Food Sci. Biotechnol. Vol. 16, No. 2, pp. 177 ~ 182 (2007)

RESEARCH REVIEW

Food Science and Biotechnology

Evaluation of Different Methods of Antioxidant Measurement

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Abstract The beneficial effects of fruits, vegetables, and beverages on human health have been attributed to their antioxidant activities. Therefore, antioxidant activity of food products is recognized as one of the important parameters in determining their functional values. Until now, antioxidant activity has been measured by various chemical and biological methods; however, many factors confound the reliability and reproducibility of measurements of antioxidant activity of food. *In vitro* methods may provide a useful indication of antioxidant activity but their results may not translate to the human biological system, while *in vivo* tests are difficult to carry out due to the intricate processes of uptake, cellular transportation, and metabolism of individual antioxidant components. Therefore, as long as these limitations exist, our best option is to measure the antioxidant activity in food directly. This review briefly summarizes currently available methods for the measurement of antioxidant activity in food and examines their respective validity.

Keywords: antioxidant, measurement, free radical, antioxidant activity

Introduction

Naturally occurring antioxidative phytochemicals in fruits and vegetables have been known to play a major role in ameliorating oxidative reactions induced by free radicals (1-3), which cause oxidative damage to biomolecules such as DNA, lipids, and protein (4-6). Damaged molecules are associated with increased risk of many human chronic diseases that include various cancer, aging, and cardiovascular diseases (3, 7). Reactive oxygen species (ROS) arise from both endogenous and exogenous process (Table 1). The ROS include superoxide anion, hydrogen peroxide, hydroxyl radical, and peroxyl radical (8). Endogenous ROS are continuously produced as byproducts of energy metabolism, by enzymatic reactions with xanthine oxidase and NO synthase, or by hormonal responses with adrenaline, dopamine, and tetrahydrofolates (9). Exogenous ROS occur during exposure to ultraviolet light or radiation, and from undesirable food components (10, 11) or environmental sources (4, 12). Of note, the ROS excess, that leads to the oxidation of cellular membrane and components. The oxidation of food products can also deteriorate the food quality such as color, taste, flavor, and texture (13-15).

Measurement of antioxidant activity has been a major focus in the kinetic study of oxidation reactions (4, 12). Recently, antioxidant activity has been considered a leading parameter for the determination of the bioactivities of food products and various metabolites in our body. Hence, the current popular methods for measurement of antioxidant activity in foods have developed concurrently with the recent advancement of functional foods. Although there are numerous ways to test antioxidant activity in biological materials and foods (16-19), there is yet a strong demand for methods that are both reliable and reproducible. This article provides a brief overview of the analytical methods of antioxidant activity commonly available for food and food products.

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Received November 27, 2006; accepted March 2, 2007

Antioxidants and antioxidant activity

An antioxidant can be defined as a compound that, when present even in low concentrations, delays or inhibits oxidation of the substrate (20, 21). Therefore, some antioxidants (Table 2) are known to play a defense role in the human body where excessive quantities of ROS lead to cellular dysfunction, or in foods where quality loss is taking place (11, 20, 22). Many polyphenolics, including phenolic acid and flavonoids, might exert antioxidant activity in biological and food systems by inhibiting or scavenging ROS (23, 24). The original mechanism of autocatalytic oxidation that was suggested by Farmer *et al.* (25) includes three primary steps :

1. Initiation

$$RH + O_2 \rightarrow R^{\cdot} + {}^{\cdot}OOH \quad (1)$$

Table 1. Reactive oxygen species

Oxygen radicals	Non-radical reactive oxygen
Oxygen, $O_2^{\cdot}$	Hydrogen peroxide, HOOH
Superoxide anion, $O_2^{\cdot-}$	Hydroperoxyde, ROOH
Hydroxyl radial, $OH^{\cdot}$	Hypochlorous acid, HOCl
Perhydroxyl radial, $HOO^{\cdot}$	Hypobromous acid, HOBr
Peroxyl radical, $RO_2^{\cdot}$	Singlet oxygen, $^1O_2$
Alkoxyl radical, $RO^{\cdot}$	Ozone, O^3
Nitric oxide, $NO^{\cdot}$	Nitrous acid, HNO_2
Nitrogen dioxide, $NO_2^{\cdot}$	Peroxynitrite, $ONOO^{\cdot}$

where, RH represents the substrate molecule: for example, a lipid, and R$^{\bullet}$ as the initial oxidized radical (equation 1). The oxidation of the lipid generates a reactive allyl radical (R$^{\bullet}$) that can react with oxygen to form a peroxyl radial (ROO$^{\bullet}$) (26)

2. Propagation

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (2)$$
$$ROO^{\bullet} + R_1H \rightarrow ROOH + R_1^{\bullet} \quad (3)$$

The peroxyl radicals are the chain carriers of the reaction that can further react with hydroperoxides (ROOH) (equation 2 and 3). In turn, ROOH break down to a wide range of compounds, including aldehydes, alkyl formates, alkoxyl radical (RO$^{\bullet}$), and radicals (R$^{\bullet}$) (27)

3. Termination

$$R^{\bullet} + R^{\bullet}\,R \rightarrow R \quad (4)$$
$$R^{\bullet} + ROO^{\bullet} \rightarrow ROOR \quad (5)$$
$$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + O_2 \quad (6)$$

Termination reaction refers to the combination of radicals and antioxidants to form non-radical products (equation 4, 5, and 6).

Antioxidants have been divided into primary and secondary groups (28). The primary antioxidant group, or the single oxygen quenching group, AH, present in low concentrations, may delay or inhibit the initiation step by reacting with peroxyl or alkoxyl radicals (9). The secondary or preventive antioxidant group retards the rate of oxidation. This might be accomplished in a number of singlet oxygen quenching reactions (29). The primary antioxidant group includes natural compounds such as phenolic acid, and flavonoids or synthetic compounds, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (6). The secondary antioxidant group often broadly includes metal chelators such as ethylenediaminetetraacetic acid (EDTA) and antioxidant enzyme cofactors including Se and coenzyme Q_{10}. (30)

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet} \quad (7)$$
$$R^{\bullet} + AH \rightarrow RH + A^{\bullet} \quad (8)$$
$$RO^{\bullet} + AH \rightarrow ROH + A^{\bullet} \quad (9)$$

The antioxidant free radical (A$^{\bullet}$) may interrupt the chain propagation step (equation 10 and 11) by forming peroxyl antioxidant compounds (4).

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet} \quad (10)$$
$$A^{\bullet} + ROO^{\bullet} \rightarrow ROOA \quad (11)$$

Common assays to evaluate the antioxidant measurement

1. 2-2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical method

The ABTS test has been popularized for the determination of antioxidant activity. This assay was first suggested by Miller *et al.* (31) to test biological samples and has recently been applied to food products and plant extracts. ABTS is oxidized to the colored nitrogen-centered radical cation, ABTS$^{\bullet+}$, which has an absorption maximum at 600-734 nm and can be easily determined by a spectrophotometer. The original ABTS method was based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce the radical cation (9, 32). An improvement on this method is the decolorization technique, in that the radical is generated directly in a stable form prior to its reaction with antioxidants. Stable ABTS$^{\bullet+}$ reacts actively with the hydrogen donor such as phenolics and then is converted to a non-colored form of ABTS (31, 33).

Results of this assay can be expressed by comparison with standard amounts of the antioxidants, such as Trolox and ascorbic acid. The Trolox equivalent antioxidant capacity (TEAC) is equal to the millimole concentration of a Trolox solution that contains the antioxidant capacity equivalent to a 1.0 mM solution of the substance (34). The TEAC reflects the relative ability of hydrogen or electron donation antioxidants to scavenge the ABTS radical cation compared with that of Trolox (31). The TEAC value characterizes the capability of the tested sample to react with ABTS$^{\bullet+}$ rather than to inhibit the oxidative process. Many antioxidants, such as phenolics, ascorbic acid, and carotene, react with ABTS$^{\bullet+}$ at different rates (35). Thus, the result of TEAC is expected to be dependent on the time of incubation as well as on the ratio of sample quantity to ABTS$^{\bullet+}$ concentration.

Kim *et al.* (36) suggested using vitamin C as a reference antioxidant instead of Trolox in the ABTS test. Their argument is that Trolox is not a natural compound found in foods (37), while vitamin C is a natural nutrient that has a strong antioxidant capacity in our daily diet. Therefore, the assay of antioxidant capacity using the ABTS assay expressed as vitamin C mg/100 g equivalent (VCEAC) has merit.

2. 2, 2-Diphenyl-1-picryl-hydrazyl (DPPH) free radical method

The stable free radical DPPH$^{\bullet}$ reacts with hydrogen donors absorbs at 515 nm (38). It can be easily determined by the UV-Vis spectroscopy. Recently, several researchers suggested two different protocols; the first one determines the rate of DPPH decay observed after the addition of the antioxidant sample (19, 38) and the second one measures the amount of DPPH scavenged by a tested sample. Antioxidant activity can be determined by monitoring the decrease in the absorbance. Results are reported as the EC_{50}: that is, the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% (38-40). Sanchez-Moreno *et al.* (41) suggested using the combination of kinetic and static approaches to characterize the antiradical efficiency. The time taken to reach the steady state to EC_{50} concentration (T_{EC50}) was suggested.

This DPPH assay is simple and inexpensive to use and also demonstrates good repeatability. However, the color interference from the samples that have pigments such as anthocyanins may lead to underestimation of antioxidant activity (19). Because anthocyanins' maximum absorbance is at 475-485 nm, it interferes easily with the DPPH chromogen, which has an absorbance at 515 nm (19, 41).

3. Oxygen radical absorbance capacity (ORAC) method

The ORAC method is designed to determine the loss of

Table 2. Important antioxidant in fruits and vegetables[1)]

Compound	Typical food
Vitamin C	Vegetables, fruits
Vitamin E	Grains, nuts, oils
β-Carotene and lycopene	Vegetables, tomatoes
Xanthophylls	Citrus fruits, sea foods, red peppers
Flavonols	Vegetables, fruits, berries
Flavones	Vegetables, citrus fruits
Flavanones	Citrus fruits
Anthocyanidins	Berries, colored fruits
Catechins	Tea, wines

[1)]Extensive literature on the antioxidants may be found in Rice-Evans *et al.* (1), Herrmann (56), and Miller *et al.* (57).

fluorescein (3',6'-dihydrosyspiro[isobenzofuran-1[3*H*], 9'[9*H*]-xan-then]-3-one) by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) as a tool for antioxidant assessment (42, 43). The ORAC procedure uses AAPH as a peroxyl radical source, which is relevant to biological systems because the peroxyl radical is the most abundant free radical (44). The fluorescence decay of fluorescein refers to the extent of its transfer reaction with the peroxyl radical (45). The protective effect against free radicals by individual antioxidant or food products is determined by assessing the area under the decay curve of the sample compared to that of the blank in which no antioxidant is present (43). Antioxidant activities of some beverages and fruits were reported as the ORAC in micromoles of Trolox equivalents (46-48).

4. Ferric reducing antioxidant power (FRAP) method

The FRAP assay is based on the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) by antioxidant compounds (49). This method involves a single electron reaction between Fe^{3+} and a single electron donor antioxidant (43). Usually, this assay cannot be used with a biological sample. Most of samples having intense color may interfere in the test. The FRAP assay depends on the reduction of Fe^{3+} by an antioxidant at a low pH of 3.6 (43). Therefore, the FRAP assay actually determines the reducing potential based on the ferric ion instead of the antioxidant activity. As such, it may not qualify as a method that directly measures the total antioxidant activity in food. Nonetheless, the FRAP assay has the benefits of being relatively simple and inexpensive.

Comparison of various methods for antioxidant activity

Some of the most common methods utilized for determining antioxidant activity in foods have been described thus far. However, there is substantial disagreement among scientists who arrive at differing results on the same commodity of food. The main reason for this is that different investigators measure different properties of the antioxidants. There is currently no gold standard method with respect to antioxidant determination, because each method has its own advantages and disadvantages (Table 3). Another reason for the discrepancy in published data is the disagreement among scientists in expression of antioxidant units as indicated (Table 4). Since food is a complex mixture of various compounds, it is impossible to express total antioxidant capacity by a single method measuring one specific antioxidant. In addition, the absolute values of antioxidant activity reported vary from one study to another, even when the same test is used (50).

Table 3. The properties of the different methods to determine for antioxidant activity *in vitro*[1)]

Method	Advantages	Disadvantages
ABTS[2)]	Applicable to both aqueous and organic phase, fast reaction	Very sensitive with temperature and light
	Using standard as vitamin C, hence can be expressed on a weight basis as natural source	Extra step to thermally generate free radical from ABTS salt
	Stable to pH hence can be used to study pH effect on activity	Not standardized, hence hard to compare values across laboratories
DPPH[3)]	Simple to use and inexpensive	Dissolved in organic solvents
	Reaching steady state quickly	Sensitive pH and slow reaction
	Good repeatability	Color interference causing underestimation of antioxidant activity
ORAC[4)]	To uses biologically relevant free radicals	Using standard as Trolox, hence can not be expressed on a weight
	To integrate both degree and time of antioxidant reaction	basis as natural source
	Various free radical generators can be used	
FRAP [5)]	Being expressed in ascorbic acid equivalents	Oxygen electrode, which may not maintain stable during measurement
	Easy and inexpensive	SH-group containing antioxidant are not detected

[1)]Extensive literature on the methods mentioned may be found in Roginsky and Alegria (58), and Kim and Lee (20).
[2)]ABTS stands for 2-2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid).
[3)]DPPH stands for 2, 2-diphenyl-1-picrylhydrazyl.
[4)]ORAC stands for oxygen radical absorbance capacity.
[5)]FRAP stands for ferric reducing antioxidant power.

Table 4. Various expression of antioxidant activity evaluated by different assays[1)]

Method	Expression of antioxidant activity
ABTS[2)]	TEAC (mM Trolox equivalent to 1 mM test substrate)
	VCEAC (mg vitamin C equivalent of 100 mg sample weight)
DPPH[3)]	Percentage inhibition
	EC_{50}, concentration to decrease concentration of test radical by 50%
	T_{EC50}, time to decrease concentration of test free radical by 50%
ORAC[4)]	μmol of Trolox equivalent of 1 g sample
FRAP[5)]	Absorbance of Fe^{2+} at 593 nm produced by antioxidant reduction of corresponding tripyridyltriazine Fe^{3+} complex

[1)]Extensive literature on the expressions mentioned may be found in Cao *et al.* (46), van den Berg *et al.* (37), and Re *et al.* (32).
[2)]ABTS stands for 2-2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid).
[3)]DPPH stands for 2, 2-diphenyl-1-picrylhydrazyl.
[4)]ORAC stands for oxygen radical absorbance capacity.
[5)]FRAP stands for ferric reducing antioxidant power.

As shown in Table 5, antioxidant activity in some vegetables collected from the previous reports, are expressed in various units, which leads to difficult comparison of that of the same (Table 5).

Among several methods described above, assays using ABTS and DPPH have shown to be more popular and easier to use (51-55). The ABTS test has the extra flexibility and can be used at a wide range of pHs and is thus more attractive when measuring different foods in different pH range (10). In contrast, DPPH is sensitive to pH and slow in reaction time (38). In addition, ABTS is soluble not only in hydrophilic solvents but also in hydrophobic solvents, and can therefore be used widely. However, major disadvantage in assay using ABTS radicals are that the radical is very sensitive with temperature and light and that the extra step is required to generate free radicals from ABTS salt (33).

Table 5. Level of antioxidant activity in some vegetables measured on various assays using different unit expression

Vegetables	VCEAC[1)] (mg VCE/g)	ORAC[2)] (μmol TE/g)	FRAP[3)] (μmol TE/g)
Broccoli	0.30±1.24	126±42	41±11
Carrots	0.11±0.44	60±15	3160±7
Cabbage	0.58±2.52	61±21	39±17
Cauliflower	0.16±0.43	102±28	61±12
Green peeper	0.52±1.08	154±60	157±58
Onion	0.21±0.09	85±23	17±4
Radish	0.29±0.64	115±36	86±29
Snap beans	0.01±0.04	79±37	20±13
Spinach	0.35±2.13	152±26	64±13
Tomatoes	0.29±1.60	67±13	56±8

[1)]VCEAC stands for vitamin C equivalent antioxidant capacity. The results are expressed as mg vitamin C equivalent per g based on the weight (mg VCE/g). Extensive literature on the expressions mentioned may be found in Chun *et al.* (59).
[2)]ORAC stands for the oxygen radical absorbance capacity. The results are expressed as μmol Trolox equivalent per g based on the weight (μmol TE/g). Extensive literature on the expressions mentioned may be found in Ou *et al.* (43).
[3)]FRAP stands for ferric reducing antioxidant power. The results are expressed as μmol Trolox equivalent per gram based on the weight (μmol TE/g). Extensive literature on the expressions mentioned may be found in Ou *et al.* (44).

ORAC and FRAC are based on either single electron transfer reaction or a hydrogen atom transfer reaction between an oxidant and free radical. The ORAC value represents the peroxyl radical (ROO·) scavenging activity (41). The major advantage of ORAC is that both inhibition time and the degree of inhibition (46), whereas other methods use either the inhibition time at a fixed degree or the inhibition degree at a fixed time. The ABTS, DPPH, and FRAC methods can provide the most rapid results and requires inexpensive equipment. Since ORAC method uses Trolox as a standard, it is difficult to relate directly to common natural foods as opposed to the vitamin C equivalent antioxidant capacity (VCEAC) expressed by vitamin C mg/100 g equivalent.

The FRAC assay measures only the Fe^{3+} reducing ability with no direct free radical's involvement, therefore, it is different from the ABTS, DPPH, and ORAC assays. As mentioned, the antioxidant activity against free radicals does not necessarily match its ability to reduce Fe^{3+} to Fe^{2+} (42).

Conclusion

Common assay methods of antioxidant activity in foods have been discussed and compared for their validity. Among them, the method that uses ABTS as a target radical and vitamin C as a standard appears to be the one applicable to a wide range of foods and beverages. In order to measure antioxidant activities accurately, one must consider many variables associated with the targeted free radicals and variable reaction mechanisms on individual antioxidants. It is recommend that various additional factors associated with the reactions, such as extraction solvents, temperature, light, and time, be considered in order to have a reliable result of antioxidant measurement and more than two antioxidant assays be used to evaluate antioxidant activity expressed as the same unit for its results.

Acknowledgments

This research was supported by the Korea Research

Foundation Grant funded by the Korean Government (MOEHRD), (KRF-2006-F00025).

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