1 Nomenclature and general classification of antioxidant activity/capacity assays

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1.1 Introduction

In the last three decades, significant changes have been made to the definition of "antioxidants." These changes have largely reflected the tremendous advances in food science, nutrition, and molecular and cell biology. Antioxidants are no longer mere chemical substances that make a food last longer or phytochemicals such as polyphenols and carotenoids that show stronger antioxidant activity/capacity (AOA/TAC) than vitamin C or E in a chemical reaction. Antioxidants were broadly defined as "any substance that, when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate" (Halliwell & Gutteridge 1995) in 1995, but later the word "oxidation" was altered to "oxidative damage" that suggests an *in vivo* biological process: "any substance that delays, prevents or removes oxidative damage to a target molecule"(Halliwell 2007). Most recently, Apak et al. (2016a) gave a more specific definition: "natural or synthetic substances that may prevent or delay oxidative cell damage caused by physiological oxidants having distinctly positive reduction potentials, covering reactive oxygen species (ROS)/reactive nitrogen species (RNS) and free radicals (i.e. unstable molecules or ions having unpaired electrons)." These definitions demonstrate the roles of antioxidants at cellular levels in humans as they are related to oxidative stress and free radicals and further to potential health effects in humans.

Oxidative stress (OS), defined as the imbalance between prooxidants and antioxidants, is characterized by the inability of endogenous antioxidants to counteract the oxidative damage on tissues and organisms owing to overproduction of cellular ROS/RNS that are highly reactive and can cause oxidative modification of biological macromolecules, such as lipid, protein, and DNA, leading to tissue injury, accelerated cellular death (Trevisan et al. 2001), and various diseases such as atherosclerosis, diabetes mellitus, chronic inflammation, neurodegenerative disorders, cardiovascular disease, Alzheimer's disease (Smith et al. 2000), mild cognitive impairment (Guidi et al. 2006), Parkinson's disease (Bolton et al. 2000), and certain types of cancer. OS from ROS/RNS is important in the etiology of these chronic diseases. Abundant evidence suggests that antioxidants play a pivotal role in the

Measurement of Antioxidant Activity & Capacity: Recent Trends and Applications, First Edition.
Edited by Resat Apak, Esra Capanoglu, and Fereidoon Shahidi.
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maintenance of human health and prevention and treatment of these diseases because of their ability to reduce OS. Measuring the AOA/TAC of foods and biological samples is therefore not only crucial for assuring the quality of functional foods and nutraceuticals, but more importantly for efficacy of dietary antioxidants in the protection and treatment of oxidative stress-related diseases.

Many AOA/TAC assays have been developed over the years, based on different chemical, physicochemical, and biochemical mechanisms. While the mechanisms of some assays are clearly understood, some are complex systems with multiple modes of action. Several attempts have been made to categorize the various AOA/TAC assays (López-Alarcón & Denicola 2013; Niki 2010), but thus far there is no unified and standardized system for the nomenclature and classification of these assays. This chapter intends to find a way to reconcile the different views and provides a relatively simplified approach to the nomenclature and general classification of various AOA/TAC assays currently in use for the assessment of AOA/TAC in diets and biological fluids.

1.2 Nomenclature of antioxidant activity/capacity assays

The concept of AOA/TAC may be traced back to its origin in chemistry and then its applications in food science, in biology and medicine, and in nutrition and epidemiology. Many terms have been used for this concept over the years, including antioxidant activity (Rice-Evans et al. 1995), antioxidant capacity (Sies 1999), antioxidant power (Benzie & Strain 1996), and antioxidant potential (Jovanovic et al. 1995), to mean almost the same thing – the ability of a compound or a mixture of compounds to prevent or stop oxidative reactions occurring to another molecule. Other terms such as total antioxidant performance (Hollman et al. 2011), antioxidant effect (Talegawkar et al. 2009) and antioxidant status (Bouanane et al. 2009) have also been used, albeit relatively less widely.

Meanwhile, regardless of these terminologies, even more names have been given to the assay methods used to measure antioxidant activity or similar terms crowned with the word "total". Because these AOA/TAC assays have their origin in chemistry, the majority of the currently used methods are seriously limited in that they preclude meaningful application to *in vivo* conditions, so critical appraisal is needed to reassess the inherent flaws in the nomenclature and classification of these assays (Sies 2007). Also, due to the large number of different assay methods, comparison of different foods or the physiological effects of different foods can be very challenging, and often one compares apples with oranges. A systematic approach to this is critically important to the food, functional food and nutraceutical, and dietary supplement industries, and to better understanding of the relationship between diet and chronic diseases. Most of the current AOA/TAC assays are named based on the reactants, the reaction mechanism and/or the corresponding techniques, and these are summarized in Table 1.1.

1.3 Classification of antioxidant activity/capacity assays

In terms of classification of AOA/TAC assays, Niki (2010) classified the antioxidant assays into *in vitro* and *in vivo* according to their applications, while Sahu and Saxena (2013) proposed two systems based on the mechanisms of action: hydrogen atom transfer (HAT)- and

Table 1.1 Nomenclature of antioxidant activity/capacity assays.

Name of assay	Abbreviations
Hydrogen atom transfer-based assays	HAT-based assays
Oxygen radical absorbance capacity assays	ORAC assays
Total radical-trapping antioxidant parameter assay	TRAP assay
Total oxyradical scavenging capacity assay	TOSC assay
Crocin bleaching assay	–
Single electron transfer-based assays	SET-based assays
Ferric reducing antioxidant power FRAP assay	FRAP assay
Cupric reducing antioxidant capacity assay	CUPRAC assay
Ferricyanide-Prussian blue assay	–
Ce(IV) reducing antioxidant capacity assay	CERAC assay
Cr(VI) reducing antioxidant capacity assay	CHROMAC assay
Acidic potassium permanganate chemiluminescence	–
Cyclic voltametry-based assay	CV based assay
Differential pulse-based assay	DPV based assay
Square-wave voltametry-based assay	SWV based assay
Dropping mercury electrode-based assay	DME based assay
Silver nanoparticles-based assay	SNPAC-based assay
Gold nanoparticles-based assay	AuNPs-based assay
2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)/Trolox-equivalent antioxidant capacity assay	ABTS/TEAC assay
2,2-diphenyl-1-picrylhydrazyl radical scavenging assay	DPPH radical scavenging assay
N,N-dimethyl-p-phenylenediamine dihydrochloride radical scavenging assay	DMPD radical scavenging assay
Galvinoxyl radical scavenging assay	–
(2,6-di-tert-butyl-4-(4′-methoxyphenyl) phenoxyl radical) scavenging assay	–
Luminol-based chemiluminescence	–
Nitroblue tetrazolium-based chemiluminescence	NBT-based chemiluminescence
Electron spin resonance (ESR) spin trapping method	(ESR) spin trapping method
Hydrogen peroxide scavenging assay	–
Hydroxyl radical scavenging assay	–
Hypochlorous acid scavenging assay	–
Singlet oxygen scavenging assay	–
Nitric oxide radical scavenging assay	–
Peroxynitrite anion scavenging assay	–
Peroxyl radical scavenging assay	–
β-Carotene bleaching assay	–
Iodometric hydroperoxide measurement	–
Ferric thiocyanate and ferric xylenol orange assays	–
Ultraviolet measurement of conjugated dienes	–
Thiobarbituric acid reactive substances assay	TBARS assay
Electrophoretic mobility shift assays	–
Real-time PCR (RT-PCR) method	RT-PCR method
Western blot method	–
Enzyme-linked immunosorbent assay	ELISA
High performance liquid chromatography	HPLC
Gas chromatography	GC
Mass spectrometric methods	MS
Electrochemiluminescence	–
Chemometric methods	–

–, no abbreviations are commonly used.

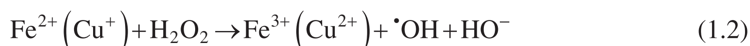
single electron transfer (SET)-based assays. López-Alarcón and Denicola (2013) further divided the *in vitro* assays into chemical- and cell-based assays. Most of these classifications tend to be limited and overly simplified, but several excellent comprehensive reviews on the classification of AOA/TAC assays have been published recently, and readers are referred to those for detailed information. (Apak et al. 2016a,b,c; Shahidi & Zhong 2015). Shahidi and Zhong (2015) suggested that the AOA/TAC assays can be grouped into two categories: "measuring of the current state of oxidation in model systems" and "radical scavenging assays," while Apak et al. (2016a,b,c) suggested a system based on mechanisms of antioxidant actions and technologies. This contribution therefore intends to serve as a brief summary of these recent proposals.

Antioxidants have traditionally been divided into two classes: primary or chain-breaking antioxidants and secondary or preventive antioxidants (Madhavi et al. 1995). Thus, an antioxidant may act directly by scavenging reactive species or inhibiting their generation or may also act indirectly by upregulating endogenous antioxidant defenses (Halliwell 1995; Halliwell et al. 1995). Direct antioxidant actions occur through various mechanisms such as ROS/RNS scavenging activities, HAT, SET, reducing power, metal chelation and preventing lipid peroxidation. The chain-breaking mechanisms are represented by:

$$FR^{\bullet} + AH \rightarrow FRH + A^{\bullet} \quad (1.1)$$

where $FR^{\bullet}$, AH, FRH, and $A^{\bullet}$ stand for free radicals, antioxidants, lipids/proteins/DNA molecules, and antioxidant radicals, respectively. Reaction (1) is considered the basis of the classic mechanism of action of antioxidants and indicates that they can transfer either a hydrogen atom ($H^{\bullet}$) and an electron (e^-) or both to inhibit the radical initiation or propagation steps. Thus, these non-enzymatic chain-breaking AOA/TAC assays are commonly classified as HAT- and SET-based assays for measuring ROS/RNS scavenging activity. AOA measurement methods, such as oxygen radical absorbance capacity (ORAC), total peroxyl radical trapping antioxidant parameter (TRAP), total oxyradical scavenging capacity (TOSC), and crocin bleaching, are usually competitive HAT-based assays, whereas TAC assays are usually non-competitive and work on SET and mixed-mode (SET/HAT) mechanisms (Apak et al. 2016a). The reducing power of the antioxidants, an important indicator of their antioxidant activities, is SET-based.

Secondary (or preventive) antioxidants retard or prevent lipid oxidation. Redox active metals, such as iron (Fe) and copper (Cu), undergo redox cycling reactions, generate reactive radicals (such as superoxide radical anion and nitric oxide) in biological systems (Finley et al. 2011), and play important roles in many physiological functions. Meanwhile, chelator antioxidants can inhibit Fenton-type reactions by sequestering transition metal ions, such as Fe^{2+} and Cu^+, through the reaction:



$$Fe^{2+}\left(Cu^{+}\right) + H_2O_2 \rightarrow Fe^{3+}\left(Cu^{2+}\right) + {}^{\bullet}OH + HO^{-} \quad (1.2)$$

Essentially, a preventive antioxidant operates through a neutralization reaction between an antioxidant (Lewis base) and a metal ion (Lewis acid), without involving the donation of hydrogen atoms ($H^{\bullet}$) or electrons (e^-) by the antioxidant (Apak et al. 2016a). Thus, these preventive antioxidant assays measure transition metal ion chelating ability.

On the other hand, physiological antioxidant actions in a biological system are not only about scavenging free radicals but also include upregulation of detoxifying enzymes and

antioxidant defense, modulation of gene expression and redox cell signaling at the cellular level. Bioassays based on cell culture and analyses of biomarkers arising from the redox control system and cell signal transcription factors are an important and emerging new class of AOA/TAC assays.

An integrated approach to the existing complex classification systems has been taken in this chapter and as a result, the existing AOA/TAC assays are classified into the following five categories: (1) HAT-based assays; (2) SET-based assays; (3) mixed-mode (HAT/SET) assays; (4) *in vivo* antioxidant activity/capacity assays; and (5) miscellaneous methods. This classification is summarized in Table 1.2, and a brief explanation of these assays is given below.

1.3.1 Hydrogen atom transfer-based assays

Hydrogen atom transfer-based assays measure the capability of an antioxidant to quench free radicals by donating a hydrogen atom (H). The HAT mechanisms of antioxidant action can be demonstrated in the following reaction in which the hydrogen atom (H) of a phenol (ArOH) is transferred to a peroxyl radical:

$$ROO^{\bullet} + AH/ArOH \leftrightarrow ROOH + A^{\bullet}/ArO^{\bullet} \quad (1.3)$$

where the aryloxyl radical ($ArO^{\bullet}$) formed from the reaction of phenol (ArOH, an antioxidant) with a peroxyl radical is stabilized by resonance. AH denotes the protected biomolecules. An effective phenolic antioxidant must react faster than biomolecules (protected molecule) with free radicals to have a protective effect against oxidation of the latter (Apak et al. 2007). HAT-based antioxidant assays normally use a fluorescent probe which can also react $ROO^{\bullet}$ like the antioxidants, therefore the antioxidant activity can be determined from competition kinetics by measuring the fluorescence decay curve of the probe in the absence and presence of an antioxidant, that is, by integrating the area under these curves (Cao et al. 1995; Huang et al. 2005). Typical examples of HAT-based assays include the ORAC, TRAP, and TOSC assays and crocin bleaching test (Demirci Çekiç et al. 2013; Huang et al. 2005).

1.3.1.1 *ORAC assay*

The ORAC assay measures the radical chain-breaking AOA by monitoring the inhibition of oxidation by peroxyl radical, which is evaluated from the area under the curve (AUC) of the kinetic profiles of the target molecule (TM) consumption. This assay originally used 2,2′ azobis (2-methylpropionamidine) dihydrochloride (AAPH) as a free radical generator and β-phycoerythrin as TM, but the latter has been mostly replaced by fluorescein in recent years. The new ORAC assay also uses trolox (a hydrosoluble vitamin E analog) as a standard, and ORAC value as AOA is expressed in trolox equivalent (TE) (Cao & Prior 1999; Cao et al. 1993).

1.3.1.2 *TRAP assay*

The TRAP assay is based on the ability of antioxidants to inhibit the reaction between peroxyl radicals and a target probe, which was originally the consumption of O_2 (as probe) in the peroxidation process induced by thermal decomposition of ABAP (2,2′-azobis(2-amidinopropane)

Table 1.2 General classifications of antioxidant activity/capacity assays

Classifications			Assays	References
Chemical-based antioxidant activity/ capacity assays	HAT-based assays		ORAC assay	Apak et al. 2016b; Cao & Prior 1999; Cao et al. 1993
			TRAP assay	Apak et al. 2016b; Wayner et al. 1985
			TOSC assay	Apak et al. 2016b; Regoli & Winston 1999
			Crocin bleaching assay	Apak et al. 2016b; Bors et al. 1998
	SET-based assays	Spectroscopic methods	Folin-Ciocalteu assay	Apak et al. 2016a; Singleton et al. 1999
			FRAP assay	Apak et al. 2016a; Benzie & Strain 1996
			CUPRAC assay	Apak et al. 2016a; Özyürek et al. 2007, 2009, 2010; Bektaşoğlu et al. 2006; Çelik et al. 2010
			Ferricyanide-Prussian blue assay	Apak et al. 2016a; Gorinstein et al. 2006
			Assays involving strongly oxidizing reagents (Ce(IV), Cr(VI), and Mn(VII) assays)	Apak et al. 2016a; Ozyurt et al. 2007; Işık et al. 2013; Popović et al. 2012
		Electrochemical methods	Cyclic voltammetry (CV) based assay Differential pulse (DPV) based assay Square-wave voltammetry (SWV) based assay Dropping mercury electrode (DME) based assay	Apak et al. 2016a; Blasco et al. 2007; Novak et al. 2009; Magarelli et al. 2013; Bartosz 2003, 2006; Gorjanović et al. 2010, 2012
		Nanotechnological methods	SNPAC based assay AuNPs-based assay	Apak et al. 2016a
	Mixed-mode (SET/HAT) assays		ABTS/TEAC assay	Apak et al. 2016b
			DPPH radical scavenging assay	Apak et al. 2016b; Papariello & Janish 1966; Brand-Williams et al. 1995; Gómez-Alonso et al. 2003; Hogg et al. 1961; Plank et al. 2012
			DMPD radical scavenging assay	Apak et al. 2016b; Beltran et al. 2009; Corral-Aguayo et al. 2008
			Galvinoxyl radical scavenging assay [2,6-di-tert-butyl-4-(4′-methoxyphenyl) phenoxyl radical] scavenging assay	Apak et al. 2016b; Nagaoka et al. 2013
In vivo antioxidant activitiy assays	Cellular antioxidant activity (CAA) assay		Cellular antioxidant activity (CAA) assay with different cell lines	Apak et al. 2016c; Wolfe & Liu 2007; Peng et al. 2016; Zhang et al. 2016
	Biomarkers of oxidative stress and related methods		ROS and RNS-superoxide anion radical scavenging assay; luminol-based chemiluminescence; nitroblue tetrazolium (NBT)-based chemiluminescence; electron spin resonance (ESR) spin trapping method); hydrogen peroxide scavenging assay; hydroxyl radical scavenging assay; hypochlorous acid scavenging assay; singlet oxygen scavenging assay; nitric oxide radical scavenging assay; peroxynitrite anion scavenging assay; peroxyl radical scavenging assay	Apak et al. 2016c; Özyürek et al. 2010; Bourdon & Blache 2001; White & Bursey 1964; Pou et al. 1989; Li 2013; von Kruedener et al. 1995; Martinez et al. 2006; Kumaran & Karunakaran 2006; Kooy et al. 1994; Chandna et al. 2012

		Lipid peroxidation products Malondialdehyde (MDA); thiobarbituric acid reactive substances (TBARS); 4-hydroxynonenal (4-HNE); F2-isoprostanes (8-iso-PGF2); lipid hydroperoxidation - Hexanoyl - Lys adduct (HEL); oxidized low-density lipoprotein; hydrocarbons (ethane, pentane); carbonyl-crotonaldehyde (CRA); F4-neuroprostanes (F4-NPs) β-carotene bleaching assay; iodometric hydroperoxide measurement; ferric thiocyanate and ferric xylenol orange assays; ultraviolet measurement of conjugated dienes; colorometric assay; fluorometric assay; ELISA; HPLC; GC- MS	Apak et al. 2016b; Shah et al. 2014; Niki & Yoshida 2005; Marco 1968; Lea 1931; Sattler et al. 1994; Sinnhuber & Yu 1958; Gutteridge & Halliwell 1990; Jiang et al. 1992; Musiek et al. 2005; Bevan et al. 2003; Santilli et al. 2015a
		Nucleic acid oxidation products 8-Hydroxy-2′deoxyguanosine (8-OHdG); DNA break - comet assay, flow cytometry; 8-hydroxyadenine (8-OHA); 2,6-diamino- 4-hydroxy-5-formamidopyrimidine (Fapy-guanine); 4,6-diamino-5-formamidopyrimidine (Fapy-adenine); 5-hydroxycytosine (5-OHC); 5-hydroxyuracil (5-OHU); hymine glycol; 8-hydroxyguanosine (8-OHG); miRNA - HPLC, GC-MS; LC-MS; ELISA; RT-PCR	Santilli et al. 2015a
		Protein oxidation products Carbonyl; 3-nitrotyrosine; protein thiol; glutamic semialdehyde; aminoadipicsemialdehyde; carboxyethyl-lysine; carboxymethyl-lysine - colorometric, ELISA, HPLC, GC-MS	Shah et al. 2014; Chang et al. 2014; Sultana & Allan 2013
		Carbohydrate oxidation products (AGEs; RAGE)	Sultana & Allan 2013; Santilli et al. 2015b
		Antioxidant enzymes Superoxide dismutase(SOD), glutathione peroxidase (GSH), catalase (CAT), glutathione reductase (GSH-RX), xanthine oxidase - colorometric, fluorometric, ELISA, HPLC	Halliwell 1991; Sies 1991; Lozovoy et al. 2013; Zitka et al. 2012; Townsend et al. 2003; Bae et al. 2002; Agouni et al. 2009; Silva et al. 2011; Schewe et al. 2002
	Redox signaling mechanism underlying antioxidant actions	Electrophoretic mobility shift assays, real-time PCR (RT-PCR) and Western blot for the evaluation of gene and protein expressions Indirect assessment of antioxidant activity using biomarkers such as cell signaling transcription factors from the Nrf-2 and NF-κB pathways	Lü et al. 2010; Forman et al. 2010; Arredondo et al. 2010; Shen et al. 2006; Tanigawa et al. 2007; Biswas et al. 2005; Xie et al. 2011
Miscellaneous methods	Hybrid methods between chromatography and *in vitro* assays	Online HPLC-DPPH method Online HPLC-FRAP Online HPLC-ABTS	Dapkevicius et al. 2001; Koleva et al. 2000; He et al. 2010

dihydrochloride). The lag time of O_2 uptake, that is, the induction period, can be quantitatively measured and used to express TAC of plasma samples in TRAP value (Wayner et al. 1985). This method has since been modified into many versions by using a broader range of probes, initiators, and end-point measurements, for example, AAPH and peroxidase enzymes were used as initiators, and fluorescein, dichlorofluorescein diacetate (DCFH-DA) or luminol as endpoint measurements (Apak et al. 2016b).

1.3.1.3 *TOSC assay*

The TOSC assay is based on the inhibition of ethylene formation (a control reaction is monitored by headspace gas chromatography (GC)) in the presence of antioxidant compounds that compete with α-keto-γ-methiolbutyric acid (KMBA) for ROS. This assay uses an AUC of ethylene concentration versus the reaction time (up to 300 min) (Regoli & Winston 1999). Use of GC prevents this method from being developed as a high-throughput assay.

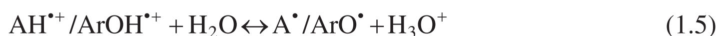
1.3.1.4 *Crocin bleaching assay*

This assay is based on the competitive kinetic reaction of an antioxidant and crocin, a naturally occurring carotenoid derivative. β-Carotene is also used but due to the fact that decolorization of β-carotene at 470 nm can occur by multiple pathways, crocin is often used to avoid misinterpretation of the results (Bors et al. 1990).

1.3.2 Single electron-transfer-based assays

Single electron transfer-based assays, also called ET (electron transfer) assays, detect the capability of an antioxidant to transfer one electron to reduce metal ions, carbonyls, and radicals (Wright et al. 2001). The SET mechanisms of antioxidant action can be summarized by the following reactions:

$$ROO^{\bullet} + AH/ArOH \rightarrow ROO^{-} + AH^{\bullet+}/ArOH^{\bullet+} \quad (1.4)$$



$$AH^{\bullet+}/ArOH^{\bullet+} + H_2O \leftrightarrow A^{\bullet}/ArO^{\bullet} + H_3O^{+} \quad (1.5)$$

$$ROO^{-} + H_3O^{+} \leftrightarrow ROOH + H_2O \quad (1.6)$$

where the reactions are relatively slower than those of HAT-based assays, and are solvent and pH dependent (Apak et al. 2016a). The aryloxyl radical ($ArO^{\bullet}$) is subsequently oxidized to the corresponding quinone (Ar = O) (Gupta 2015). The more stabilized the aryloxyl radical is, the easier will be the oxidation from ArOH to Ar = O due to reduced redox potential (Gupta 2015). The antioxidant action in these assays is often simulated with a suitable fluorescent or colored probe instead of peroxyl radicals.

Spectroscopic SET-based assays, including Folin-Ciocalteu (FC) assay, ferric reducing antioxidant power (FRAP) assay, cupric reducing antioxidant capacity (CUPRAC) assay and Ferricyanide-Prussian blue assay, measure the capacity of an antioxidant in the reduction of an oxidant, which changes color when reduced. The degree of color change is correlated to the concentration of TAC. Furthermore, electrochemical and nanotechnological methods also belong to SET-based assays.

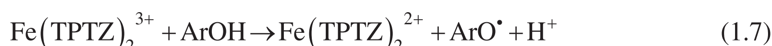
1.3.2.1 *Spectroscopic methods*

1.3.2.1.1 Folin-Ciocalteu assay

The FC method is based on the oxidation of phenol compounds in alkaline (carbonate) solution with a molybdotungstophosphate heteropolyanion reagent, yielding a colored product which absorbs at 750–765 nm (Singleton et al. 1999). Because of the specific relatability with phenolics, this method is also often used for total phenolic content (TPC) estimation.

1.3.2.1.2 FRAP assay

The FRAP assay is based on the reduction of Fe^{3+} to Fe^{2+} by antioxidants in the presence of tripyridyltriazine tridentate ligand, forming a colored complex with Fe^{2+}.



$$\mathrm{Fe(TPTZ)_2}^{3+} + \mathrm{ArOH} \rightarrow \mathrm{Fe(TPTZ)_2}^{2+} + \mathrm{ArO}^{\bullet} + \mathrm{H}^+ \quad (1.7)$$

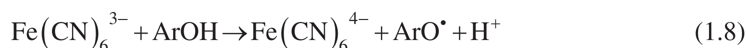
TPTZ denotes the 2,4,6-tripyridyl-S-triazine ligand, and the absorption maximum wavelength (λ_{max}) of the Fe(II) complex is at 593 nm (Benzie & Strain 1996). This method has been widely used to assess the reducing power of an antioxidant in both food and biological fluid samples.

1.3.2.1.3 CUPRAC assay

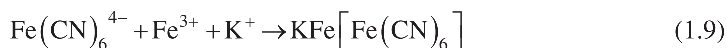
The CUPRAC assay is based on measuring the absorbance of the CUPRAC chromophore, Cu(I)-neocuproine (Nc) chelate, formed as a result of the redox reaction of antioxidants with the CUPRAC reagent, bis(neocuproine) copper(II) cation (Cu(II)-Nc). The absorbance is recorded at 450 nm (Apak et al. 2004). This assay is also branched into various modified methods of AOA/TAC associated with Cu(II) – Cu(I) reduction in the presence of a selective Cu(I)-stabilizing ligand, neocuproine (2,9-dimethyl-1,10-phenanthroline) (Bektaşoğlu et al. 2006; Çelik et al. 2010; Özyürek et al. 2007, 2009, 2010).

1.3.2.1.4 Ferricyanide-Prussian blue assay

The Ferricyanide-Prussian blue assay is based on the following reactions:



$$\mathrm{Fe(CN)_6}^{3-} + \mathrm{ArOH} \rightarrow \mathrm{Fe(CN)_6}^{4-} + \mathrm{ArO}^{\bullet} + \mathrm{H}^+ \quad (1.8)$$



$$\mathrm{Fe(CN)_6}^{4-} + \mathrm{Fe}^{3+} + \mathrm{K}^+ \rightarrow \mathrm{KFe}\left[\mathrm{Fe(CN)_6}\right] \quad (1.9)$$

The ferricyanide reagent is first incubated in ($H_2PO_4^-/HPO_4^{2-}$) buffer at pH 6.6 with antioxidants (at 50 °C for 20 min), and the reduction product, ferrocyanide, combines with the later added Fe^{3+} to produce Prussian blue, which is detected at λ_{max} 700 nm (Gorinstein et al. 2006). This method is also referred to as the reducing power assay (Yen & Duh 1993).

1.3.2.1.5 Assays involving strongly oxidizing reagents

A simple, sensitive, and low-cost indirect spectrophotometric method was developed to evaluate the Ce(IV) reducing antioxidant capacity (CERAC) of plant extracts, which is based on the oxidation of antioxidants with Ce(IV) sulfate in dilute sulfuric acid at room temperature. The spectrophotometric determination of the remaining Ce(IV) at 320 nm was performed after all antioxidants in solution were oxidized (Ozyurt et al. 2007).

In addition, the Cr(VI) reducing antioxidant capacity (CHROMAC) assay involves the reduction of chromate(VI) with antioxidants to Cr(III) in acidic solution at pH 2.8 for 50 min. The remaining Cr(VI) was spectrophotometrically measured with 1,5-diphenylcarbazide (DPC) at 540 nm (Işık et al. 2013).

The acidic $KMnO_4$ spectrophotometric assay was reported as a measure of the reducing capacity of antioxidants (Cacig et al. 2006). The discoloration of potassium permanganate is proportional to the concentration of antioxidants; therefore by measuring absorbance at 535 nm, the reducing power can be extrapolated (Popović et al. 2012).

1.3.2.2 *Electrochemical methods*

Direct electrochemical sensing methods for *in vitro* AOA have been reviewed by Blasco et al. (2007). Among these assays, cyclic voltametry (CV), differential pulse (DPV) and square-wave voltametry (SWV) based assays have been employed to investigate the electrochemical behavior of phenolic compounds in different food samples in conjunction with carbon, diamond, and graphite electrodes (Bartosz 2003; Magarelli et al. 2013; Novak et al. 2009).

Prieto-Simon (2008b) reported that electroanalytical biosensor-originated AOA/TAC assays are based on the reduction of hazard caused by $O_2^{\bullet-}$, and they essentially involved the use of Cyt *c* heme protein, SOD enzyme or DNA as a biosensor.

The recently developed direct current polarographic assay for AOA estimation is based on the measurement of anodic current obtained by dropping mercury electrode (DME) in hydrogen peroxide solution upon the addition of antioxidant compounds (Gorjanović et al. 2010, 2012).

1.3.2.3 *Nanotechnological methods*

Nanotechnological methods of colorimetric TAC assay usually have the advantage of either the formation or enlargement of noble metal (Au, Ag, etc.) nanoparticles, abbreviated as AuNPs or AgNPs, upon reaction of Au(III) or Ag(I) salts with antioxidant compounds (Apak et al. 2016a).

1.3.3 Mixed-mode (HAT/SET) assays

Not all AOA/TAC assays can be clearly classified into a HAT- or SET-based mechanism. In fact, some assays use both modes. These mixed-mode assays are generally based on the scavenging of a stable radical chromophore (such as 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$)) or fluorophore by antioxidants, in which HAT, ET, and proton-coupled electron transfer (PCET) mechanisms may play different roles to varying extents, depending on the corresponding reaction conditions (such as pH and solvent) (Apak et al. 2016a). Mixed-mode (HAT/SET) assays mainly include ABTS/TEAC (trolox equivalent antioxidant capacity) assay, DPPH radical scavenging assay, and DMPD (*N*, *N*-dimethyl-*p*-phenylenediamine dihydrochloride) radical scavenging assay.

1.3.3.1 *ABTS/TEAC assay*

ABTS/TEAC assays use intensely colored cation radicals of $ABTS^{\bullet+}$ as a colorimetric probe accepting hydrogen atoms or electrons supplied by antioxidant compounds. The antioxidant capacity is measured as the ability of the test compound to decrease $ABTS^{\bullet+}$ color by

intercepting initial oxidation and preventing $ABTS^{\bullet+}$ production or reacting directly with the preformed radical cation. Results of these assays may vary greatly, even for the same compound, as the reaction depends largely on the oxidizing agent used to generate the stable colored radical (Apak et al. 2016b).

1.3.3.2 *DPPH radical scavenging assay*

The stable chromogen radical $DPPH^{\bullet}$, first reported by Blois (1958), was used for quantitating antioxidant content based on the thiol-containing amino acid cysteine. Later it was used as a phenol reagent or a reference point (Brand-Williams et al. 1995; Gómez-Alonso et al. 2003; Papariello & Janish 1966). Reaction with DPPH was adapted for measuring radical quenching kinetics (Hogg et al. 1961) and since then, numerous variations of the DPPH assay, by modifying protocols and time for following the reaction as well as for calculating relative antioxidant action by reaction stoichiometry, have been reported (Brand-Williams et al. 1995). This has been accepted as an official method by the AOAC for food and beverage samples (Plank et al. 2012).

1.3.3.3 *DMPD radical scavenging assay*

Similar to other stable radicals, DMPD is converted to the colored $DMPD^{\bullet+}$ radical cation in the presence of ferric iron or reactive species such as hydroxyl radicals. In the DMPD assay, an antioxidant molecule transfers a hydrogen atom (or an electron) to $DMPD^{\bullet+}$, causing rapid decolorization of the solution (manifested by an absorbance decrease at $\lambda_{max} = 505$ nm) with a stable endpoint that can be calculated as AOA (Beltran et al. 2009; Corral-Aguayo et al. 2008).

1.3.3.4 *Other radical scavenging assays*

Other radical probes used for free radical-scavenging activity measurement are Fremy's salt (galvinoxyl radical, potassium nitrosodisulfonate) and the more recently developed aroxyl radical (2,6-di-*tert*-butyl-4-(4′-methoxyphenyl) phenoxyl radical) methods (Nagaoka et al. 2013).

1.3.4 *In vivo* antioxidant activity assays

The above-mentioned chemical-based non-enzymatic AOA/TAC assays are relatively easy, less costly and widely adopted but they cannot be compared, nor can their results be directly and accurately extrapolated to *in vivo* performance. More importantly, the physiological antioxidant action is not only about scavenging free radicals but also includes upregulation of detoxifying enzymes, promotion of overall antioxidant defense, modulation of gene expression and redox cell signaling in a biological system. Cell-based antioxidant assays (CAA) have since been developed to reflect biological relevance (López-Alarcón & Denicola 2013).[9] CAA as an intermediate or indirect testing method for AOA/TAC has advantages over animal models and human studies due to its convenience, low cost, and physiological relevance. Cellular antioxidant status is governed by many biochemical processes, of which many biomarkers can be used in the CAA approach.

1.3.4.1 *Cellular antioxidant activity assay*

The CAA assay was developed for measuring the AOA of antioxidants in dietary supplements and foods (Wolfe & Liu 2007). CAA uses 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as a fluorescence probe which is bioavailable and can be taken up by the cells. Different cell lines including Caco-2 have been used in CAA (Peng et al. 2016). Once inside the cell, DCFH-DA is deacetylated by cellular esterases to form 2′,7′-(DCFH) which is consequently oxidized by peroxyl radicals generated from a highly bioavailable molecule AAPH or H_2O_2 to form a fluorescent compound, dichlorofluorescein (DCF), within the cells. We preferred the use of H_2O_2 as it represents real OS in the cells (Peng et al. 2016; Zhang et al. 2016). Potential antioxidants that could quench peroxyl radicals can therefore inhibit the generation of fluorescent DCF, from which the AOA/TAC can be assessed by measuring the degree of decrease in cellular fluorescence.

1.3.4.2 *Biomarkers of oxidative stress*

Many biologically active molecules are biofactors that can induce and generate OS and thus can be used as biomarkers in AOA assays. By-products of OS or oxidative damages caused by OS, or components that control OS or reduce oxidative damage, can also be used as biomarkers. Detailed information on OS biomarkers can be found in Chapter 9. A brief summary of commonly used biomarkers of OS with respect to the classification of AOA/TAC assays is presented below.

1.3.4.2.1 ROS and RNS

Reactive oxygen species (ROS) is a collective term that mostly refers to oxygen radicals: superoxide ($O2^{\bullet-}$), hydroxyl ($^{\bullet}OH$), peroxyl ($ROO^{\bullet}$), and alkoxyl ($RO^{\bullet}$), but also includes certain non-radicals such as HOCl, ozone (O_3), peroxynitrite ($ONOO^-$), singlet oxygen ($^1O_2$), and H_2O_2 that are either oxidizing agents or easily converted into radicals (Apak et al. 2016a). Similarly, RNS include nitric oxide radical ($^{\bullet}NO$), $ONOO^-$, nitrogen dioxide radical ($^{\bullet}NO_2$), other oxides of nitrogen, and products arising from NO reaction with $O_2^{\bullet-}$, $RO^{\bullet}$, and $ROO^{\bullet}$ (Wiseman & Halliwell 1996). Although ROS/RNS are vital in normal cell metabolism and thus important to our health, excess may cause oxidative stress-related diseases (Bourdon & Blache 2001; Niki 2010). ROS/RNS scavenging-based assays (Apak et al. 2016c; Li 2013; Kooy et al. 1994; Kumaran & Karunakaran 2006; Martinez et al. 2006; Özyürek et al. 2009; Pou et al. 1989; von Kruedener et al. 1995; White & Bursey 1964) are based on a reaction between the reactive species (generated enzymatically or by redox-active chemical reagents) and a probe. The extent of conversion of the probe is a measure of ROS/RNS concentration, and its attenuation indicates the scavenging activity of the antioxidants (Apak et al. 2016a). Because neither ROS and RNS are readily detected in the circulation system, and most of the current probes cannot be used in humans, other biomarkers have to be explored (Chandna et al. 2012).

1.3.4.2.2 Lipid peroxidation products

Lipid peroxidation products including isoprostanes (IsoPs), hydroxynonenal (HNE), and malondialdehyde (MDA), and other products such as lipid hydroperoxides (LOOHs), oxysterols and total hydroxyoctadecadienoic acids (tHODE), have been successfully used as biomarkers for antioxidant capacity *in vivo* (Niki & Yoshida 2005; Shah et al. 2014). In addition,

different AOA assays for estimating lipid peroxidation have been developed, such as β-carotene bleaching assay (Marco 1968), iodometric hydroperoxide measurement (Lea 1931; Sattler et al. 1994), thiobarbituric acid-reactive substances (TBARS) assay (Sinnhuber & Yu 1958), ultraviolet (UV) spectroscopic measurement of conjugated dienes (Gutteridge & Halliwell 1990), ferric thiocyanate and ferric xylenol orange assays (Jiang et al. 1992). GC, high performance liquid chromatography (HPLC) coupled to mass spectrometry (GC-MS, LC-MS), enzyme-linked immunosorbent assays (ELISA), and radioimmune assay (RIA) have been used to analyze these biomarkers (Bevan et al. 2003; Musiek et al. 2005; Santilli et al. 2015a). TBARS, expressed as MDA equivalents, can also be conveniently quantified by colorimetric assay based on the reaction between MDA and thiobarbituric acid (TBA), and commercial ELISA kits are available (Bevan et al. 2003).

1.3.4.2.3 Nucleic acid oxidation products

Oxidation of DNA occurs on several nucleic acid bases, among which guanine is most vulnerable. 8-Hydroxyguanosine (8-OHG) and 8-hydroxy-2-deoxyguanosine (8-OHdG) are the most common oxidation products of guanine bases, and have been used as biomarkers of RNA and DNA damage. 8-OHG and 8-OHdG in various biological samples are analyzed by HPLC, GC-MS, LC-MS, and ELISA. Mitochondrial microarray chips and real-time PCR-based bioassays have also been developed for the detection of mtDNA damage and hence indirectly AOA (Santilli et al. 2015a).

1.3.4.2.4 Protein oxidation products

Oxidation of proteins produces protein carbonyls and nitrotyrosine, a product of tyrosine nitration in protein molecules. These compounds, such as 3-nitrotyrosine (3-NO2-Tyr), are chemically stable and widely used biomarkers of protein oxidation (Chang et al. 2014; Shah et al. 2014). Spectrophotometry, GC- or LC-MS, and immunochemical techniques have been used for the analysis of these oxidation products in biological samples as indirect indicators of AOA of antioxidants (Sultana & Allan 2013). Use of tyrosine nitration products or other intermediates for nitration *in vivo* is still in its infancy as the precise mechanism of formation and relationship with diseases are not clearly known.

1.3.4.2.5 Carbohydrate oxidation products

Carbohydrates are relatively less reactive than other biomolecules such as proteins, lipids, and DNA. However, advanced glycation endproducts (AGEs), a group of molecules that result from the non-enzymatic reaction of reducing sugars with certain amino groups of proteins or other biomolecules, can be produced especially when under OS. AGEs values can be potentially useful biomarkers for evaluation of antioxidants and prediction of their effect on chronic diseases (Santilli et al. 2015b; Sultana & Allan 2013).

1.3.4.2.6 Antioxidant enzymes and radical producing enzymes

Different from the non-enzymatic antioxidants such as vitamins C and E, which rely on exogenous sources such as diet, antioxidant enzymes are intrinsic to the biological system and play pivotal roles in reducing OS and repairing damage. Antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), xanthine oxidase (XO), glutathione-related enzymes (glutathione peroxidase, GPx; glutathione reductase, GR; glutathione S-transferase, GST), thioredoxin reductase, and heme oxygenase (Halliwell 1991; Lozovoy et al.

2013; Sies 1991). The ratio of reduced glutathione to its oxidized form glutathione disulfide (GSH/GSSG) has also been suggested as a reliable biomarker for whole-body antioxidant index of diseases, and thus the effect of antioxidants (Bae et al. 2002; Townsend et al. 2003; Zitka et al. 2012).

In addition, expression or inhibition of radical producing enzymes such as NADPH oxidases (Nox), particularly its Nox1 and/or Nox4 isoforms, and nitric oxide synthases (NOS) have also been used to evaluate the activity of natural antioxidants (Agouni et al. 2009; Schewe et al. 2002; Silva et al. 2011).

1.3.4.3 *Redox signaling mechanism underlying antioxidant actions*

Rapid advancement in cell biology and antioxidant research has provided evidence that apart from radical scavenging ability, antioxidants can also inhibit the activity or expression of intracellular antioxidant enzymes by modulating the expression of associated genes and modifying cell signaling pathways (Lü et al. 2010). Measuring these biomarkers helps explain the lack of correlation between *in vitro* AOA/TAC and *in vivo* effects of antioxidants.

Cellular OS accompanies increased levels of endogenous oxidants such as H_2O_2 and $^{\bullet}OH$ which could act as secondary messengers and trigger a cascade of intracellular responses, resulting in increased expression of antioxidant enzymes that regulate the cellular redox status (Forman et al. 2010). Nuclear factor κ B (NF-κB) and nuclear factor E2-related protein 2 (Nrf-2) compartmentalized in cytosol and nucleus are the two redox control-associated transcription factors. Many biomarkers of the two cascades have been identified and used to evaluate *in vivo* AOA/TAC, and detailed discussion is beyond the scope of this chapter. NF-κB is a group of inducible transcription factors that is involved not only in redox control but, more importantly, in regulating immune and inflammatory responses and protecting cells from OS and other stresses. Nrf-2 is a redox-sensitive transcription factor that is activated by an oxidative signal in the cytoplasm that causes its translocation to the nucleus. Several cell model assays have been developed to assess antioxidant activity by measuring the activation of Nrf-2 using electrophoretic mobility shift assays, real-time PCR (RT-PCR), and Western blot (Arredondo et al. 2010; Shen et al. 2006; Tanigawa et al. 2007). Similarly, inhibition of NF-κB activation which renders an antiinflammatory/antioxidant response has also been adopted in cell models (Biswas et al. 2005; Xie et al. 2011). For this reason, indirect assessment of antioxidant activity using cell signaling transcription factors from the Nrf-2 and NF-κB pathways also provides information on the antiinflammatory activity of antioxidants.

1.3.5 Miscellaneous methods

1.3.5.1 *Hybrid methods between chromatography and in vitro assays*

A hybrid method combining HPLC and DPPH was developed for simultaneously detecting antioxidant compounds of plants and assessing antioxidant activity. The online HPLC-DPPH method provides a highly effective screening tool for the identification of natural antioxidants (Dapkevicius et al. 2001; Koleva et al. 2000). Since then, online HPLC-FRAP, HPLC-ABTS, and other similar methods have been developed and applied to the search for antioxidants and assessment of their AOA/TAC (He et al. 2010; Zhang et al. 2015).

1.4 Conclusions

The nomenclature and classification of AOA/TAC assays are challenging as numerous assay methods with different mechanisms have been developed for different purposes. Requirements for AOA/TAC in a food system are different from that of a biological system, therefore not all can be used in the evaluation of antioxidants in both. The inability of some *in vitro* AOA/TAC assays to reflect actual *in vivo* activity is a particularproblem as *in vitro* assays are often insufficiently sensitive for biological samples, and they lack physiological and biological relevance. While we intended to divide the methods into the above discussed five categories, this is by no means perfect, and further improvement is expected. Nevertheless, it is the authors' intention that this chapter provides an overview for those interested in antioxidant research.

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