in a crude extract, the total phenol content and compositional data of the extract should be reported in order to compare samples. Include a reference compound in the study such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ), or one of the tocopherol homologues. Use the same molar concentration of tested components (e.g., phenolics) as the reference compound.

6. Use a food lipid source with a consistent source of natural endogenous antioxidants or prooxidants for all analyses because the antioxidant being tested can interact with endogenous antioxidants and pro-oxidants to alter oxidation kinetics. Because the biological sources from which foods are derived can exhibit great differences in antioxidant and pro-oxidant concentrations due to factors such as seasonal, geographical, genetic, and age variations, it is best to use a single source of the material to evaluate antioxidants. In some cases, variations in antioxidant concentrations can be minimized by isolating purified oils (triacylglycerols) that have been stripped of all minor oil constituents.

7. pH can affect oxidative reactions by influencing pro-oxidant (e.g., iron solubility increases with decreasing pH) and antioxidant (the pH can alter the charge of antioxidants, which can affect solubility and chelation capacity) activity. The pH of oxidation models should therefore be similar to the food of interest.

8. Standardize time and conditions to determine if the antioxidant is effective. Express antioxidant effectiveness as differences in induction period, percent inhibition of compound formation at a set time, rates of compound formation or decomposition, I_{50} (antioxidant concentration to achieve 50% inhibition), or percent loss or retention of antioxidant. These calculations should be conducted on data obtained from the early stages of oxidation because the concentrations of oxidation products in later stages will cease to increase or can even decrease. Statistical evaluation should be used to determine differences between controls (no added antioxidants) and samples.

MEASURING ANTIOXIDANT EFFECTIVENESS IN EDIBLE OILS

Vegetable Oils. The methods methods are for the evaluation of antioxidant efficacy in vegetable oils such as soybean, canola, sunflower, corn, cottonseed, rice bran, olive, and peanut oils. Protocols are for vegetable oils and for purified oils (triacylglycerols) that have been stripped of all minor oil constituents.

Methods. *1. Oxidation Conditions for Oils. a. Autoxidation: AOCS Oven Storage Test for Accelerated Aging of Oils (Cg 5-97; 7) Conducted at 60 °C in the Dark.* Sampling during the oxidation process should be done at set times such as every 24 h depending on the oil stability. A defined endpoint, relative to the goal of the experiment, should be set prior to initiation of the project. For example, a typical endpoint is the number of hours to reach a peroxide value of 20 mequiv/kg.

b. Photooxidation: AOCS Light Exposure Procedure for Oils (Cg 6-02; 7) Conducted at 4200 lx (600 ft-c) at 25 °C or Other Specified Condition. Sampling during the oxidation process should be done at set times such as every 4, 8, 12, or 24 h depending on the oil stability.

2. Measuring Oxidation Levels in Oils. a. Primary Oxidation Products by Peroxide Value (AOCS Cd 8b-90; 7) or Conjugated Dienes (AOCS Ti 1a-64; 7).

b. Secondary Oxidation Products [Volatile Compounds by Gas Chromatography with Static or Dynamic Headspace Snalyses (AOCS Cg 4-94; 7) or Anisidine Value (AOCS Cd 18-90; 7)]. The types of volatile compounds are related to the fatty acid composition of the oil. For example, hexanal is derived from linoleic acid and propanal from linolenic acid; other volatile compounds can also be monitored. Sensory evaluation of odor or flavor is measured according to AOCS Cg 2-83 (*7*).

Marine and Highly Unsaturated Oils. Polyunsaturated fatty acids (PUFA), especially highly unsaturated fatty acids (HUFA) of the omega-3 family in marine and algal oils, are susceptible to rapid oxidative deterioration. Oxidation of oils generally depends on their degree of unsaturation, as represented by double-bond index or methylene bridge index (*8*), position of HUFA in the triacylglycerol molecule and the composition and spectrum of minor components (*9*, *10*). Methodologies provided for vegetable oils are also applicable to marine and other highly unsaturated oils. However, other methodologies that may not lend themselves for use for vegetable oils may also be useful. As an example, encapsulated marine oils may be assessed by changes in their fatty acid composition during storage (*11*). Marquez-Ruiz et al. (*12*) also evaluated the oxidation of microencapsulated fish oils by a combination of adsorption and size exclusion chromatography. The procedure employed demonstrated that polymers provided a better picture of the progress of oxidation as compared to polyene index and thiobarbituric acid reactive substances (TBARS).

Shahidi et al. (*13*) examined the oxidative stability of cod liver and seal blubber oils using standard procedures and nuclear magnetic resonance. Although the latter procedure proved to be useful, this method requires standardization. Cho et al. (*14*) also found that tocopherol content and lipid composition were important factors that could explain the oxidative stabilities as evaluated by measuring changes in PUFA. The presence of phospholipids in the oils enhanced their stability, and this was influenced by phospholipid composition. Meanwhile, the oxidative stability of marine oils was evaluated in the presence of green tea extracts. The presence of chlorophyll was found to overwhelm the antioxidant activity of constituent catechins (*15*). However, the same extracts showed an antioxidant effect in a fish meat model system (*16*).

In a series of studies, Hamam and Shahidi (*17*, *18*) reported the inclusion of capric acid into algal oils containing HUFA. Incorporation of capric acid into these oils, despite lowering the degree of unsaturation in the resultant products, compromised their stability as determined by conjugated diene and TBARS analyses. Removal or alteration of endogenous antioxidants such as tocopherols was considered to be the underlying explanation for this observation.

MEASURING ANTIOXIDANT EFFECTIVENESS IN OIL-IN-WATER EMULSIONS

An emulsion consists of two immiscible liquids (usually oil and water), with one liquid being dispersed as small (diameter = 0.1−100 mm) spherical droplets in the other liquid. Food emulsions can exist as oil-in-water emulsions where oil is dispersed in water or water-in-oil emulsions where water is dispersed in oil. Emulsions are thermodynamically unstable because of the positive free energy needed to increase the surface area between oil and water phases (*19*, *20*). For this reason emulsions tend to separate into a layer of oil (lower density) on top of a layer of water (higher density) with time. To form emulsions that are kinetically stable for a reasonable period (a few weeks, months, or even years), chemical substances known as emulsifiers must be added prior to homogenization. Emulsifiers are surface-active molecules that adsorb to the surface of freshly formed droplets during homogenization, forming a protective membrane that prevents the droplets from coming close enough together to aggregate. The most common emulsifiers used in the food industry are surface-active proteins (e.g., casein, whey, soy, and egg), phospholipids (e.g., egg or soy lecithin), and small molecule surfactants (e.g., Spans, Tweens, fatty acids).

An emulsion can be considered to consist of three regions: the interior of the droplets, the continuous phase, and the interfacial membrane. The interfacial membrane consists of a narrow region surrounding each emulsion droplet that consists of a mixture of oil, water, and emulsifier molecules. Typically, the interfacial membrane has a thickness of a few nanometers and often makes up a significant proportion of the total number of molecules present in the droplet (*21*). The various molecules in an emulsion partition themselves among these three different regions according to their polarity and surface activity. Nonpolar molecules are located predominantly in the oil phase, polar molecules in the aqueous phase, and surface-active molecules at the interface. The precise molecular environment of a molecule may have a significant effect on its chemical reactivity. Therefore, the nature of the emulsion droplet interfacial membrane would be expected to be extremely important in lipid oxidation reactions because it could dictate how lipids (e.g., unsaturated fatty acids and lipid hydroperoxides) could interact with aqueous phase prooxidants (e.g., transition metals and reactive oxygen species). The physical location of antioxidants in emulsions is also critical in their chemical reactivity because they are most effective when they are at or near the physical location of the oxidative reactions.

When antioxidant activity in emulsions is evaluated, it is important to utilize emulsion systems similar to those found in foods. Below are several factors to consider in the design of emulsions for antioxidant evaluation.

1. The interfacial membrane of emulsion droplets is critical in oxidation kinetics. For example, anionic emulsion droplet charge will attract transition metals to increase metal−lipid interactions and thus increase lipid oxidation rates (*22*, *23*). Charged emulsion droplets can also attract oppositely charge antioxidants to change their partitioning behavior. Another property of emulsion interfacial membranes that can affect lipid oxidation is thickness, with thick membranes decreasing metal−lipid interactions, thus decreasing lipid oxidation rates (*24*). Therefore, in the selection of an emulsifier for the evaluation of antioxidants in foods it would be best to use an emulsifier with similar properties to what is used in the food of interest. If synthetic nonfood grade emulsifiers are used, they should have characteristics (charge, headgroup size,and critical micelle concentrations) similar to those of food emulsifiers.

2. The type of oxidizable lipid used in emulsion studies is also very important. Triacylglycerols make up the large majority of food lipids and thus are the best source of oxidizable lipids to use for the evaluation of antioxidants in emulsions. Use of free fatty acids should be avoided because their acid group will

migrate to the emulsion interfacial region, where they will make the emulsion droplet more a negative. Fatty acid/emulsifier models that do not have triacylglycerols will produce mixed micelle systems and not emulsion droplets. These micelles do not have the lipid core observed in typical oil-in-water emulsions. Antioxidant will not partition into these mixed micelles in the same manner as an emulsion containing triacylglycerols.

3. Several emulsifiers such as Tween can form hydroperoxides during storage. Emulsifier hydroperoxides can promote the oxidation of unsaturated fatty acids and antioxidants (*25*, *26*). Thus, emulsifiers low in hydroperoxides (<15 μmol/g) should be used for antioxidant studies.

4. Many antioxidants in emulsions will partition into both the lipid and water phases of emulsions. This partitioning behavior is critical in the activity of the antioxidant. Measurement of partitioning behavior can provide important insight into antioxidant behavior and can help predict if an antioxidant will be effective in oil from an emulsion.

5. When emulsifiers (surfactants) are used to make emulsions, they will migrate to the emulsion droplet surface until it becomes saturated. After interface saturation, the excess emulsifier will partition into the aqueous or lipid phase. In the case of low molecular weight surfactants, micelles can be produced (at concentrations about the critical micelle concentration). These surfactant micelles can facilitate the transfer components form one phase to another. In oil-in-water emulsions, surfactant micelles have been found to increase the partitioning of antioxidants out of lipid emulsion droplets into the aqueous phase (*27*). Excess proteins will also partition into the aqueous phase, where they can chelate pro-oxidative metals and scavenge free radicals (*28*, *29*). Because of the ability of excess emulsifiers to alter the physical location of antioxidants and the activity of pro-oxidants, it is advisable to use the minimal concentration of surfactant necessary to stabilize the emulsion. Alternately, excess surfactant can be removed from oil-in-water emulsions by repeated washing of the emulsion (*29*).

6. Emulsions must be stable (e.g., resistant to creaming, flocculation, and/or coalescence) during the entire length of the oxidation study. Use environmental conditions (e.g., pH, ionic strength, and temperature) at which the emulsion is stable. The size of the emulsion droplet is not a major factor in lipid oxidation rates (*30*). However, droplet size will affect the physical stability of the emulsion. Therefore, emulsions should be prepared with similar droplet sizes (≤ 1.0 μm), which can be verified by laser light scattering (*31*).

Methods. *1. Oxidation Conditions for Emulsion.* Prepare an emulsion by sonication or homogenization. Lipid-soluble antioxidants should be added to the lipid prior to emulsification, whereas water-soluble antioxidants can be added to the finished emulsion. If multiple emulsions are being made, measure particle size to determine that the droplets in all emulsions have similar sizes. Use emulsifiers low in hydroperoxides. Use the minimal concentration of emulsifier needed to stabilize the emulsion or remove excess emulsifier after emulsification by washing techniques.

Oxidation should be conducted at temperatures of <60 °C. Sampling during the oxidation process should be done at set times (e.g., every 6−24 h depending on the stability of the oil in the emulsion). Decrease storage temperature if oxidation rates are too fast or if the emulsion becomes physically unstable.

2. Measuring Oxidation Levels in Oils. a. Measure primary oxidation products by peroxide value (*26*). Conjugated dienes (AOCS Ti 1a-64; *7*) can also be measured on oil extracted from the emulsion.

b. Measure secondary oxidation products using techniques such as volatile compounds by gas chromatography with static or dynamic headspace analyses (AOCS Cg 4-94; *7*), anisidine value (AOCS Cd 18-90; *7*), or thiobarbituric acid reactive substances (TBARS; *32*). TBARS are suitable when using oils with fatty acids with three or more double bonds and emulsions that are low in carbohydrates or other interfering substances.

MEASURING ANTIOXIDANT EFFECTIVENESS IN MUSCLE FOODS

Muscle tissue is composed of a multitude of endogenous components that can either accelerate (prooxidants) or inhibit (antioxidants) oxidative processes. The concentrations and activity of pro-oxidants and antioxidants in muscle foods can change dramatically during processing and storage as can be seen from the examples below. Ascorbate, glutathione, and tocopherol decrease in concentration with increased storage time (*33*). Ascorbate can be antioxidative or pro-oxidative depending on the concentration of lipid hydroperoxides and low molecular weight metals present (*34*, *35*). Heme proteins (e.g., hemoglobin and myoglobin) are converted to met and ferryl oxidation states during storage, which increases their ability to promote lipid oxidation (*36*). Low oxygen partial pressure accelerates browning and lipid oxidation due to the rapid rate of met heme protein formation that occurs at oxygen partial pressures between 3 and 10 mmHg (*37*). The concentration of hemin and low molecular weight metals (e.g., copper and iron) increases with storage, which can accelerate oxidative processes (*38*). Copper ions were found to accelerate oxymyoglobin oxidation (*39*). Physical changes also occur with increased storage time and processing steps (e.g., comminution), which bring previously segregated reactants together. Minced trout muscle was considered to be susceptible to rancidity due to the dispersion of blood pigments in the flesh caused by the mechanical destruction of the tissue (*40*). Cooking will cause denaturation of proteins, increase the concentration of low molecular weight metals, and alter lipid phases. These changes may cause an antioxidant that is effective in the raw state to have a different efficacy in the cooked state. The mechanism of lipid oxidation and hence the effect of added antioxidants may change depending on animal species because hemoglobin from bovine and avian species promoted the oxidation of lipids much less effectively than fish hemoglobins (*41*). Below are some factors to consider when antioxidant efficacy and the mechanisms by which antioxidants inhibit lipid oxidation in muscle food systems are assessed.

1. The oxidative stability of skeletal muscle can show large animal-to-animal variations. Therefore, when antioxidants are assessed in either muscle food or muscle food models, it is best to pool together muscle from several animals to minimize these animal−animal variations.

2. There is a perception that muscle foods with high fat contents are especially susceptible to lipid oxidation, whereas those with lower fat contents should be more stable. Phospholipids are present at ~1% of the tissue weight regardless of fat content, whereas the remaining lipid comprises mostly triacylglycerols (e.g., neutral lipids). Phospholipids are considered to be the primary substrate for lipid oxidation reactions that lead to rancidity despite their low percentage in muscle (*42*). This is because phospholipids possess ~100 times more surface area than triacylglycerols on a weight basis and the fact that fatty acids of phospholipids are more unsaturated than those in triacylglycerols. However, it is possible that fat content could affect antioxidant activity because nonpolar antioxidants could partition into the triacylglycerols, where they would be unable

to protect membrane phospholipids. Therefore, antioxidant evaluation should be conducted in muscle samples with similar fat contents and the fat concentration should be similar to that of the processed muscle food of interest.

3. Partitioning of antioxidants into either the membrane phospholipids or triacylglycerols can also be dependent on how the antioxidant is added to the muscle sample. Tocopherol was preferentially incorporated into the membrane fraction if the antioxidant was added to lean muscle before the addition of triacylglycerols (*43*). Triacylglycerols are routinely added to lean tissues to improve palatability in the final product. The polarity of the antioxidant carrier can also control antioxidant distribution. The optimal dielectric constant for tocopherol incorporation into the membrane fraction when added to lean tissue before the triacylglycerols was ~21, which is achieved using a combination of ethanol and n-butanol carrier (*44*).

4. Surface tissue (1−2 mm) should be removed and assayed when intact steaks or fillets are analyzed. Oxygen penetrates 1−4 mm into muscle foods (*45*). Because the interior portion is mostly anaerobic in post-mortem muscle, a large majority of the oxidation will occur at the surface.

Model Systems To Assess Antioxidant Effectiveness. *1. Ground Muscle Foods.* Ground muscle foods can be an excellent way to evaluate antioxidant effectiveness. Unfortunately, these methods are often time-consuming because endogenous antioxidants provide a substantial lag phase prior to the formation of oxidation products. This long lag phase can mean that spoilage by microbial growth can occur prior to oxidative rancidity. In addition, determining mechanisms of antioxidant action can be difficult in muscle foods due to the complex and dynamic nature of muscle tissue (e.g., depletion of antioxidants and formation of pro-oxidants during storage). Thus, in some cases antioxidant evaluation may be more effective in model systems containing pro-oxidants so that analysis can be completed before microbial degradation (see below).

a. Cooked Ground Muscle Foods. Thermal processing causes rapid development of lipid oxidation in muscle foods, which has been termed "warmed-over flavor". Therefore, cooked muscle foods can provide an excellent model for the evaluation of antioxidant effectiveness. Unfortunately, the activity of antioxidants in cooked muscle foods does not always match the antioxidant activity in raw products; therefore, this test should be used only if the products of interest are thermally processed.

The evaluation of antioxidants in cooked muscle foods can be accomplished by adding the antioxidants to ground muscle and then placing the samples (10−20 g) into test tubes and cooking in a water bath at 90 °C. A thermometer can be placed into one or two samples to monitor internal temperature, and then samples can be removed when the desired degree of doneness is reached (65−80 °C). Samples are then placed in cold tap water to cool, removed from test tubes, and stored at refrigeration temperatures. Samples should be analyzed for lipid oxidation products one or two times a day for up to 4 days depending on the oxidative stability of the muscle food.

To evaluate antioxidants in muscle foods, antioxidants or additives may be added to the system and TBARS evaluated over a set period of time after cooking (*46*−*48*). Use of headspace volatiles for the evaluation of the oxidative state of muscle foods is also commonplace (*49*, *50*).

b. Raw Ground Muscle Foods. Depending on the oxidative stability of the muscle foods, microbial spoilage can often occur prior to oxidative rancidity. One exception to this problem is certain species of fish (e.g., mackerel) in which lipid oxidation is faster than microbial spoilage. Therefore, in most cases antioxidant evaluation needs to be conducted under freezing conditions. Many muscle foods such as sausages are salted. Addition of salt (1−2%) to ground muscle accelerates lipid oxidation even under frozen conditions. The evaluation of antioxidants in ground, salted muscle foods can be performed with 2−4 months of frozen storage depending on the oxidative stability of the skeletal muscle. The evaluation of antioxidants in ground, unsalted muscle foods will take substantially longer, with storage of >1 year often needed to observe lipid oxidation.

Table 1. DPPH Radical Scavenging Ability of Quercetin and Propyl Gallate at Various Concentrations

	% DPPH scavenging activity			
	1 μM	2.5 μM	5 μM	10 μM
quercetin	12.1	29.5	58.4	94.1
propyl gallate	14.1	30.2	54.4	90.5

2. Washed Muscle Systems. An alternative approach to assessing antioxidants in ground muscle is to use a model system consisting of washed skeletal muscle as the lipid substrate. Washing the skeletal muscle removes aqueous antioxidants and pro-oxidants. The remaining washed tissue contains myofibrillar proteins and membrane phospholipids (*51*). This matrix is advantageous in that it physically resembles the structure of muscle more than isolated cell membranes. Extensive research evaluating antioxidants in a washed cod muscle system has been conducted (*52*−*54*). A washed cod system has several advantages in that it is high in polyunsaturated fatty acids, providing measurable lipid oxidation rates in a reasonable amount of time, and is mostly devoid of pro-oxidative heme and mitochondria, a source of reactive oxygen species. Washed beef, pork, or chicken muscle could also be used, but they would have slower development of oxidation products and be more difficult to obtain in heme- and mitochondria-free states.

Hemoglobin or myoglobin is added to the washed cod to promote the oxidation of the lipids. Bovine hemoglobin (Sigma-Aldrich Co.) can be used as a convenient source of heme protein, but the pH of the washed cod muscle system must be no higher than 5.7 for reasonable rates of lipid oxidation during iced storage. The hemoglobin concentration should be ~12 μmol/kg of washed cod. Commercially prepared bovine hemoglobin is mostly oxidized, which can be less pro-oxidative compared to reduced hemoglobin. Reduction of the methemoglobin with sodium dithionite in a nitrogen atmosphere followed by gel filtration can be done to prepare reduced hemoglobin. Hemoglobin prepared from the blood of rainbow trout is an alternative source of a highly pro-oxidative and reduced heme protein (*55*). The ability of added antioxidants to inhibit heme protein-mediated lipid oxidation is then assessed during iced storage. Fe^{3+}-ADP in the presence of NADH was found to be weakly pro-oxidative compared to hemoglobin on an equimolar iron basis in washed cod muscle (*56*). Hemoglobin was a far superior pro-oxidant compared to inorganic iron in washed pork muscle (*57*). The preformed lipid hydroperoxide content in the muscle should be monitored because this can affect rates of lipid oxidation (*54*).

It is tempting to assess "antioxidant activity" using non-lipid free radical scavenging assays and extrapolate the findings to activity in muscle foods. We found that quercetin and propyl gallate scavenged DPPH radicals with nearly equivalent efficacy (**Table 1**) but that propyl gallate was a better inhibitor of lipid oxidation in washed cod muscle containing added hemoglobin

Table 2. Ability of Quercetin and Propyl Gallate To Inhibit Hemoglobin-Mediated Lipid Oxidation in Washed Cod Muscle during 2 °C Storage

	μmol of TBARS/kg of washed cod at day						
	0	1	2	3	5	7	9
washed cod + Hb	4.8	5.9	15.8	103.7	86.3	88.3	85.2
washed cod + Hb + quercetin	3.3	3.7	3.4	3.0	5.9	19.6	52.3
washed cod + Hb + propyl gallate	2.8	3.0	2.4	2.4	3.1	5.9	9.7

(**Table 2**). This indicated that the DPPH assay was a poor predictor of antioxidant efficacy in muscle systems.

Quercetin and propyl gallate were added at 37 μmol/kg of washed cod (**Table 2**). Trout hemoglobin (Hb) was added at 12 μmol/kg of washed cod. The pH was adjusted to 6.3. Each antioxidant was dissolved in a mixture of ethanol/water (1:1) prior to the addition to washed cod (1% carrier). The carrier was also added to control samples (washed cod + Hb).

3. Isolated Muscle Membranes. A microsomal membrane fraction from muscle can also be utilized as a substrate for lipid oxidation and antioxidant studies (*58*). Additions of $FeCl_3$, NADH, and ADP effectively stimulated the formation of lipid oxidation products in the microsomes. A membrane-bound reductase converts ferric (Fe^{3+}) iron to its active ferrous (Fe^{2+}) form. NADH is a cofactor for the reductase, and ADP chelates the iron. The chelation of ADP to iron increases the solubility of the metal and changes the oxidation−reduction potential so that the oxidation of lipids is promoted. The pH value in muscle model systems should be in the range of that of post-mortem muscle tissues (pH 5.5−7.0). Limitations in the use of microsomes include the requirement of freshly slaughtered tissue for membrane isolation and the lack of myofibrillar proteins in the preparation.

Measuring Oxidation Levels in Muscle Tissue and Model Muscle Systems. Lipid hydroperoxides should be measured as indicators of primary lipid oxidation products (*59*). Organic solvents that are stabilized with amylene and used to extract lipids from the muscle should be avoided. Amylene interferes with the ferric thiocyanate assay for lipid hydroperoxides (*60*). Lipids can be extracted from muscle tissue according to a number of procedures (*61*, *62*).

A portion of the lipid hydroperoxides that form in the early stages of lipid oxidation are broken down to form the low molecular weight volatile compounds (secondary products) that impart rancidity. Various low molecular weight aldehydes, alkenals, and nonvolatile precursors of these substances react with 2-thiobarbituric acid, resulting in chromogens termed thiobarbituric acid reactive substances (TBARS) that can be determined after acid extraction (*63*). TBARS have been found to increase with increasing rancid odor and warmed-over flavor development in raw and cooked muscle, respectively, provided the starting materials were of high initial quality (*64*, *65*). Good correlations between TBARS and headspace volatiles (e.g., hexanal, pentanal) were determined in cooked turkey during 4 °C storage (*66*). TBARS have also been correlated with the sensory quality of pork (*67*). Loss of redness can be used to assess the progress of hemoglobin-mediated lipid oxidation in washed fish muscle (*68*). There was a lack of changes in fatty acid composition when fresh and rancid muscle tissues from Atlantic mackerel were compared (*69*). This indicates that a very small percentage of the total lipid needs to be oxidized in order for rancid odor to be detected.

Blood plasma supplemented with hemoglobin had lower lipid peroxide values during 2 °C storage compared to samples not supplemented with hemoglobin, which suggested hemoglobin was inhibiting lipid oxidation; however, when secondary oxidation products were also measured, TBARS and rancid odor were higher in the hemoglobin-containing samples (*70*). To explain these findings, it is believed that the added hemoglobin effectively broke down lipid hydroperoxides in the system, which resulted in the formation of TBARS and rancidity. This further emphasizes the need to measure both primary and secondary lipid oxidation products to properly assess mechanisms of lipid oxidation and the effect of antioxidant treatments in muscle food systems.

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