JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

REVIEWS

Antioxidant Assays for Plant and Food Components

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Recently, research on natural antioxidants has become increasingly active in various fields. Accordingly, numerous articles on natural antioxidants, including polyphenols, flavonoids, vitamins, and volatile chemicals, have been published. Assays developed to evaluate the antioxidant activity of plants and food constituents vary. Therefore, to investigate the antioxidant activity of chemical(s), choosing an adequate assay based on the chemical(s) of interest is critical. There are two general types of assays widely used for different antioxidant studies. One is an assay associated with lipid peroxidations, including the thiobarbituric acid assay (TBA), malonaldehyde/high-performance liquid chromatography (MA/HPLC) assay, malonaldehyde/gas chromatography (MA/GC) assay, β-carotene bleaching assay, and conjugated diene assay. Other assays are associated with electron or radical scavenging, including the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, ferric reducing/antioxidant power (FRAP) assay, ferrous oxidation−xylenol orange (FOX) assay, ferric thiocyanate (FTC) assay, and aldehyde/carboxylic acid (ACA) assay. In this review, assays used recently were selected for extended discussion, including discussion of the mechanisms underlying each assay and its application to various plants and foods.

KEYWORDS: Antioxidant assay; 2,2′-azinobis(3-ethylbenzothiazoline-6 sulfonic acid) (ABTS) assay; β-carotene bleaching assay; 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay; ferric reducing/antioxidant power (FRAP) assay; thiobarbituric acid (TBA) assay

INTRODUCTION

An antioxidant is defined as a molecule capable of slowing or preventing the oxidation of other molecules (*1*), whereas a biological antioxidant has been 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" (*2*). It is well-known that oxidation damages various biological substances and subsequently causes many diseases. Accordingly, there are many reviews on the relationships between oxidative damages and various diseases including cancer (*3*), liver disease (*4*), Alzheimer's disease (*5*), aging (*6*) arthritis (*7*), inflammation (*8*), diabetes (*9, 10*), Parkinson's disease (*11, 12*), atherosclerosis (*13*), and AIDS (*14*). As a result, many diseases have been treated with antioxidants to prevent oxidative damage.

Well-known natural antioxidants, such as vitamin E (α-tocopherol), vitamin C, and polyphenols/flavonoids, have been investigated for their possible use to prevent the diseases described above (*15*). Vitamin E therapy is reportedly effective in decreasing oxidative stress and the levels of erythrocyte osmotic fragility in patients on dialysis (*16*). One review reports that vitamin E therapy had beneficial effects on patients with diabetes (*17*). Clinical and research evidence on the effects of vitamin C on cancer and cardiovascular disease were also well discussed in a recent review (*18*). Recently, polyphenols/flavonoids found in plants have begun to receive much attention among researchers as a new natural antioxidant. In particular, the "French paradox", a report which suggested that the drinking of red wine has been linked to the low incidence of coronary heart disease (CHD) in France (*19*), triggered active research on the relationship between red wine constituents and CHD. The situation in France was paradoxical in that there was high intake of saturated fat but low mortality from CHD. Therefore, it was hypothesized that some phenolic compounds, such as polyphenols, flavonoids, and anthocyanins, in red wine play an important role in the prevention of CHD. Later, the antioxidant activity of wine phenolic compounds was confirmed (*20*), and possible mechanisms for the protective role of antioxidants, including flavonoids, phenolic compounds, and other phytochemicals, in wine and plant foods were summarized (*21*). Recently, research associated with natural antioxidants has increased dramatically in various fields, including food chemistry, food biology, natural plant chemistry, medicinal plants,

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10.1021/jf803537k CCC: $40.75
Published on Web 01/30/2009

Table 1. Selected Studies on Natural Antioxidants

natural substance	testing method	active compounds	reference
	Roots		
yacon root	DPPH, β-carotene/deoxyribose	chlorogenic acid, tryptophan	*28*
Cornus capitat Adventitious roots	DPPH, TBA	ellagic acid derivatives	*29*
licorice root (*Glycyrrhiza glabra*)	TBA	hispaglabridins A and B, glabridin	*30*
licorice root (*Glycyrrhiza uralensis*)	TBA, MA/GC	glycyrrhizin	*31*
Morinada elliptica root	TBA, FTC	anthraquinones	*32*
Paeonia lactiflora root	DPPH	(+)-catechin	*33*
ginger root	DPPH	6-gingerdiols	*34*
	Vegetables		
cherry tomatoes	FRAP	chalconaringenin, rutin, ascorbic acid, chlorogenic acid, lycopene	*35*
tomato paste	MA-TBA/HPLC	lycopene	*36*
red spinach (gangeticus)	TBA	not reported	*37*
bitter melons (*Momordica charantia*)	conjugated diene	gallic acid, gentisic acid, catechin, chlorogenic acid, epicatechin	*38*
potato peel	DPPH	phenolic compounds	*39*
sweet potato	DPPH	anthocyanins	*40*
soybean, mung bean, kidney beans, azuki beans	aldehyde/carboxylic acid, MA/GC	eugenol, maltol, benzyl alcohol	*41, 42*
	Herbs and Spices		
thyme (*Thymus zygis* L.)	TBA, FRAP	thymol, carvacrol, terpinene	*43*
rosemary	conjugated diene	carnosoic acid	*44*
bird chili (*Capsicum frutescenes* Linn.)	FRAP, ABTS, DPPH	phenolic compounds	*45*
rapeseed and pine bark	2,4-DNP/spectrophotometer	phenolic compounds	*46*
pepper	TBA, DPPH	arbutin, magnoflorine	*47*
sage	DPPH	caffeoyl fructofuranosyl glucopyranoside	*48*
eucalyptus leaves	MA/GC, aldehyde/carboxylic acid	1,8-cineole, benzaldehyde, benzyl alcohol	*49*
clove bud	MA/GC, aldehyde/carboxylic	eugenol, eugenyl acetate	*50*
	Fruits		
cloudberry (*Rubus chamaemorus*)	TBA	not reported	*51*
Dillenia indica fruit	DPPH, β-carotene/linoleate	phenolics	*52*
cinnamon, turmeric, golden thread	DPPH, FRAP, FTC	cinnamaldehyde, curcumin, berberine	*53*
buntan (citrus)	DPPH, β-carotene/linoleic acid	sitosterol, limonin	*54*
grape and wines	β-carotene/linoleic acid	phenolic compounds	*20*
raspberry	DPPH	ellagic acid, phenolic compounds, vitamin C	*55*
guava leaf	ABTS, FRAP	phenolic compounds	*56*
	Teas		
Brazilian green tea	DPPH	catechins	*57*
green tea	TBA	(−)-epigallocatechin gallate	*58*
rooibos tea	ABTS	flavonoids	*59*
green tea, oolong tea, black tea	aldehyde/carboxylic acid	heterocyclic compounds	*60*
	Cereals		
oat	β-carotene/linoleic acid	phenolic acids	*61*
buckwheat	β-carotene/linoleic acid	flavonoids	*62*
rice	DPPH	quinolone alkaloid	*63*
green barley leaves	TBA, MA/GC	saponarin, lutonarin	*64*
			65
	Miscellaneous		
brewed coffee	aldehyde/carboxylic acid	heterocyclic compounds	*66, 67*
beer	aldehyde/carboxylic acid	volatile compounds	*68*
kurosu (rice vinegar)	DPPH	ferulic acids, sinapic acid	*69*
balsamic vinegar	ABTS	polyphenols	*70*
red wine (Vini Novelli)	ABTS	polyphenols	*71*

and biochemistry. For example, there have been numerous articles on the subject of natural antioxidants published in the *Journal of Agricultural and Food Chemistry*: 158 articles associated with antioxidant studies published in 2008 (Vol. 56, Issues 1−24).

Many reviews and monographs evaluating various antioxidant assays have been published (*22*−*25*). Also, the scavenging mechanisms of antioxidants toward reactive oxygen species (ROSs) are well documented (*26*, *27*). Therefore, to investigate the antioxidant activities of chemical(s), deciding on an adequate assay based on the chemical(s) of interest is crucial. Essentially, there are two kinds of antioxidant tests: one is a straight chemical method, which involves analytical instruments such as a spectrophotometer, gas chromatograph, and HPLC, as well as gas chromatograph/mass spectrometer and HPLC/mass spectrometer. The other involves a biological assay, such as ELISA. This review focuses, however, mainly on the chemical methods of antioxidant testing. Because the reviews cited above do not describe the details of chromatographic methods, this review also focused on the assays using gas chromatography and HPLC.

Table 1 shows selected recent papers on antioxidants found in plants investigated using different assays. **Table 2** shows antioxidant studies on multiple numbers of samples from various medicinal plants.

ASSAYS ASSOCIATED WITH LIPID PEROXIDATIONS

Lipid Peroxidation and Secondary Products Used for Antioxidant Assays. The oxidative degradation of lipids (lipid peroxidation) has been studied from various perspectives, such as its roles in the alteration of foods, thermal oxidation, autoxidation, and oxidation with ROS. The basic mechanisms of lipid peroxidation can be found in many reference books (*85*−*88*). One of the most well-known mechanisms is that of

Table 2. Antioxidant Studies on Multiple Numbers of Samples from Various Plants

plant	no. of samples	testing method	active or major constituents	reference
essential oils	12	DPPH, FRAP, TBA	not reported	*72*
Indian medicinal plants	133	ABTS, DPPH, FRAP	phenolic acids, tannins, flavonoids, curcuminoids, coumarins, lignans	*73*
medicinal plants	70	FRAP, DPPH, ABTS	phenolic compounds	*74*
fruits and vegetable byproducts	11	DPPH, TBA	polyphenols	*75*
vegetables	43	β-carotene/linoleic acid	polyphenols	*76*
sorghum varieties	50	ABTS	proanthocyanidins, flavan-4-ol 3-deoxyanthocyanidins	*77*
Chinese medicinal plants	112	ABTS	phenolic compounds	*78*
Bolivian plants	54	DPPH	phenolic compounds	*79*
Chinese medicinal herbals	68	FRAP, DPPH	phenolic compounds	*80*
Korean salad plants	13	DPPH	phenolic compounds	*81*
medicinal plants	45	FRAP	phenolic compounds	*82*
plants from Cordoba (Argentina)	41	FRAP	phenolic compounds	*83*
essential oils	13	aldehyde/carboxylic acid, DPPH, MA/GC	limonene, benzyl acetate, myristicin	*84*

unsaturated fatty acids, such as linoleic acid, linolenic acid, arachidonic acid, and various ω-3 fatty acids (*89*−*92*).

Figure 1 shows proposed mechanisms of lipid peroxidation and formation of typical secondary oxidation products (*93*). Lipid peroxidation is initiated by ROS (*94*). Some typical ROS are superoxide ($O_2^{\bullet}$), singlet oxygen $^1O_2$, triplet oxygen ($^3O_2$), ozone (O^3), hydroxyl radical ($^{\bullet}OH$), alkoxyl radical ($RO^{\bullet}$), and peroxyl radical ($ROO^{\bullet}$). ROS abstract a hydrogen atom from a methylene group of an unsaturated fatty acid and subsequently form free radicals such as a peroxyl radical (*95*). Once these free radicals are formed, lipid peroxidation progresses and, consequently, lipids produce various so-called secondary oxidation products, shown in **Figure 1**. Some of these secondary oxidation products formed from lipids have been used as biomarkers to investigate their roles in the diseases mentioned above. Even though numerous lipid peroxidation products have been identified, only a few chemicals have been satisfactorily used as biomarkers of oxidative damages.

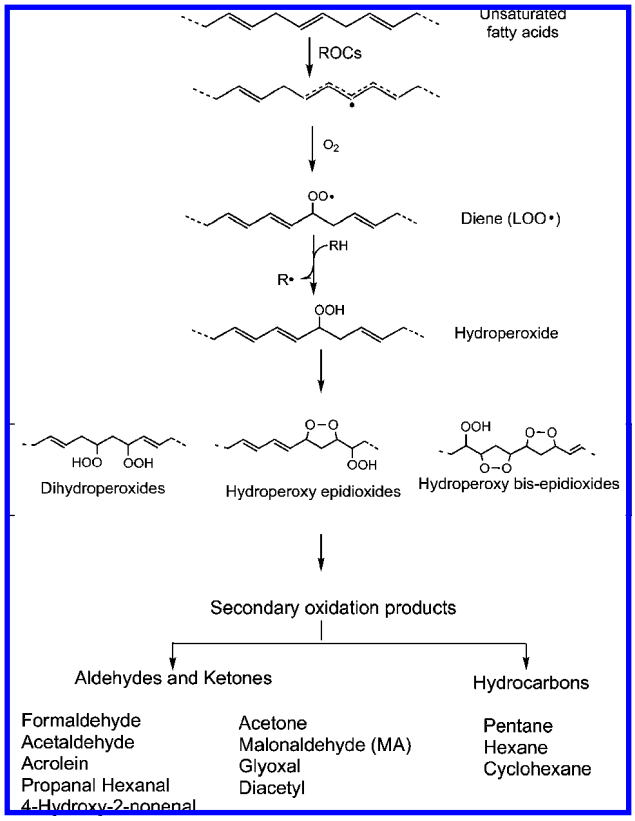

Figure 1. Proposed mechanisms of lipid peroxidation and formation of typical secondary oxidation products.

Most antioxidant tests associated with lipid peroxidation involve the monitoring of hydroperoxides or a specific oxidative secondary product. There have also been many reviews that summarize various antioxidant assays involved in the investigation of lipid peroxidation (*23*, *24*, *96*−*98*).

Thiobarbituric Acid (TBA) Assay. Among lipid peroxidation products used for antioxidant assays, MA has been most widely used to evaluate the antioxidant activity of chemical(s) in lipid peroxidation systems (*99*, *100*). In particular, MA is a useful biomarker to investigate the final stage of lipid peroxidation. However, it is extremely difficult to analyze MA in a lipid sample because it is very soluble in water and tends to present as a polymer in an aqueous solution. In the late 1950s, estimation of 2-deoxy sugars was conducted using the MA−TBA product (**Figure 2**), which was monitored by a colorimeter at UV absorption of 535 nm (*101*). Later, this method was used with a spectrophotometer to assess antioxidant effectiveness in pharmaceutical oils (*102*). Consequently, the MA−TBA assay became one of the most popular assays for studies related to lipid peroxidation, and it is still currently used widely to evaluate antioxidant activities of various natural products. Studies of plants and their components using the TBA assay that have appeared recently are studies of extracts from rosemary, green tea, grape seed, and tomato (*103*), phenolic compounds of *Salvia virgata* Jacq. (Lamiaceae) (*104*), hexane extracts of eight *Cephalaria* (Dipsacaceae) species (*105*), extract from fruit of Mengkudu (*Morinda citrifolia* L.) (*106*), and sage and rosemary essential oils (*107*).

However, TBA reacts with many different carbonyl compounds formed from lipid peroxidation including one shown in **Figure 2**, and their TBA adducts absorb the same UV wavelength absorbed by the MA−TBA adduct. Therefore, the TBA assay is not specific to MA, and the result is an overestimation of MA concentration (*108*−*110*). Later, total carbonyl compounds reacted with TBA came to be called TBA reacting substances (TBARS).

A typical detailed TBA assay procedure is as follows: Various concentrations of testing samples (generally 10−500 μg/mL) are added to an aqueous solution (2 mL) containing 200 μL of Tris-buffer (pH 7.4), 300 μL of 1 M KCl, 400 μL of 1% SDS, 10 μL of cod liver oil (this can be any kind of lipid such as linolenic

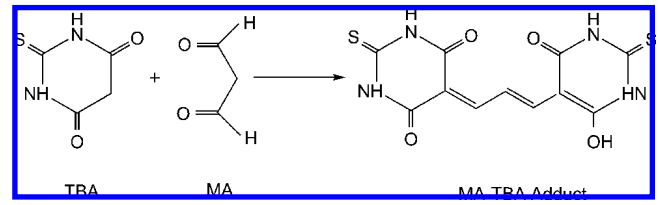

Figure 2. Formation of TBA−MA adduct.

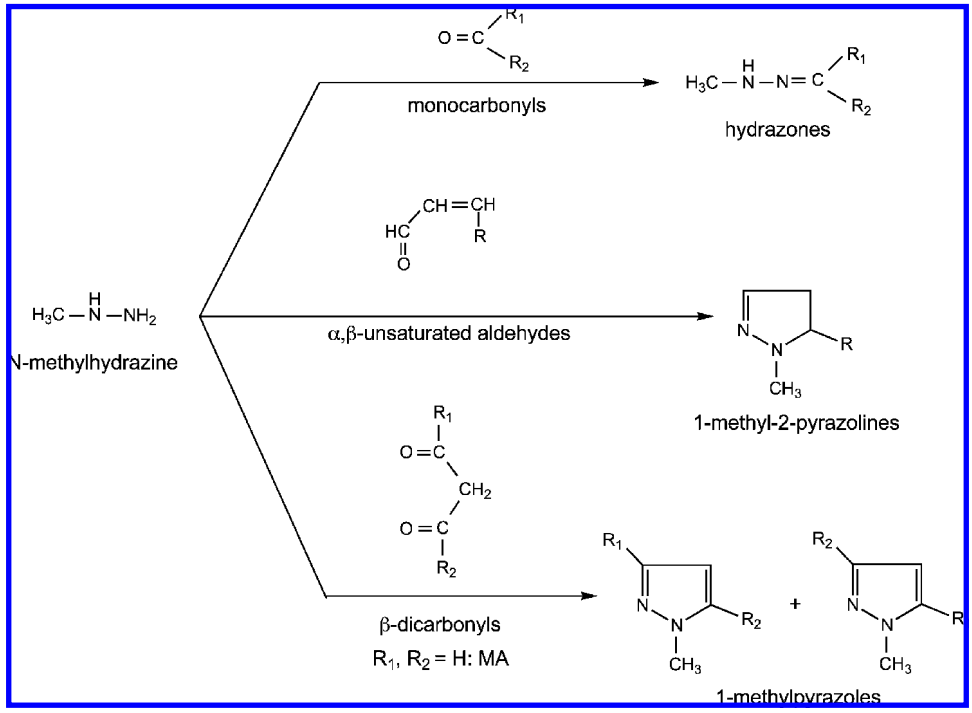

Figure 3. Reaction schemes of *N*-methylhydrazine and three different types of carbonyl compounds.

acid, arachidonic acid, or ω-3 fatty acids), 40 μL of 1.0 μM $FeCl_2$, and 20 μL of 0.5 μM H_2O_2 in a brown nontransparent vial (to avoid any oxidation caused by UV irradiation). The sample vial is then incubated for 18 h at 37 °C with shaking. After the incubation, oxidation is terminated by adding 50 μL of 4% BHT in ethanol solution, and 2 mL of the TBA reagent (0.67% TBA, TCA, 1% SDS, 5 N HCl) is added to the sample. The sample is heated at 80 °C for 1 h and then cooled in an ice bath for 10 min. A blank sample is prepared following the same procedure without a test sample. The TBA−MA adduct formed is measured using a spectrophotometer at 532 nm (A_{532}). A known antioxidant such as BHT, vitamin A, or vitamin C is used as a positive control in the assay.

Malonaldehyde/High-Performance Liquid Chromatography (MA/HPLC) Assay. To determine the exact amount of MA formed from lipid peroxidation, HPLC has been applied as a more specific method for MA−TBA adduct analysis. In particular, use of the HPLC method was recommended for biological samples, such as serum and plasma upon lipid peroxidation (*111*−*113*). There have been many reports of achieving satisfactory analysis of MA−TBA adduct by HPLC in various samples, such as cell suspensions and liver with a 25 cm × 4.6 mm C_{18} (5 μm particle size) column and a methanol/ammonium acetate buffer (40:60) mobile phase (*114*), rat spinal cord with a 15 cm × 4.6 mm (3 μm) C_{18} column and a methanol/10 mM sodium acetate (2:1) mobile phase (*115*), and human seminal plasma by a 15 cm × 4.6 mm (5 μm) C_{18} column and a methanol/potassium phosphate buffer (42:58) mobile phase. The one drawback of this method is that the sensitivity of a spectrophotometric detector is somewhat low compared with that of GC detectors. However, the MA−TBA adduct is not applicable to GC due to its low volatility. On the other hand, the recent development of LC/MS can achieve a higher efficiency of HPLC analysis on the MA−TBA adduct (*116*, *117*). For example, MA formed in oxidized linoleic acid was successfully analyzed by LC/MS using a 15 cm × 2 mm (5 μm) SGE C_{18} column or a 25 cm × 4.6 mm (5 μm) SGE C_{18} column and 0.1% aqueous acetic acid/ methanol mobile phase with a gradient mode (*118*). In this paper, the structure of the MA−TBA adduct was characterized and *m*/*z* 280 or 264 was selected by the first quadrupole for subsequent MS/MS analysis. Because fewer sample preparation steps are required than with the GC method, the LC/MS method is expected to become the mainstream of MA−TBA adduct analysis for antioxidant tests involved in lipid peroxidation.

Malonaldehyde/Gas Chromatography (MA/GC) Assay. Even though LC/MS will likely continue to be the major method for MA−TBA adduct analysis, this system is still significantly expensive, and it is not easy for an individual laboratory to obtain. An MA derivative (**Figure 3**), which can be analyzed specifically by a GC equipped with a nitrogen−phosphorus detector (NPD), has been prepared and used for antioxidant testing. This method was developed at first to analyze volatile carbonyl compounds, including MA, formed in corn oil and beef fat upon UV irradiation (*119*). The parts per million levels of volatile carbonyl compounds formed from oxidized lipids were analyzed using this derivative with a GC equipped with a 50 m × 0.25 mm i.d. bonded phase DB-Wax column and an NPD. Subsequently, various antioxidant studies on lipid peroxidation systems have been conducted using this assay. The lipids/antioxidant used were cod liver oil/saporanin (*120*), ω-3 fatty acids/saporanin (*121*), blood plasma/probucol and saporanin (*122*), blood plasma/volatiles from clove and eucalyptus (*123*), and calf thymus DNA/flavonoids (*124*). One of the major drawbacks of the GC method is that the sample preparation steps require somewhat tedious procedures. For example, a liquid−liquid continuous extraction—6 h required to obtain satisfactory recovery—was used to isolate the MA derivative (1-methylpyrazole) at the time when this method was developed. However, use of a solid phase extraction to recover the MA derivative improved the sample preparation process significantly (*124*, *125*).

A typical detailed MA/GC assay procedure is as follows: Various concentrations of the samples (generally 10−500 μg/mL) are added to an aqueous solution (5 mL) containing 10 μL of cod liver oil (this can be any kind of lipids such as linolenic acid, arachidonic acid, or ω-3 fatty acids), 0.05 M Tris buffer (pH 7.4), 0.5 μM H_2O_2, 1.0 μM $FeCl_2$, 0.75 mM KCl, and 0.2% of SDS. The sample solution is put in a 20 mL test tube, vortex

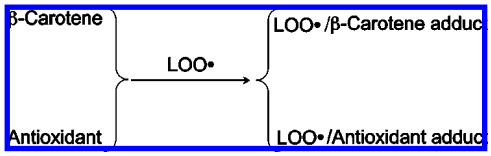

Figure 4. Formation of adducts from β-carotene and antioxidant with a lipid peroxide radical.

mixed for 10 s, and incubated at 37 °C for 18 h in a water bath with shaking. The sample test tube is covered with aluminum foil during incubation to avoid photo-oxidation. A control sample is prepared following the same procedure without a test sample. After incubation, a 4% ethanol solution of BHT (50 μL) is added to stop the oxidation. MA formed in the sample is derivatized to 1-methylpyrazole by adding 10 μL of *N*-methylhydrazine. Fifty microliters of 2-methylpyrazine stock solution (4 mg/mL) is added as a gas chromatographic internal standard. MA formed is quantified as 1-methylpyrazole by a gas chromatograph with an NPD. A known antioxidant such as BHT, vitamin A, or vitamin C is used as a positive control of the assay.

***β*-Carotene Bleaching Assay.** It has long been known that β-carotene reacts with the peroxyl radical to produce β-carotene epoxides (*126*). Therefore, β-carotene has received attention as a radical scavenger or antioxidant (*127*). Later, an antioxidant assay using β-carotene combined with lipids, such as linoleic acid, was established. As shown in **Figure 1**, lipids, such as linoleic acid, form a peroxyl radical (LOO$^{\bullet}$) in the presence of ROS and O_2. This peroxyl radical reacts with β-carotene to form a stable β-carotene radical as shown in **Figure 4**; subsequently, the amount of β-carotene reduces in a testing solution (*127*). If an antioxidant is present in a testing solution, it reacts competitively with the peroxyl radical (*128*). Therefore, antioxidant effects are easily monitored by bleaching the color of a test solution with a spectrophotometer at 470 nm, which is the typical absorbance by β-carotene.

Antioxidant activities of various phenolic compounds were determined using the β-carotene bleaching assay (*129*). In this study, linoleic acid was selectively oxidized with lipoxygenase. Among 18 phenolic compounds tested by this method, quercetin exhibited the greatest antioxidant activity, which was confirmed by the DPPH assay. A more comprehensive study of the antioxidant activities of 42 flavonoids was reported using the β-carotene bleaching method, in which linoleic acid was oxidized by heat treatment (*130*). Quercetin also exhibited strong antioxidant activity by this method, and the result was consistent with the one obtained by DPPH assay. In addition to studies on phenolic compounds, this method has been used for antioxidant studies on various plants and their components as shown in **Tables 1** and **2**.

Conjugated Diene Assay. The term conjugated diene is defined as a moiety with two double bonds separated by a single bond. This kind of moiety does not normally occur in unsaturated fatty acids. However, a conjugated diene is readily formed from a moiety with two double bonds separated by a single methylene group, which occurs most commonly in polyunsaturated fatty acids, by the action of ROS and oxygen as shown in **Figure 1** (formation of monohydroperoxide). Once a conjugated diene is formed, it can be monitored spectrophotometrically using its characteristic absorption at 234 nm (*96*). The antioxidant effect of test substances can be evaluated by monitoring the conjugated diene formation. The major drawback of this method is that many biological and natural compounds have significant absorbance around 234 nm, which, consequently, interferes with absorption by a conjugated diene. Therefore, this

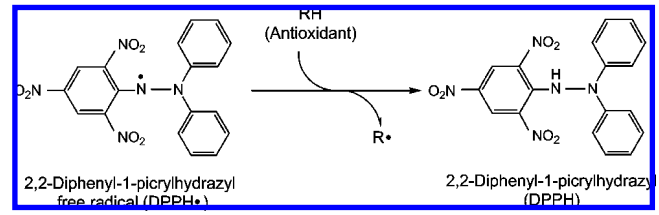

Figure 5. Reaction between DPPH$^{\bullet}$ and antioxidant to form DPPH.

method has not been applied in studies of natural or biological substances as frequently as the β-carotene bleaching assay. However, if a simple fatty acid, such as linoleic acid, is used, this method is useful because of its simplicity. Also, this method can be used for investigation of the early stage of lipid peroxidation.

This assay has been used with various other antioxidant assays (TBA, MA/GC, DPPH, and ABTS) for studies of natural food sources including fruits and vegetables (*131, 132*), herbs and spices (*133*), teas (*60*), and honeys (*134*). The antioxidant activity of an extract of an edible marine red alga was evaluated by conjugated diene assay using linoleic acid and fish oil (*135*). This method tends to be used in combination with a nonlipid system, such as DPPH and ABTS assays.

ASSAYS ASSOCIATED WITH ELECTRON AND RADICAL SCAVENGING

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay. Recently, the DPPH assay has become quite popular in natural antioxidant studies. One of the reasons is that this method is simple and highly sensitive. This assay is based on the theory that a hydrogen donor is an antioxidant. **Figure 5** shows the mechanism by which DPPH$^{\bullet}$ accepts hydrogen from an antioxidant. DPPH$^{\bullet}$ is one of the few stable and commercially available organic nitrogen radicals (*27*). The antioxidant effect is proportional to the disappearance of DPPH$^{\bullet}$ in test samples. Various methods of monitoring the amount of DPPH$^{\bullet}$ in the antioxidant test system have been reported: electron spin resonance spectroscopy (ESR)/plant powders (*136*), NMR/catechins (*137*), and UV spectrophotometry/polyphenols (*129*). However, monitoring DPPH$^{\bullet}$ with a UV spectrometer has become the most widely and commonly used method recently because of its simplicity and accuracy. DPPH$^{\bullet}$ shows a strong absorption maximum at 517 nm (purple). The color turns from purple to yellow followed by the formation of DPPH upon absorption of hydrogen from an antioxidant. This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed. Therefore, the antioxidant effect can be easily evaluated by following the decrease of UV absorption at 517 nm. To standardize the results from various studies, the Trolox equivalent (TE) unit has been used. Trolox is a commercial water-soluble vitamin E. The antioxidant activity of a sample is expressed in terms of micromoles of equivalents of Trolox per 100 g of sample (TE/100 g). Results have also been reported as EC_{50}, which is the amount of antioxidant necessary to decrease the initial DPPH$^{\bullet}$ concentration by 50% (*138*).

This method was introduced as an easy and accurate method for use in fruit and vegetable juice extracts (*139*). Therefore, numerous studies on antioxidants present in plants have been conducted using the DPPH assay, including fruits and vegetables (*39, 52, 140, 141*), medicinal plants (*29, 53, 73, 142*), cereals and beans (*45, 143, 144*), spices and herbs (*145, 146*), and tea and leaves (*57, 147−149*). Some unique studies on the antioxidant activities of alga (*150*) and mushroom (*151*) were also performed using this method.

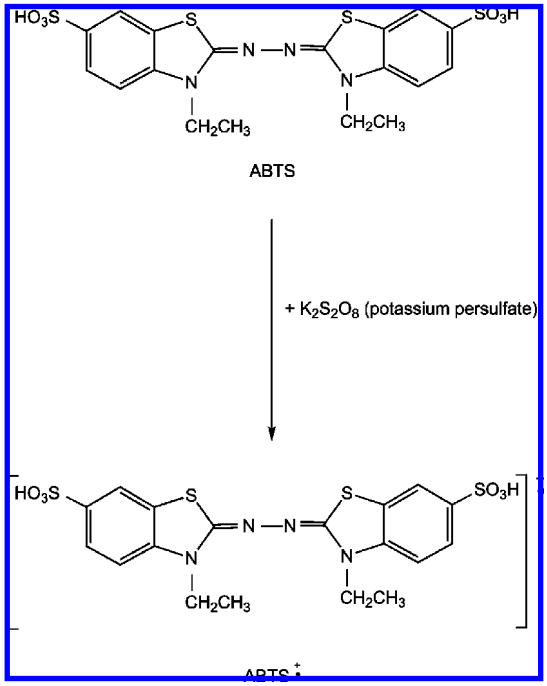

Figure 6. Formation of stable ABTS radical from ABTS with potassium persulfate.

Recently, almost 90% of antioxidant studies use the DPPH assay combined with other assays as shown in **Tables 1** and **2**.

2,2′-Azinobis (3-ethylbenzothiazoline-6 sulfonic acid) (ABTS) Assay. The ABTS assay, which is also called the ABTS radical assay, has been widely used to evaluate antioxidant activities of components in foods and beverages due to its applicability in aqueous and lipid phases (*27*). The original ABTS assay was based on the activation of metmyoglobin by hydrogen peroxide in the presence of ABTS (*152*). In the improved version of this assay, a stable ABTS radical cation, which has a blue-green chromophore absorption, was produced by oxidation of ABTS with potassium persulfate prior to the addition of antioxidants as shown in **Figure 6** (*153*). The antioxidant activity of the natural products, including carotenoids, phenolic compounds, and some plasma antioxidants, is determined by the decolorization of the ABTS, by measuring the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm (*154*). The absorbance of the reaction mixture of ABTS and an antioxidant is compared to that of the Trolox standard, and the results are expressed in terms of Trolox equivalent antioxidant capacity (TEAC) (*153*).

This method has been applied to investigating the antioxidant activities of many natural products as shown in **Tables 1** and **2**, including fruits and vegetables (*56, 155*), medicinal plants (*73, 156*), wines and grapes (*157*), cereals (*158, 159*), beverages (*160, 161*), and essential oils (*162, 163*).

Ferric Reducing/Antioxidant Power (FRAP) Assay. The mechanism associated with this method is shown in **Figure 7**. This method was first developed to quantitate ascorbic acid in serum or plasma (*164, 165*). When a Fe^{3+}−TPTZ complex is reduced to the Fe^{2+} form by an antioxidant under acidic conditions, an intense blue color with absorption maximum develops at 593 nm. Therefore, the antioxidant effect (reducing ability) can be evaluated by monitoring the formation of a Fe^{2+}−TPTZ complex with a spectrophotometer. The FRAP assay gives fast, reproducible results, and the only drawback of this method is that the testing system must be aqueous. Therefore, the reference antioxidant must be a water-soluble

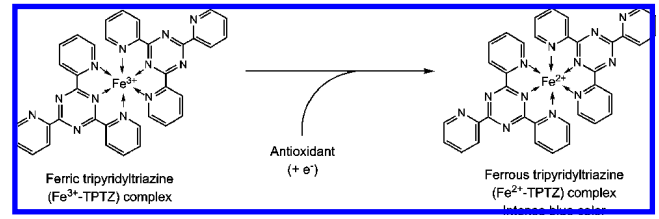

Figure 7. Formation of (Fe^{2+}−TPTZ) complex from (Fe^{3+}−TPTZ) complex by antioxidant.

one, such as ascorbic acid, uric acid, or Trolox. There are only a few reports of antioxidant studies conducted with this method alone. However, many studies on plants and foods have used this method in conjunction with other antioxidant assays as shown in **Tables 1** and **2**.

A recent study using the FRAP assay on extracts from 70 medicinal plants, such as *Melissae folium*, *Spiraea herba*, and *Uvae ursi folium*, reported that the results were consistent with those obtained by DPPH and ABTS assays (*74*). As well as other assays, this assay has been used in many studies including fruits and vegetables (*35, 166−168*), cereal (*169*), essential oil (*170*), beans (*171*), and Maillard reaction products (*172*).

Ferrous Oxidation−Xylenol Orange (FOX) Assay. This assay was originally developed to determine levels of lipid hydroperoxides in biological systems such as plant tissues. The mechanism associated with this method is shown in **Figure 8**. Ferrous ion is oxidized by an oxidant, such as hydroperoxides (refer to **Figure 1**) to form ferric ion, which is subsequently treated with xylenol orange (XO) reagent to give a ferric−XO complex (blue-purple color). This complex has a strong UV absorption at 550 nm. The formation or presence of hydroperoxides in a sample can easily be monitored by a spectrophotometer. Therefore, this method is still used widely to determine hydroperoxides in various biological samples including egg yolk (*173*), lipoxygenase activity in plant extract (*174*), and plant tissue (*175*).

In this assay, a hydroperoxide formed from a lipid (most commonly linoleic acid) oxidizes a ferrous ion to a ferric ion, which can be monitored as a ferric−XO complex at 550 nm. The antioxidant activity of a test substance can be evaluated as an inhibitory effect on hydroperoxide formation or by its ability to donate an electron to ferric ion. There are only a few studies on natural antioxidants using this assay. For example, the role of vitamin C supplement in milk was studied using this assay (*176*). An interesting finding from the study with this assay was a report that cholesterol might act as an antioxidant in lens membranes (*177*).

Ferric Thiocyanate (FTC) Assay. The mechanisms associated with this assay are the same as those of the FOX assay. The difference is that a ferric ion formed by an oxidant from a ferrous ion is monitored as a thiocyanate complex by a spectrophotometer at 500 nm (*178*). **Figure 9** shows the mechanisms of this assay. As in the case of the FOX assay, most studies used linoleic acid as a hydroperoxide source. The inhibitory effect toward oxidation from ferrous ion to ferric ion by antioxidants is evaluated by monitoring the formation of ferric thiocyanate complex. This assay is simple and highly reproducible. The one drawback of this assay is that if any chemical with UV absorption around 500 nm is present, the results are overestimated or not reliable. This is true for any other assays using a spectrophotometer.

This assay has been used to investigate natural antioxidants in combination with other assays, such as the TBA assay for fruit of mengkudu (*106*), with the DPPH assay for teas (*179*), with DPPH and ABTS assays for essential oils (*163*), and with

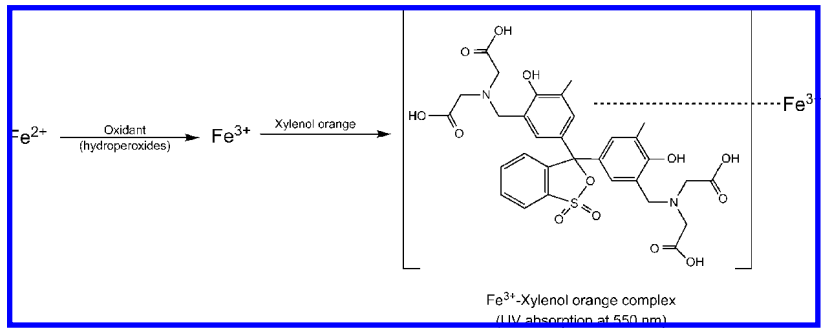

Figure 8. Formation of Fe^{3+}—xylenol orange complex from Fe^{2+} with oxidant followed by xylenol orange treatment.

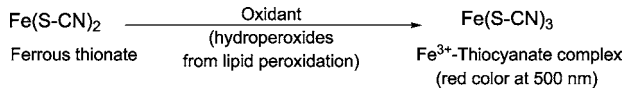

Figure 9. Formation of Fe^{3+}—thiocyanate complex from Fe^{2+}—thiocyanate complex by hydroperoxide.

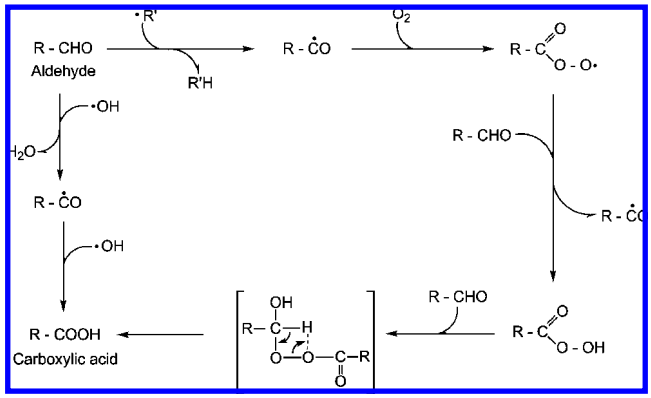

Figure 10. Proposed conversion mechanisms from alkylaldehyde to alkylcarboxylic acid in the presence of reactive radicals.

DPPH, ABTS, and FRAP assays for cauliflower (*180*).

Aldehyde/Carboxylic Acid (ACA) Assay. This assay has been used by only a limited number of researchers (**Table 1**). However, this assay is convenient for evaluating the effects of antioxidants against slow oxidation phenomena occurring over prolonged periods of time, such as the shelf life of foods. **Figure 10** shows the proposed conversion mechanisms from alkylaldehyde to alkylcarboxylic acid in the presence of reactive radicals (*181*). This conversion occurs stoichiometrically, and the reduction of aldehyde or formation of carboxylic acid in a dichloromethane solution is easily monitored by gas chromatography (*182*). A 3% hexanal in dichloromethane solution is most commonly used, and oxidation of hexanal is induced by heat, O_2, or H_2O_2. One drawback of this method is that the test sample must be lipid soluble.

This assay has been used to investigate the antioxidant activities of essences from plants, including beans (*41*), herbs and spices (*133*), essential oils (*84*, *183*), and tea tree oil (*184*) as well as extracts from beverages and foods, such as beer (*68*), teas (*60*), brewed coffee (*66*, *67*), and Maillard reaction products (*185*).

SUMMARY

Studies on antioxidants present in plants and foods have come to be one of the most popular topics in the area of food and agriculture today. Accordingly, many assays for the investigation of antioxidant activity have been developed and applied. The majority of studies have used assays with spectrophotometry, such as TBA, β-carotene bleaching, conjugated diene, DPPH, ABTS, FRAP, FOX, and FTC. However, some spectrophotometry assays have problems with substances exhibiting UV wavelengths similar to that of the test chemical, overall causing interference of the chemical being tested. Therefore, more specific and selective assays, such as MA/HPLC and MA/GC assays, have been developed. Generally, it is recommended to use at least two different types of assays. One is to monitor the early stage of lipid peroxidation, such as β-carotene bleaching, conjugated diene, or FTC, whereas the other is to monitor the final stage of lipid peroxidation, such as TBA, MA/HPLC, or MA/GC. It is generally recognized practice to use two different methods for the investigation of antioxidant activities of samples. Therefore, recent studies on the antioxidant activities of plants and their components have typically used more than two different methods. It is also recommended that a combination of assays for scavenging electron or radical, such as DPPH, ABTS, ACA, or FRAP, and for the assays associated with lipid peroxidations be used.

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Received for review November 11, 2008. Revised manuscript received January 2, 2009. Accepted January 3, 2009.

JF803537K