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Measurement of antioxidant activity

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ABSTRACT

Antioxidants play an important role in food preservation by inhibiting oxidation processes and contributing to health promotion rendered by many dietary supplements, nutraceuticals and functional food ingredients. Antioxidant activity can be monitored by a variety of assays with different mechanisms, including hydrogen atom transfer (HAT), single electron transfer (ET), reducing power, and metal chelation, among others. Understanding the principle mechanisms, advantages and limitations of the measurement assays is important for proper selection of method(s) for valid evaluation of antioxidant potential in desired applications. This contribution provides a general and up-to-date overview of methods available for measuring antioxidant activity and the chemistry behind them.

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1. Introduction: lipid oxidation and antioxidants

Lipid oxidation is a major cause of food quality deterioration, and has been a challenge for manufacturers and food scientists alike. Lipids are susceptible to oxidative processes in the presence of catalysts such as heat, light, enzymes, metals, metalloproteins and microorganisms, giving rise to the development of off-flavours in foods and loss of essential amino acids, fat-soluble vitamins and other bioactive molecules (Shahidi & Zhong, 2005). When occurring in the human body, it is a cause for oxidative stress and thought to exert destructive cellular effects associated with pathophysiology of a number of diseases and health conditions, including inflammation, atherosclerosis and ageing, among others (Dalton, Shertzer, & Puga, 1999; Davies, 2000; Floyd & Hensley, 2002; Kruidenier & Verspaget, 2012). Lipids in living organisms undergo oxidation reactions during normal aerobic metabolism (Beckman & Ames, 1998). Unsaturated fatty acids in membrane phospholipids and cholesterol, especially LDL-cholesterol, are generally the major reactants affected by such reactions, causing irreversible cellular and tissue damage.

Oxidation of lipids may be via autoxidation, photooxidation, thermal oxidation and enzymatic oxidation, most of which involve free radicals and/or other reactive species as the intermediate (Shahidi, 2000; Vercellotti, St. Angelo, & Spanier, 1992). Autoxidation, defined as the spontaneous reaction of atmospheric oxygen with lipids, is the most common process leading to oxidative deterioration/damage in food and biological systems (Shahidi & Zhong, 2005). The process can be accelerated at higher temperatures as experienced during deep fat frying, which is referred to as thermal oxidation, with increased formation of free fatty acids and polar matters, foaming, colour and viscosity (Perkins, 1992). Photooxidation involves excitation of photosensitizer and energy transfer to lipid molecules or oxygen. Enzymes such as lipoxygenases also catalyse oxidation of fatty acids and are usually inactivated

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by thermal processing of food. It has been widely accepted that lipid oxidation occurs via a free radical (e.g. lipid radicals, R•; alkoxyl, RO•; peroxyl, ROO•; and hydroxyl, •OH) chain reaction mechanism. The chain reaction proceeds through three stages of initiation, propagation, and termination that lead to a series of complex chemical changes (Shahidi & Zhong, 2005). More detailed pathways of lipid oxidation have been elucidated and explained elsewhere by Schaich (2005) and Shahidi and Zhong (2010). In general, lipid oxidation produces lipid hydroperoxides and conjugated dienes (or trienes) as primary oxidation products, which are unstable and further break down to a wide range of secondary oxidation products, including alcohols, aldehydes, ketones, hydrocarbons, volatile organic acids and epoxy compounds, among others, some of which give rise to a bad odour at very low threshold values and are responsible for the "off-flavour" notes of oxidatively deteriorated foods. A variety of polymeric products is also generated during the termination stage of the oxidation reaction as a result of radical–radical coupling or radical–radical disproportionation. The formation of polymers accounts for increased viscosity of highly oxidized vegetable oils after deep frying. Polymeric products produced from membrane phospholipid oxidation lead to reduced fluidity of the membranes, affecting membrane transport and cell signalling, as observed in Alzheimer's disease (Lyras, Cairns, Jenner, & Jenner, 1997).

Lipid oxidation has long been classified as the major deterioration process affecting both the nutritional and sensory quality of foods, especially lipid-based products (Yanishlieva & Marinova, 2001). It occurs during harvesting, processing and storage of foods, and causes chemical spoilage, resulting in rancidity and/or deterioration of the nutritional value, colour, flavour, texture and safety of the food products. In edible fats and oils such as butter, margarine, and salad/cooking oils, oxidative rancidity observed as the obvious manifestation of "off-flavour" development is the major cause of food wastage (Scott, 1997). Lipid oxidation is also responsible for quality deterioration of meat (Min & Ahn, 2005) and dairy products (Collomb & Spahni, 1996), fruits and vegetable crops. While lipid oxidation in food poses challenge to food quality preservation, oxidative stress *in vivo* renders destructive and lethal effects by oxidizing membrane lipids, cellular proteins, enzymes and DNA, thus disrupting the cells (Winrow, Winyard, Morris, & Blake, 1993). Owing to its detrimental effects on food quality and human health, concerns about lipid oxidation have been addressed in the food and feed as well as the health care industries, leading to intensive efforts to improve oxidative stability of oils and fats by different means.

Oxidation of fats/oils and lipid-containing products may be prevented by excluding the initiator and promoter elements during processing and storage. This explains why packaging of highly oxidation-susceptible products usually avoids heat and uses materials that are not metal or transparent, while under vacuum or inert gas. When used as nutraceuticals or pharmaceuticals, the highly unstable polyunsaturated lipid components are protected by encapsulation. The membrane of the capsule protects the core material from undesirable effects of light, moisture and oxygen, thus increasing the shelf-life of encapsulated materials (Shahidi & Han, 1993). Nanoencapsulation technique has attracted much attention as an effective and efficient delivery method for bioactive ingredients and drugs such as fish oils and omega-3 polyunsaturated fatty acid (PUFA) concentrates. In spite of the many advantages of nanoencapsulation, toxicity issues related to possible overdosing remains to be a major concern.

Among the many methods employed for controlling lipid oxidation, use of antioxidants is the most effective, convenient and economical means. Food manufacturers use antioxidants to stabilize food lipids and thus prevent quality deterioration of the products. Antioxidants are also used in health related areas for disease risk reduction and health promotion due to their ability to protect the body against oxidative damage. Antioxidants are substances that when present in food or in the body at very low concentrations, delay, control or prevent oxidative processes leading to food quality deterioration or initiation and propagation of degenerative diseases in the body. Antioxidants that fit in this definition include free radical scavengers, singlet oxygen quenchers, inactivators of peroxides and other reactive oxygen species (ROS), metal ion chelators, quenchers of secondary oxidation products and inhibitors of pro-oxidative enzymes, among others (Shahidi & Zhong, 2007). These substances exert their inhibitory effect against oxidation processes through different mechanisms and with varied activities. They are broadly classified by their mechanism of action as primary and secondary antioxidants. Primary antioxidants such as tocopherols and some phenolic compounds inhibit the chain reaction of oxidation by acting as hydrogen donors or free radical acceptors and generation of more stable radicals. The inhibition reaction is considered to be in competition with the propagation step of lipid oxidation and yields stable products that will not initiate new free radicals or bring about a rapid oxidation via a chain reaction (Nawar, 1996). Secondary antioxidants prevent or retard oxidation by suppressing the oxidation promoters, including metal ions, singlet oxygen, pro-oxidative enzymes and other oxidants. Reducing agents can reduce lipid peroxides and related oxidants through redox reactions and are also referred to as oxygen scavengers. Some secondary antioxidants such as ascorbic acid can regenerate primary antioxidants by replenishing hydrogen atom, thus inhibiting depletion of important primary antioxidants. Other secondary antioxidants promote decomposition of hydroperoxides into non-radical species, or absorb UV radiation thus protecting lipids from UV-induced photooxidation. An in-depth discussion of antioxidant mechanisms has already been provided elsewhere (Shahidi & Zhong, 2010).

Antioxidants may occur naturally in plants, animals and microorganisms or may be synthesized by chemical means. Higher plants and their constituents provide a rich source of natural antioxidants, such as tocopherols and polyphenols which are found abundantly in spices, herbs, fruits, vegetables, cereals, grains, seeds, teas and oils. Antioxidants from marine origin such as algae, fish/shellfish and marine bacteria, have also been considered (Amarowicz, Karamac, & Shahidi, 1999; Athukorala et al., 2003; Shahidi & Amarowicz, 1996). In addition, by-products from the food and agricultural industries have been explored for their potential use as antioxidants. For example, hulls, shells and skins of nuts and cereals, citrus peels and seeds, canola meal and fish viscera extracts have been found to possess antioxidant activity (Cumby, Zhong, Naczk, & Shahidi, 2008; Liyana-Pathirana, Dexter, & Shahidi, 2006; Shahidi, Alasalvar, & Liyana-Pathirana, 2007). Naturally occurring antioxidants can

be isolated from their source material as pure compounds for possible use in nutraceutical/pharmaceutical applications. Alternatively, plant or animal extracts containing a cocktail of antioxidant substances with potential synergistic effects are used directly to inhibit *in vitro* and *in vivo* oxidation.

Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and *tert*-butylhydroquinone (TBHQ), have been used as potent compounds in food preservation for decades due to their low cost and bland flavour. However, they have fallen out of favour due to consumers' demand for "clean label", all natural ingredients. Safety concerns have been raised about synthetic antioxidants (Sherwin, 1990; Wattenberg, 1986) and their use is limited and regulated by the Food and Drug Administration (FDA), necessitating the search for effective antioxidants from natural sources.

The effectiveness of antioxidants is generally influenced by a number of factors, including their structural features, concentration, temperature, type of oxidation substrate and physical state of the system as well as presence of pro-oxidants and synergists (Yanishlieva-Maslarova, 2001). The chemical structure of an antioxidant determines its intrinsic reactivity towards free radicals and other ROS and hence the antioxidant activity. Efficiency of antioxidants also depends on their concentration and location in the system (e.g. distribution at interface) (Shahidi & Zhong, 2011; Zhong & Shahidi, 2012a). The reaction kinetics plays an important role in their short-term or long-term protection against oxidation, and involves the rate at which an antioxidant reacts with a specific oxidant, the thermodynamics of the reaction and the extent to which the antioxidant reacts (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002). All these factors need to be considered when assessing the efficiency and selecting a suitable antioxidant for a specific application.

Methods and tools used for measuring antioxidants activity have advanced remarkably during the last few decades. Early methods measure the efficacy of antioxidants against the formation of particular species of oxidation products and therefore are based on lipid oxidation measurement. To date, various chemical assays coupled with highly sensitive and automated detection technologies are employed for evaluation of antioxidant activity through particular mechanisms, for example, scavenging activity against certain types of free radicals or ROS, reducing power, and metal chelation, among others. Oxidation substrates or target probes have also been extended from food model systems to chemical compounds, biological materials, cell lines, and even live tissues. Methods used for measuring antioxidant activity are discussed in the sections below which vary in types of oxidation substrates, oxidants, reaction mechanisms and conditions, detection technologies and expression of results.

2. Chemical assays

2.1. *Radical/ROS scavenging methods*

A large group of assays are available for direct measurement of hydrogen atom or electron transfer from potential antioxidants to free radicals in simple "lipid free" systems. The antioxidant activities reported by this group of methods are generally associated with their scavenging capacity against specific types of radical species, some of which may be artificial and biologically irrelevant. Therefore, they have been criticized for not reflecting the situation in an oxidizing food or an *in vivo* situation. However, the hydrogen atom or electron donating capacity data obtained by these methods provide important information on their intrinsic antioxidant potential with minimum environmental interference. These assays require no lipid substrate and usually employ a chemical system containing an oxidant (free radicals or other ROS), an oxidizable probe (not necessary for some assays), and antioxidants under investigation. Depending on the chemical reactions involved, these assays fall into two categories of hydrogen atom transfer (HAT) reaction-based assays and single electron transfer (ET) reaction-based assays. Antioxidants can scavenge radicals or other ROS (e.g. hydrogen peroxide and lipid peroxides) by HAT and ET leading to the same end results, regardless of the mechanism involved, although kinetics and potential for side reactions vary (Prior, Wu, & Schaich, 2005). Proton-coupled ET and HAT reactions may also occur in parallel, and in this case the dominating mechanism in a given system is dependent on antioxidant structure and properties, solubility and partition coefficient, as well as the solvent system (Prior et al., 2005). Antioxidant activities are either expressed as inhibition against ROS-mediated oxidation of the probe, or equivalents of a selected reference antioxidant such as trolox, ascorbic acid or another compound. Oxidation of the probe is measurable by various detection technologies, which are advancing with availability of up-to-date instrumentation. These include spectrophotometric, fluorometric, chemiluminescent, EPR (electron paramagnetic resonance), FT-IR (Fourier transform-infrared), NMR (nuclear magnetic resonance), and amperometric methods, among others. Chemical assays (summarized in Table 1) for antioxidant activity measurement are simple, quick, and usually automated, and are widely used in initial screening and evaluation of novel antioxidant compounds or extracts of natural products/by-products.

2.1.1. *Oxygen radical absorbance capacity (ORAC) assay*

The ORAC assay measures the radical chain breaking ability of antioxidants by monitoring the inhibition of peroxyl radical-induced oxidation. Peroxyl radicals are the predominant free radicals found in lipid oxidation in foods and biological systems under physiological conditions. Hence, ORAC values are considered by some to be of biological relevance as a reference for antioxidant effectiveness. In this assay, the peroxyl radical produced by a generator reacts with a fluorescent probe resulting in the loss of fluorescence, which is recorded with a fluorometer. A set of fluorescence decay curves can be constructed in the absence or presence of antioxidants and the net integrated area under the decay curves (area gain in the presence of antioxidants compared to that of a blank run without antioxidants) can be calculated as an indicator of the peroxyl radical scavenging capacity of the antioxidants. A standard antioxidant, usually trolox, is used as reference and ORAC values of the tested antioxidants are reported as trolox equivalents. The ORAC method measures hydrogen atom donating ability of antioxidants, and is therefore an HAT-based method. The assay is readily automated using a multichannel liquid handling system coupled with a fluorescence microplate reader.

Table 1 – Major reactive oxygen species (ROS) scavenging- and redox potential-based assays.

Name of assay	Dominating mechanism	Oxidant	Probe	Detection	Reference
ROS scavenging assays					
ORAC	HAT	Peroxyl radical generated by AAPH	Fluorescein	Fluorometry	Prior et al., 2005
Chemiluminescence	HAT	Hydrogen peroxide	Luminol	Fluorometry	Papadopoulos et al., 2003
DPPH scavenging	ET	DPPH radical	DPPH radical	Spectrophotometry or EPR	Blois, 1958
TEAC	ET	ABTS radical cation	ABTS radical cation	Spectrophotometry	Miller et al., 1993
Redox potential assays					
FRAP	ET	Fe^{3+}	Ferricyanide	Spectrophotometry	Molina-Diaz et al., 1998
CUPRAC	ET	Cu^{2+}	Neocuproine	Spectrophotometry	Apak et al., 2004
Ag^{+} reducing	ET	Ag^{+}	Ag nanoparticle	Surface plasmon resonance	Özyürek et al., 2012
Au^{3+} reducing	ET	Au^{3+}	Au nanoparticle	Cyclic voltammetry	Andreu-Navarro et al., 2011
CERAC	ET	Ce^{4+}	Indigo carmine dye	Spectrophotometry	Ozyurt et al., 2010
CHROMAC	ET	Cr^{6+}	Cr^{3+} complex	Spectrophotometry	Isik et al., 2013

AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid.

Commonly used peroxyl radical generators in ORAC assays include a group of azo compounds such as the lipophilic AIBN (α,α-azobisisobutyronitrile), ABAP (2,2-azobis(2-amidinopropane) hydrochloride) and AMVN (2,2′-azobis(2,4-dimethylnaleronitrile)) and the hydrophilic AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride) (Becker, Nissen, & Skibsted, 2004). Reaction of peroxyl radical generation is simplified as given below:

$$R{-}N{=}N{-}R \xrightarrow{O_2} N_2 + 2ROO^{\bullet}$$

The azo compound AAPH, for example, undergoes thermal decomposition in the presence of oxygen to yield peroxyl radicals as oxidants that further react with the probe and antioxidant. Generation of peroxyl radical is sensitive to temperature, and has been reported to be one of the major contributing factors that interferes with the results and inconsistency of the assay (Prior et al., 2005). It is therefore very important to control and monitor the temperature during the assay. However, this issue has been well mitigated by development of highly automated fluorescence plate readers equipped with an incubator. Incubation of the reaction buffer at 37 °C prior to the dissolution of AAPH is recommended to reduce the intra-assay variability (Prior et al., 2005).

A number of fluorescent materials has been identified and used as probes for ORAC assay. B-phycoerythrin (B-PE), a protein isolated from *Porphyridium cruentum*, was the fluorescent probe initially employed in the early ORAC studies until it was found to suffer from several disadvantages. Being a natural component derived from red algae of various species, it shows large lot-to-lot variability in reactivity with peroxyl radicals. In addition, its nonspecific binding to polyphenol antioxidants and photobleaching by excitation light can all introduce interference to the reaction and hence false interpretation of antioxidant activity (Huang, Ou, & Prior, 2005; Prior et al., 2005). Alternative synthetic non-protein probes have been identified as replacement of B-PE, among which fluorescein is the most frequently selected fluorescent probe for ORAC assay in decades. However, fluorescein has been demonstrated to undergo undesired fluorescence quenching and side reactions (Apak et al., 2013), and novel fluorescent probes have been proposed. A long wavelength fluorophore Nile blue was used as alternative probe to determine ORAC values of fruit juices and wines, which were in good agreement with results obtained by fluorescein method (Godoy-Navajas, Aguilar-Caballos, & Gomez-Hens, 2011). Barahona, Chandia, Encinas, Matsuhiro, and Zuniga (2011) compared ORAC values measured by using c-phycocyanin and fluorescein probes for soluble polysaccharides from seaweeds. Even though use of c-phycocyanin probe resulted in higher ORAC values than fluorescein in general, the relative ORAC values and trend among different samples were similar for both probes. Ortiz et al. (2012) investigated the use of pyrogallol red probe for ORAC assay, which showed good linearity, precision and accuracy. Guclu, Kibrislioglu, Ozyurek, and Apak (2014) reported ORAC values of liver and kidney samples measured using *p*-aminobenzoic acid (PABA) as the fluorescent probe. The authors suggested replacement of fluorescein with PABA in ORAC assay for amino acids, plasma and thiol-type antioxidants as well as albumin, as inconsistent results have been reported for thiols such as glutathione and cysteine.

Although it was originally developed for measurement of hydrophilic antioxidants, ORAC assay has also been adapted to detect both lipophilic and hydrophilic antioxidants by altering the radical source and solvent. Huang, Ou, Hampsch-Woodill, Flanagan, and Deemer (2002) developed and validated a modified ORAC assay for lipophilic antioxidants using randomly methylated β-cyclodextrin (RMCD) as solubility enhancer. RMCD at 7% in an acetone/water (50:50, v/v) mixture was suggested to efficiently solubilize tocopherols and other lipophilic phenolic antioxidants in phosphate buffer. This modified ORAC assay, using cyclodextrin as solubilizer, has been successfully employed for measuring ORAC values of tocopherols, tocotrienols, γ-oryzanol, resveratrol, lipophilized tea catechins, and various food and biological samples (Huang et al., 2002; Lucas-Abellan, Mercader-Ros, Zafrilla, Gabaldon, & Nunez-Delicado, 2011; Prior et al., 2003; Zhong, Ma, & Shahidi, 2012).

It has been argued by Nkhili and Brat (2011) that further modification of ORAC method was needed to eliminate the effect of metal ions in the test systems on measured ORAC values of antioxidants. Antioxidants can form complexes with metal ions, leading to underestimated peroxyl radical scavenging capacity. The metal chelator, ethylenediaminetetraacetic acid (EDTA) can be helpful to mitigate the interference, and significantly increased measured ORAC values were reported (Nkhili & Brat, 2011).

Other similar HAT-based methods sharing the same principle and common features of ORAC assay include total radical-trapping antioxidant parameter (TRAP) and crocin bleaching assays (Shahidi & Zhong, 2007). Similar to ORAC, TRAP and crocin bleaching consist of an azo compound as peroxyl radical generator (e.g. AAPH or ABAP), a molecular fluorescence (e.g. R-phycoerythrin or dichlorofluorescin diacetate) or UV–vis probe (crocin), and an antioxidant. They both collect kinetic curves of fluorescence or absorbance decay for quantification of antioxidant activity, and express results as trolox equivalents. Like the ORAC assay, they are all readily adapted to high-throughput automated methodologies using microplate reader. However, all these three assays suffer from one limitation, i.e. naturally existing pigments and fluorophores in antioxidant extracts that may interfere with absorbance and fluorescence readings.

Despite its demonstrated usefulness in the evaluation of antioxidant activity, USDA website removed information relevant to the listing of foods based on their ORAC values. The reason given was that they did not realistically reflect the *in vivo* situation (USDA, 2010).

2.1.2. *Chemiluminescence assay*

In addition to peroxyl radicals, several other ROS contribute to oxidative stress of food and biological systems and are targets of antioxidant action. Singlet oxygen ($^1O_2$), superoxide radical anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide are highly reactive oxygen-derived species that are short-lived and difficult to evaluate. Highly sensitive and reproducible methods have been developed for measurement of antioxidant activity in quenching these ROS, such as the chemiluminescence assay for hydrogen peroxide quenching capacity.

Chemiluminescence is a phenomenon observed when a chemical reaction produces an electronically excited species that either luminesces (direct chemiluminescence) or transfers the energy to a fluorophore that generates emission (indirect chemiluminescence). A majority of chemiluminescence assays for antioxidant activity is direct chemiluminescence-based and generally consists of a chemiluminescent species, an oxidant (hydrogen peroxide) in the presence or absence of a metal or enzymatic catalyst, and an antioxidant or extract. A variety of chemiluminescent reagents such as luminol, lucigenin, oxalates, pyrogallol or Cypridina luciferin, among others have been proposed, with luminol being the most commonly used aqueous chemiluminescent reagent (Bezzi, Loupassaki, Petrakis, Kefalas, & Calokerinos, 2008; Papadopoulos, Triantis, Yannakopoulou, Nikokavoura, & Dimotikali, 2003; Stepanyan, Arnous, Petrakis, Kefalas, & Calokerinos, 2005). Luminol reacts with the potent oxidizing agent hydrogen peroxide to yield 3-aminophthalate in an excited electronic state, which is a light-producing emitter. Antioxidants can quench hydrogen peroxide by hydrogen atom donation and inhibit hydrogen peroxide-induced chemiluminescence. Therefore, this assay is based on the competition between antioxidants and luminol for hydrogen peroxide, and presence of antioxidants results in a decrease in light emission intensity. The antioxidant of interest is tested over a range of concentrations, and antioxidant activity is usually expressed as EC50, i.e. the effective concentration that reduces chemiluminescence by 50%.

Although hydrogen peroxide is the oxidizing agent used in chemiluminescence assays, it is not the only form of ROS reacting with luminol and being quenched by antioxidants. In many cases, a catalyst is added to magnify the chemiluminescence signal, by converting the relatively weak oxidant hydrogen peroxide into stronger ROS superoxide radical anion and hydroxyl radical. A metal (e.g. iron, copper, manganese, and cobalt) containing compound or enzyme can catalyse production of hydroxyl radical and superoxide radical anion from hydrogen peroxide through Fenton reaction. Even though adding catalyst can significantly increase chemiluminescence signal of luminol and linearity of the assay, it may complicate the interpretation of the results by introducing multiple ROS due to preference of antioxidants to scavenge particular ROS (Giokas, Vlessidis, & Evmiridis, 2007; Sariahmetoglu, Wheatley, Cakici, Kanzik, & Townshend, 2003). Given this concern, the hydrogen peroxide–metal catalyst–chemiluminescent reagent is still the most frequently used system for this assay. Oxidation and chemiluminescence of luminol may also be enhanced by using enzymes such as horseradish peroxidase and sensitizers such as *p*-iodophenol (Minioti & Georgiou, 2008) as in enhanced chemiluminescence. However, limitations with luminol-based antioxidant assays have also been reported. Luminol molecule is negatively charged and its signal may be quenched by non-antioxidant quenchers in the sample, leading to overestimation of antioxidant activity (Kruglov et al., 2010). The excited luminol molecule may also be quenched by antioxidants by a non-antioxidant mechanism, which interferes with the interpretation of their ROS-scavenging capacity (Zargoosh, Ghayeb, Aeineh, & Qandalee, 2014).

Different sources of chemiluminescent species have been explored for their potential use in the evaluation of antioxidants of varying hydrophilicity (or lipophilicity). For example, lucigenin–hydrogen peroxide chemiluminescence in 2-propanol has been developed to evaluate the activity of both hydrophilic and lipophilic antioxidants (Christodouleas, Fotakis, Papadopoulos, Yannakopoulou, & Calokerinos, 2009). While

alternative chemiluminescent species have been explored, indirect chemiluminescence methods have also become an option for antioxidant assessment. Zargoosh et al. (2014) reported peroxyoxalate–hydrogen peroxide–imidazol–fluorophore system for evaluation of antioxidants in olive oils and honey samples. A furan dicarboxylate derivative was used as the fluorophore. The mechanism is believed to involve highly energetic intermediates from peroxylate–hydrogen peroxide reaction that can transfer the energy and excite the fluorophore. A similar method was proposed by Nikokavoura, Christodouleas, Yannakopoulou, Papadopoulos, and Calokerinos (2011) who developed a lucigenin–hydrogen peroxide–fluorophore system for assessing various hydrophilic and lipophilic antioxidants and extracts. The fluorophore used in this method is *N*-methylacridone, which is emitted as a result of the lucigenin–hydrogen peroxide reaction.

Chemiluminescence method has been used as a sensitive, low-cost and robust tool for assessing antioxidant activity, particularly quenching capacity of hydrogen peroxide-derived ROS. More recently, it has been automated in the forms of flow-based assays, including flow-injection analysis (FIA), sequential-injection analysis (SIA), multisyringe FIA (MS-FIA) and multicommutation (Fassoula, Economou, & Calokerinos, 2011). These automated chemiluminescence assays facilitate fast screening of antioxidant activity in food and natural health products.

2.1.3. *2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay*

DPPH radical scavenging assay is among the most frequently used methods and offers the first approach for evaluating antioxidant activity. It is an ET-based method with HAT mechanism being only a marginal reaction pathway in the assay (Prior et al., 2005). DPPH is a stable chromogen radical with a deep purple colour. It is commercially available and does not need to be generated prior to the assay. The DPPH scavenging assay is based on electron donation of antioxidants to neutralize DPPH radical. The reaction is accompanied with colour change of the DPPH measured at 517 nm, and the discolouration acts as an indicator of the antioxidant efficacy. The antioxidant activity by DPPH scavenging method is often reported as EC_{50} which is defined as the effective concentration of the antioxidant necessary to decrease the initial DPPH concentration by 50%. In addition, T_{EC50} which is the time needed to reach the steady state with EC_{50} may be used. Occasionally, antiradical efficiency (AE) is reported which combines EC_{50} and T_{EC50} into one parameter according to the equation $AE = (1/EC_{50})\ T_{EC50}$. The DPPH assay is a simple technique and requires only a UV spectrophotometer or an EPR (electron paramagnetic resonance) spectrometer. However, it has been argued that the scavenging of DPPH does not mimic the radical scavenging mechanism of antioxidants in real food or biological systems due to lack of oxygen radical in the assay (Benzie & Strain, 1999). Hence, this method is largely based on the assumption that antioxidant activity is equal to its electron donating capacity or so-called reducing power. Plank et al. (2012) conducted a collaborative study to determine antioxidant activity of a variety of foods and beverages using DPPH assay and validated the method for certain types of foods but challenged its suitability for others.

Even though the DPPH assay is simple and does not require special sample treatment, its sensitivity may be affected by a number of factors, such as the type and amount of solvent used, presence and concentration of hydrogen and metal ion and freshness of DPPH reagent (Dawidowicz, Wianowska, & Olszowy, 2012; Kedare & Singh, 2011; Musa, Abdullah, Kuswandi, & Hidayat, 2013). Modifications to the assay have been proposed by different authors in an attempt to minimize the aforementioned problems and further simplify and automate the method (Kedare & Singh, 2011). Musa et al. (2013) investigated the use of DPPH dry agent as an alternative to freshly prepare DPPH in an automated microplate-based assay. The modified method was validated with the classic DPPH assay, and according to the authors, it showed excellent sensitivity while being more convenient and simple to operate with minimal solvent required.

One major limitation of the spectrophotometric DPPH assay is the overlapped spectra of compounds that absorb in the same wavelength range as DPPH. For example, anthocyanins have strong absorption over the same wavelength range (500–550 nm) as DPPH, and thus may introduce interference with the results and their interpretation. Thus, EPR spectroscopy which directly measures the concentration of DPPH radical at submicromolecular levels may be employed. The EPR detection has advantages over the classic spectrophotometric detection, not only for highly coloured samples, but also for samples appearing turbid in the chosen solvent (Gardner, McPhail, & Duthie, 1998). The DPPH radical, having an unpaired electron, generates a characteristic and distinguishable EPR spectrum in a magnetic field under microwave radiation. The peak intensity in the EPR spectrum, which is proportional to the concentration of DPPH, decays with time in the presence of antioxidants as a result of DPPH radical scavenging by the antioxidants. The rate of DPPH signal decay or signal intensity drop at an end point may serve as an indicator of scavenging capacity of the antioxidant against DPPH radical (Zhong & Shahidi, 2011).

In addition to EPR spectroscopy, scavenging of DPPH radical can be monitored by amperometric detection. At an applied voltage, DPPH radical generates a constant electric current, and the decreased amount of radicals as a result of scavenging by antioxidants leads to a drop of the amperometric signal. Thus, quantification of antioxidants is accomplished via amperometric detection of the residual concentration of unreacted DPPH radical. Amatatongchai, Laosing, Chailapakul, and Nacapricha (2012) developed an amperometric flow injection (FI) method using a carbon nanotube modified-glassy carbon electrode as the working electrode to detect the DPPH radical continuously flowing through, while antioxidant samples are injected into the carrier stream. The authors demonstrated good linearity and precision of this method for evaluation of DPPH scavenging capacity of numerous antioxidants including gallic acid, catechin, quercetin, caffeic acid and trolox. Sireerat and Schulte (2012) determined the antioxidant activity of tea infusions, fruit juices and vegetable extracts using an automated amperometric DPPH assay, in which antioxidant capacity measurements were achieved using DPPH radical as an amperometric redox indicator, with a pencil Pb working electrode, in conjunction with a Pt counter-electrode and Ag/AgCl reference electrode. This method was validated by synthetic antioxidants of known concentrations.

DPPH radical scavenging assay has also been used in combination with other techniques for particular purposes. DPPH assay can be coupled with online HPLC for rapid screening of a large number of antioxidant samples which reduces the loss of antioxidants during purification processes. Online HPLC-DPPH methods are based on post-column reaction of antioxidant eluates with DPPH radicals. Crude antioxidant samples are first subjected to HPLC (normally reversed-phase) separation, and the eluate corresponding to each peak is reacted with DPPH radical at a certain flow rate for a constant time period (e.g. 60 seconds), resulting in loss of DPPH radical and the antioxidant itself, which can be recorded by UV detector (Kedare & Singh, 2011). Some methods monitor decrease of DPPH absorbance at 517 nm (Zhang et al., 2013), while others monitor decrease of antioxidant peak area (Qiu et al., 2012) after the post-column reaction. HPLC-DPPH methods can be further coupled with identification of the components showing scavenging capacity towards DPPH radical. Nuengchamnong et al. (2005) combined separation, antioxidant activity determination and structural identification of complex antioxidant mixtures in one assay, in which the eluates after column separation are split into two flows with the major portion going through the ESI/MS/MS analysis and the minor portion reacting with DPPH radical for antioxidant activity evaluation. Qiu et al. (2012) screened and identified antioxidant components in peanut shell using DPPH-HPLC-DAD-TOF/MS method. These methods are rapid and efficient as a means of antioxidant screening; however, they require sophisticated instruments which may not be readily available in every laboratory.

More recently, the DPPH assay has been re-evaluated by Xie and Schaich (2014) for its suitability for antioxidant activity assessment through kinetic and stoichiometric approaches. The authors questioned the application of DPPH assay for ranking antioxidants, and suggested its use to distinguish the active reaction mechanism of an antioxidant, namely ET and HAT, which exhibit different reaction rates and patterns in different solvents.

2.1.4. *Trolox equivalent antioxidant capacity (TEAC) assay*

TEAC assay was first developed by Miller et al. (1993) as a simple and convenient method for total antioxidant capacity (TAC) determination. The assay measures the ability of antioxidants to scavenge the stable radical cation $ABTS^{\bullet+}$ (2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)), a blue-green chromophore with maximum absorption at 734 nm which decreases in its intensity in the presence of antioxidants. $ABTS^{\bullet+}$ can be generated from ABTS in the presence of strong oxidizing agents. Antioxidants can neutralize the radical cation $ABTS^{\bullet+}$ by either direct reduction via electron donation or by radical quenching via hydrogen atom donation, and the balance of these two mechanisms is generally determined by antioxidant structure and pH of the medium (Prior et al., 2005). Hence, although TEAC assay is usually classified as an ET-based method, the HAT mechanism also applies. The extent of discolouration of the blue-green colour, quantified as a decrease in absorbance at 734 nm, depends on the duration of reaction, the intrinsic antioxidant activity and concentration in the sample. Similar to other radical scavenging assays discussed above, TEAC assay can either monitor the absorbance decay with time, or the drop at a certain end point. When operated as an end-point assay, TEAC method requires reliable determination of end point of the reaction for quantitative evaluation of antioxidant activity (Huang et al., 2005). Results obtained by TEAC assay are reported as trolox equivalents.

In the original TEAC assay, metmyoglobin and hydrogen peroxide are used to generate an intermediate ferrylmyoglobin radical, which then reacts with ABTS to produce $ABTS^{\bullet+}$. The oxidizing agent was later replaced by peroxide or persulphate. Potassium persulphate is the most commonly used oxidant for $ABTS^{\bullet+}$ generation. Modifications of the original assay have been proposed in many aspects, including the methods for generation of $ABTS^{\bullet+}$, wavelengths for monitoring the reaction, and the means of quantification, among others (Prior et al., 2005). Durmaz (2012) prepared a ready-to-use radical powder of $ABTS^{\bullet+}$ by incubating ABTS with potassium peroxodisulphate followed by freeze-drying of the resultant radical solution. This freeze-dried radical powder was then used as a replacement for the freshly prepared radical solution and its linearity and accuracy of the modified assay was demonstrated by the authors. In another version of the assay, the radical $ABTS^{\bullet-}$ anion was used as the target radical instead of the cation. $ABTS^{\bullet-}$ was generated via oxidation of $ABTS^{2-}$ by potassium persulphate, and the radical $ABTS^{\bullet-}$ anion was reduced back by antioxidants to the colourless $ABTS^{2-}$ product (Huang et al., 2005).

The TEAC assay has been used to measure the total antioxidant activity of pure substances, body fluids and plant materials. The TEAC assay, similar to other radical scavenging methods, can be automated and adapted to microplates and flow injection techniques (Milardovic, Kerekovic, & Rumenjak, 2007). It may also be coupled with HPLC by including a post column reaction with ABTS radical cation to facilitate the search of individual antioxidants from a complex mixture. HPLC-TEAC provides an efficient and rapid method for separation and identification of bioactive compounds from the source material (Gong, Plander, Xu, Simandi, & Gao, 2011; Raudonis, Bumblauskiene, Jakstas, Pukalskas, & Janulis, 2010). Better sensitivity and precision of TEAC assay can be achieved when coupled with other detection techniques, such as amperometric (Chan-Eam, Teerasong, Damwan, Nacapricha, & Chaisuksant, 2011; Milardovic et al., 2007) and FTIR (Cerretani et al., 2010), among others. Many of these modified TEAC assays employ online enzymatic generation of $ABTS^{\bullet+}$, especially those using continuous flow systems. For example, Milardovic et al. (2007) reported the generation of $ABTS^{\bullet+}$ by glucose oxidase and peroxidase separately immobilized in tubular flow-through reactors for flow injection analysis of alcoholic beverages for their TEAC values.

The TEAC assay is simple and can be performed as a routine analysis in almost any laboratory without the need for sophisticated equipments. $ABTS^{\bullet+}$ can be solubilized in both hydrophilic and lipophilic media and is not affected by ionic strength of the medium (Arnao, 2000). However, limitations of the TEAC assay have been noted, including the effect of incubation time, antioxidant/$ABTS^{\bullet+}$ ratio and end-point determination on TEAC values (Roginsky & Liss, 2005). The limitation of the applicability of TEAC assay to rank antioxidant activity was recently reported due to its failure to evaluate radical scavenging rate (Tian & Schaich, 2013). Moreover, like the DPPH scavenging assay, the TEAC assay has also been challenged for its lack of biological relevance due to use of the artificial ABTS radical cation that is not found in food or

biological systems. Nevertheless, the usefulness of these assays for ranking of antioxidant activity of similar substrates under comparable test conditions cannot be ignored.

2.2. *Non-radical redox potential-based methods*

Antioxidants are also known as reductants. Their electron donating capability can not only allow scavenging of free radicals and other oxygen derived ROS, but also reduce higher valent elements to their lower valence state. The redox potential or reducing power of antioxidants is an important indicator of their antioxidant efficacy, and is measured through redox reaction with various metal ions, such as iron, copper, chromium, and cerium, among others. This group of methods can be classified as ET-based methods and are conducted in simple chemical assays of less biological relevance, compared to ROS scavenging measurement (see below).

2.2.1. *Ferric reducing antioxidant power (FRAP) assay*

The FRAP assay is a typical ET-based method that measures the reduction of ferric ion (Fe^{3+})–ligand complex to the intensely blue coloured ferrous (Fe^{2+}) complex by antioxidants in acidic media. Antioxidant activity is determined as increase of absorbance at 593 nm, and results are expressed as micromolar Fe^{2+} equivalents or relative to an antioxidant standard (Antolovich et al., 2002). Unlike other ET-based methods, FRAP assay is carried out under acidic pH conditions (pH 3.6) in order to maintain iron solubility and more importantly drive electron transfer. This will increase the redox potential, causing a shift in dominant reaction mechanism (Hegerman et al., 1998).

The original FRAP assay uses tripyridyltriazine (TPTZ) as the iron-binding ligand, while alternative ligands have also been employed for ferric binding, such as ferrozine for ascorbic acid reducing power evaluation (Molina-Diaz, Ortega-Carmona, & Pascual-Reguera, 1998). More recently, potassium ferricyanide has been the most popular ferric reagent used in FRAP assays. In the latter case, Prussian blue is produced as the end product which is quantified spectrophotometrically and indicates the reducing power of the antioxidants tested. Production of Prussian blue may be through two different routes with the same outcome. Antioxidants can either reduce the Fe^{3+} in the solution to Fe^{2+}, which binds the ferricyanide to yield Prussian blue, or reduce the ferricyanide to ferrocyanide, which binds the free Fe^{3+} in the solution and forms Prussian blue. The simplified scheme for these two reactions is given blow (Berker, Gueclue, Tor, Demirata, & Apak, 2010).

$$\text{Antioxidant} + Fe^{3+} \rightleftarrows Fe^{2+} + \text{oxidized antioxidant}$$

$$Fe^{2+} + Fe(CN)_6^{3-} \rightleftarrows Fe[Fe(CN)_6]^- \text{ or,}$$

$$\text{Antioxidants} + Fe(CN)_6^{3-} \rightleftarrows Fe(CN)_6^{4-} + \text{oxidized antioxidant}$$

$$Fe(CN)_6^{4-} + Fe^{3+} Fe[Fe(CN)_6]^-$$

One drawback of this ferricyanide FRAP assay is the tendency of the Prussian blue to precipitate, forming a suspension and staining the measuring cuvette. Therefore, time to add Fe^{3+} ($FeCl_3$) is essential and may introduce error to result interpretation. In order to stabilize the Prussian blue against precipitation, Berker et al. (2010) proposed addition of the surfactant sodium dodecyl sulphate, while adjusting the optimal pH to 1.7 to maintain the redox activity of ferric ion without hydrolysis. The authors suggested that this modification also allows evaluation of antioxidants whose redox potential do not exceed that of the Fe^{3+}/Fe^{2+} in conventional FRAP assay (i.e. ferric reagent two weak to oxidize the antioxidants), such as thiol type physiological nonenzymatic antioxidants. The FRAP assay was further modified by choosing water/acetone as a solvent in the absence of solubility enhancer RMCD to enable simultaneous measurement of both hydrophilic and lipophilic antioxidants, the latter of which was restricted by conventional FRAP assay (Berker, Demirata, & Apak, 2012).

The FRAP assay is simple, fast and cost-effective and does not require specialized equipment. It was originally employed to measure reducing power in plasma, but its use has been extended for assessing antioxidant activity in other biological fluids, foods, and plant extracts. However, Pulido, Bravo, and Saura-Calixto (2000) reported that FRAP results might vary depending on the analysis time as observed for the reaction between antioxidants and Fe^{3+}, which ranged from several minutes to several hours. Therefore, a single-point absorption endpoint may not represent a complete reaction, since different antioxidants require different reaction times for detection (Prior et al., 2005).

More recently, the FRAP assay has adopted electrochemical detection techniques for better sensitivity, accuracy and reproducibility. A coulometric titration method has been developed to determine the FRAP value of different antioxidant materials (Abdullin, Turova, Budnikov, Ziyatdinova, & Gajsina, 2002; Ziyatdinova, Nizamova, & Budnikov, 2011; Ziyatdinova, Salikhova, & Budnikov, 2014). In this modified FRAP method, antioxidant reacts with coulometric titrants (electrogenerated ferricyanide ions), and the quantity of electricity consumed for the titration till the end point (when initial current is resumed) serves as indicator for reducing power of the antioxidant (Ziyatdinova et al., 2014). The FRAP assay by coulometric titration has been attractive as a highly sensitive, reliable and simple test for reducing power evaluation. Another electrochemical version of the FRAP assay is the chronoamperometric measurement of antioxidant reducing power. As demonstrated by Brainina, Varzakova, and Gerasimova (2012), a shift of potential in the $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ mediator system is indirectly correlated with the reducing power of the antioxidant present. More specifically, at certain potentials (higher than 0.35 V), oxidation process takes place only forming $K_3[Fe(CN)_6]$, and concentration of $K_4[Fe(CN)_6]$ is entirely from the reaction of $K_3[Fe(CN)_6]$ with antioxidant and is proportional to the reducing power of the antioxidant (Brainina et al., 2012). This chronoamperometric method has been proposed for assessing antioxidant activity of biological samples owing to its very low detection limit (e.g. 2×10^{-6} M for ascorbic acid).

The FRAP assay, in general, as an ET-based non-radical method, has been argued to have little relationship with radical quenching process (HAT mechanism) occurring in lipid systems, and has poor correlation with other antioxidant activity measurements. It is therefore suggested that this assay could be used in combination with other methods in distinguishing dominant mechanisms for different antioxidants (Prior et al., 2005).

2.2.2. *Cupric reducing antioxidant capacity (CUPRAC) assay*

The CUPRAC assay is a copper reduction assay developed as a variant of the FRAP assay. It uses copper as the oxidant instead of iron in the FRAP assay. The method measures the reducing power of antioxidants to convert cupric (Cu^{2+}) to cuprous (Cu^{+}) ion. Similar to the FRAP assay, a ligand is employed to form a copper–ligand complex to facilitate absorbance measurement. Neocuproine (Nc; 2,9-dimethyl-1,10-phenanthroline) is the ligand commonly employed in CUPRAC assay. The Cu^{2+}–neocuproine complex can be reduced by antioxidants to Cu^{+}–neocuproine, which is a chromophore with maximum absorption at 450 nm (Apak, Güçlü, Özyürek, & Karademir, 2004).

The original CUPRAC assay has been modified in order to accommodate different samples in diverse applications. For example, acetone/water medium with the aid of methyl-β-cyclodextrin has been employed for simultaneous determination of hydrophilic and lipophilic antioxidants (Őzyürek, Bektaşoğğlu, Güçlü, Güngör, & Apak, 2008). In order to evaluate antioxidant phenolics in both free and bound forms in the food matrix without preliminary extraction and the time and labour intensive hydrolysis process, a forced solubilization of bound antioxidants by the CUPRAC reagent was proposed, which takes advantage of the surface reaction phenomenon between solid (bound antioxidants) and liquid (soluble CUPRAC reagent) material (Tufan, Celik, Őzyürek, Güçlü, & Apak, 2013). This modified method was suggested to be applicable to relatively insoluble food matrices as well as to insoluble cosmetic products, such as creams, balms, and powders.

In another modified CUPRAC assay, an optical sensor containing immobilized chromogenic redox reagent was adopted for measuring the reducing power of liquid samples without any pretreatment (Bener, Őzyürek, Güçlü, & Apak, 2010). The CUPRAC reagent Cu^{2+}–neocuproine complex was immobilized onto a cation-exchanger film of Nafion, and the complex was reduced by antioxidants to Cu^{+}–neocuproine, giving rise to changes of absorbance at 450 nm, which was monitored spectrophotometrically. Another version of optical sensor aided CUPRAC assay used a miniature reflectance spectrometer to measure reflectance changes at 530 nm instead of absorbance (Bener, Őzyürek, Güçlü, & Apak, 2013). Use of optical sensor was suggested to significantly simplify the operation, exhibit reliability and robustness, and enable the *in situ* estimation of antioxidant capacity of various food extracts and biological samples.

2.2.3. *Other reducing power assays*

Metal ions other than those derived from iron and copper have been used as target of redox reaction of antioxidants. Such sources of metal ions include silver, gold, cerium, and chromium, among others. Reduction of Ag^{+} to spherical Ag nanoparticles by antioxidants in the presence of trisodium citrate and Ag seeds produces a very intense surface plasmon resonance (SPR) absorption band of Ag nanoparticles at 423 nm, which allows quantitative detection of antioxidants (Őzyürek, Güngör, Baki, Güçlü, & Apak, 2012). A similar method was developed utilizing resonance light scattering of gold nanoparticles, a reduction product from the reaction of Au^{3+} with antioxidants in the presence of cetyltrimethylammonium bromide (Andreu-Navarro, Fernandez-Romero, & Gomez-Hens, 2011). Detection of gold nanoparticles can also be achieved by an electrochemical method, such as cyclic voltammetric detection of enlarged gold nanoparticle on electrode surface, as a result of tetrachloroauric acid reduction by antioxidants (Wang, Zhou, Zhu, Huang, & Li, 2007).

Reduction of Ce^{4+} to Ce^{3+} by antioxidants can be employed for reducing power evaluation, as in the CERAC (cerium reducing antioxidant capacity) assay. In this assay, the remaining unreacted Ce^{4+} (sulphate salt) is measured spectrophotometrically at 320 nm, which is inversely related to the reducing power of the antioxidant under investigation (Ozyurt, Demirata, & Apak, 2010). In another cerium-based assay, the unreacted Ce^{4+} reacts with a known amount of chromogenic probe indigo carmine dye followed by measuring the unreacted indigo carmine dye solution at 610 nm (Padmarajaiah et al., 2012). In the recently developed CHROMAC (chromium reducing antioxidant capacity) assay, formation of Cr^{3+} complex from reduction of Cr^{6+} complex by antioxidants gave rise to absorbance increase at 540 nm (Isik, Sahin, & Demir, 2013).

Quaranta et al. (2013) proposed an enzyme-based method for evaluating reducing power of antioxidants. This method does not involve metal ions, but rather indirectly measures the reduction of azobismethylene quinone to syringaldazine by antioxidants. The reduction product syringaldazine is reoxidized by laccase accompanied by oxygen consumption, which is measured by an optical oxygen sensor. This assay was coupled with HPLC for online monitoring and identification of antioxidant components with distinguished reducing power.

The variety of reducing power assays allows selective evaluation of a broad range of antioxidants with varying redox potential while minimizing interference from reductants out of the redox potential range. In other words, it is important to select the proper metal or non-metal oxidizing agent that is strong enough to oxidize the antioxidants of interest but not too strong to oxidize other reductants such as citric acid and reducing sugars in the food matrix.

2.2.4. *Cyclic voltammetry*

Electrochemical methods for evaluating antioxidant activity have emerged in the past decade, among which cyclic voltammetry has attracted much attention as an alternative method to conventional chemical assays. Cyclic voltammetry measures electron donation capability (redox potential) of antioxidants, which respond to a voltametric scan according to their redox potential. A cyclic voltammogram is obtained which indicates the oxidation potential of a specific antioxidant compound or functional group (usually phenyl group) and its concentration.

The antioxidant activity is determined by three major parameters: (1) the anodic peak current (I_a), usually the y-axis on a cyclic voltammogram; (2) the peak oxidation potential (E_{pa}), usually the x-axis on a cyclic voltammogram; and (3) the area under anodic current wave (Q) (Zhang et al., 2011). The E_{pa} value indicates redox potential or electron donating capability of antioxidants. The closer the E_{pa} is to zero, the higher capability of the compound to donate an electron (Ragubeer, Beukes, & Limson, 2010). The I_a as the anodic current at maximum current response reflects the concentration of the antioxidant compound in the mixture. The Q value is related to the amount of charge transferred and has been proposed as indicator of overall antioxidant activity.

When more than one antioxidant component is present in a mixture, the Q value provides the total antioxidant activity of the mixture. Polyphenols with multiple phenol rings may show multiple peaks in the cyclic voltammogram. Zhang et al. (2011) analysed antioxidant activity of 14 flavonoid compounds by cyclic voltammetry and differentiated the peaks corresponding to the sequential oxidation of the hydroxyl groups at different positions, e.g. hydroxyl groups in different aromatic rings of quercetin, isorhamnetin, catechin, epicatechin, genistein and daidzein, among others.

Cyclic voltametric measurement of antioxidant activity as a redox-based method has many advantages over conventional chemical assays, because it is rapid and simple and can be used for initial screening of antioxidants based on reducing power. Cyclic voltammetry is also clean since it does not require sophisticated chemical reagents or solvents. No special sample preparation is required for the cyclic voltametric method, and it can be used to assess samples of any hydrophilicity or lipophilicity, as well as turbidity, which are restricted in many spectrophotometric methods (Ragubeer et al., 2010). The cyclic voltametric method has been used to determine the activity of antioxidants from various origins, including edible oils (Gulaboski, Mirceski, & Mitrev, 2013), chocolate (Brcanovic et al., 2013), dry fruits (Zielinski, Zielinska, & Kostyra, 2012), and marine algae (Keyrouz et al., 2011; Ragubeer et al., 2010), among others. However, similar to some other reducing power assays, cyclic voltametric determination of antioxidant activity may experience interference from other non-antioxidant reducing substances, such as reducing sugars.

2.3. *Metal chelation capacity*

Transition metal ions are known to stimulate lipid oxidation via Fenton reaction and also by decomposing lipid hydroperoxides into more reactive peroxyl and alkoxyl radicals. Some antioxidants such as flavonoids are powerful metal chelators. These antioxidants can easily deactivate prooxidant metal ions and thus prevent or retard metal ion-induced lipid oxidation. The antioxidant property of metal chelators is assessed when a complex is formed between the antioxidant and the metal, in such a way that metal ions can no longer act as an initiator of lipid oxidation. Therefore, metal chelation capacity is also used as an indicator of antioxidant activity, usually in combination with other antioxidant assays.

In general, metal chelation capacity is determined by measuring the chelating effect of antioxidants for ferrous ion. Ferrous sulphate and ferrozine are the most commonly used sources of ferrous ion. A loss of absorbance at 485 nm (for ferrous sulphate) or 562 nm (for ferrozine) after the addition of antioxidants represents the formation of metal–antioxidant complex, and metal chelation capacity of the added antioxidant can be quantified spectrophotometrically. In most cases, metal chelation capacity of antioxidants is expressed as EDTA (ethylenediaminetetraacetic acid) equivalents. EDTA is a standard metal chelator used in many food and pharmaceutical applications. Its regulatory status as a synthetic chemical prompts its replacement with other natural metal chelating antioxidants. Metal chelation capacity has been investigated for a number of antioxidants and extracts (Chandrasekara & Shahidi, 2010; Karawita et al., 2005; Wettasinghe & Shahidi, 2002; Wijeratne, Abou-Zaid, & Shahidi, 2006; Zhong et al., 2012).

2.4. *Total phenolic content (TPC)*

Total phenolic content (TPC) is another important parameter of total antioxidant capacity (TAC), and widely used for evaluation of antioxidant extracts, including extracts from herbs, spices and fruits, cereals and legumes, among others. The Folin–Ciocalteu assay is the well-known method for determination of TPC. The Folin–Ciocalteu assay was initially designed for the analysis of proteins, which takes advantage of phenolic amino acid tyrosine in proteins (Folin & Ciocalteu, 1927). It was later adopted by Singleton, Orthofer, and Lamuela-Raventos (1999) to analyse phenolic components in wine, after which it became a routine analysis for antioxidant assessment of food and plant extracts.

The Folin–Ciocalteu assay is based on the reduction of the Folin–Ciocalteu reagent by phenolic compounds under alkaline condition. The exact chemical nature of the Folin–Ciocalteu reagent is not clearly defined, but it is believed to contain phosphomolybdic/phosphotungstic acid complexes that are reduced to yield a blue-coloured chromophore with maximum absorption at 765 nm (Magalhaes, Segundo, Reis, & Lima, 2008). The molybdenum centre in the complexes is generally accepted as the reduction site, where the Mo^{6+} ion is reduced to Mo^{5+} by accepting an electron donated by the phenolic antioxidant. Thus, the Folin–Ciocalteu assay is as an ET-based assay and associated with the reducing power of phenolic antioxidants. Gallic acid is the commonly used reference standard and results of TPC is usually expressed as gallic acid equivalents. However, TPC results are also occasionally expressed as catechin, caffeic acid, chlorogenic acid, or ferulic acid equivalents, necessitating standardization of the reported results (Karadag, Ozcelik, & Saner, 2009).

The Folin–Ciocalteu assay for TPC determination has many advantages, including its simplicity, reproducibility and robustness. It does, however, suffer from several drawbacks. First, the test is sensitive to pH, temperature and reaction time (Karadag et al., 2009), hence proper selection of reaction condition is necessary for consistent and reliable results. Secondly, overestimation of TPC is a major concern for Folin–Ciocalteu assay, due to the contribution of non-phenolic reducing agents present in the system to the reduction of the Folin–Ciocalteu reagent (Blasco, Rogerio, Gonzalez, & Escarpa, 2005; Escarpa & González, 2001). Examples of such contaminants include reducing sugars and certain amino acids. Thus, results for TPC determination may be over-estimated by an order of magnitude compared to those obtained by HPLC methods. The assay is also carried out in aqueous systems and its application for lipophilic phenolics is limited unless modifications on solvent system are applied. Berker, Olgun, Ozyurt, Demirata, and Apak (2013) proposed NaOH-containing isobutanol/water medium for simultaneous measurement of hydrophilic and lipophilic phenolics.

TPC measured by alternative methods other than traditional Folin–Ciocalteu assay have been reported. Bonanni, Campanella, Gatta, Gregori, and Tomassetti (2007) demonstrated the use of a biosensor to measure the content of phenolic components, which were oxidized to quinones by tyrosinase while

consuming oxygen. Oxygen consumption was recorded amperometrically and correlated with the content of phenolic compounds. The same authors also proposed an OXY-adsorbent method that utilizes reaction of phenolic antioxidants with hyperchlorous acid (HClO) while quantifying the TPC by measuring the unreacted excess hyperchlorous acid spectrophotometrically through a chromogenous agent (Bonanni et al., 2007).

3. Antioxidant evaluation in food model systems

Since food is a major application area of antioxidants, their performance in the food matrix is important for selection and optimized usage level of antioxidants in food. For this purpose, model food systems are used for antioxidant activity evaluation.

Normally, most assessments of antioxidant activity are performed in oil, giving sensible prediction for the activity in oil or water-in-oil emulsions, whereas the results may be misleading for oil-in-water emulsions (Gordon, 2001). Similarly, antioxidant effectiveness in meat (fresh or cooked) differs from that in oil. Localization of antioxidants in the food matrix is believed to play an important role in their functional behaviour and hence efficiency. Antioxidants of different lipophilicity have different distribution pattern in the lipid-containing food system; some are preferentially distributed in either the water or lipid compartment while others tend to be localized at the interface (Porter, 1993). The interfacial phenomenon was widely accepted to provide a strong support for the so-called "polar paradox" theory. However, this theory was recently re-visited and its limitations noted as more factors were found to contribute to the complicated antioxidant behaviour patterns (Shahidi & Zhong, 2011). Thus, effectiveness and efficacy of antioxidants are only representative when a proper model system is selected.

It should also be ensured that the starting lipid does not contain high levels of oxidation products (Decker, Warner, Richards, & Shahidi, 2005). Several oxidation products of lipids such as hexanal and 2,4-decadienal have been reported to exhibit pro-oxidative effects (El-Magoli, Karel, & Yong, 1980). Furthermore, stripping of oils may be necessary in such evaluations because the endogenous antioxidants in non-stripped oils are found to enhance the oxidative stability of test samples, thus giving rise to erroneous results about the efficacy of antioxidants under investigation (Khan & Shahidi, 2000; Povilaityte, Cuvelier, & Berset, 2001). In addition to oils and fats, lipid substrates used for testing antioxidant activity may include fatty acids, fatty acid ethyl esters or triacylglycerols, and β-carotene (Amarowicz, Naczk, & Shahidi, 2000; Shahidi, Desilva, & Amarowicz, 2003).

Determination of antioxidants activity in food model systems is usually carried out under accelerated oxidation conditions. The combination of increased temperature and oxygen supply, addition of metal catalysts, and exposure of the reactants to light can reduce the oxidative stability of the food model system and shorten the time frame of the test to a large extent. Nevertheless, the elevated temperature may bring about changes in the oxidation mechanism thus causing difficulties in the prediction of antioxidant activity at low temperatures when compared with those at high temperatures (Antolovich et al., 2002; Gordon, 2001). Therefore, high oxidation temperatures (>60 °C) are not recommended for testing antioxidant activity (Decker et al., 2005), unless similar thermal treatment is involved in the processing of the food system under investigation. After the substrate is oxidized under standard conditions, the oxidation is monitored by chemical, instrumental or sensory methods. An appropriate measure of end point is essential for assessing antioxidant activity. Analytical strategies for end point determination include measurement at a fixed time point, measurement of reaction rate, lag phase measurement, and integrated rate measurement (Antolovich et al., 2002).

3.1. *Measurement of antioxidant activity in oil systems*

Bulk oil, triacylglycerols and free fatty acids or their alkyl ester are typical food model systems used for antioxidant evaluation. Methods vary depending on the particular scenario, but they all share the common principle, i.e. assessing the current oxidative state of the model system in the presence and absence of an antioxidant. The extent that the oxidation process is inhibited by added antioxidant indicates its antioxidant activity.

The process of lipid oxidation can be monitored through a number of chemical and physico-chemical procedures, including measuring the substrate, oxidant/initiator, intermediates or final products. Quantification of the loss of reactants, depletion of oxygen (oxygen consumption) and formation of primary or secondary oxidation products may provide the most appropriate markers depending on the stage of oxidation. Methods for measuring lipid oxidation have been reviewed elsewhere (Shahidi & Zhong, 2005).

3.1.1. *Measurement of oxygen consumption*

Consumption of oxygen during the initial stages of lipid oxidation results in an increase in oil weight or a drop of headspace oxygen pressure (or concentration). Therefore, changes in oxygen consumption caused by antioxidants serve as an indicator of antioxidant activity. The oxygen consumption is usually determined by means of monitoring weight gain or headspace oxygen uptake. Heating an oil and periodically testing for weight gain is one of the oldest methods for evaluating its oxidative state (Antolovich et al., 2002). To avoid the influence of mass change by volatiles, samples can be preheated in an inert atmosphere. The induction period can be obtained by plotting weight gain against storage time. While this method offers advantages such as simple operation and low instrumentation cost, it suffers from drawbacks of compromised reproducibility associated with discontinuous heating and weighing. It was suggested that this technique may be extended to more sophisticated continuous monitoring of mass and energy changes as in thermogravimetry (TG)/differential scanning calorimetry (DSC) (Antolovich et al., 2002). This method is most suitable for evaluation of highly unsaturated oils, such as marine oils and vegetable oils containing a high content of polyunsaturated fatty acids as the oxidizable substrates.

In addition to the weight gain method, oxygen consumption can be measured directly by monitoring the drop in oxygen pressure. Using headspace oxygen method, oil samples with and without antioxidants are placed in a closed vessel containing a fixed amount of headspace oxygen at elevated

temperatures, commonly around 100 °C. The pressure reduction in the vessel which is due to the oxygen consumption is monitored continuously and recorded automatically. The induction period as the point of maximum change in rate of oxygen uptake can be calculated (Velasco & Dobarganes, 2002). Pressure change caused by oxygen consumption can be measured electronically by a commercial instrument Oxidograph equipped with pressure transducers (Velasco & Dobarganes, 2002).

Oxygen consumption may also be measured electrochemically by detecting changes in oxygen concentration. The use of a semi-automatic polarographic method has been proposed as an improvement for evaluation of lipid oxidation by determination of oxygen consumption (Genot, Kansci, & Laroche, 1994). This method is based on the use of two oxygen meters with microcathode oxygen electrodes, coupled to a computerized data collection and a processing unit.

3.1.2. *Measurement of change in oxidation substrate*

Oxidative state of an oil model system may also be assessed quantitatively by measuring the loss of initial substrates. Unsaturated fatty acids are the main reactants whose composition changes significantly during oxidation. Changes in fatty acid composition provide a direct measure of the extent of lipid oxidation and in turn antioxidant efficacy, as antioxidants exert a significant inhibitory effect on oxidation of unsaturated fatty acids. Fatty acid methyl esters (FAME) are commonly used for determination of fatty acid composition by gas chromatography (GC). Similar to the oxygen consumption method, it is recommended that oils rich in highly unsaturated oils be used as the model system.

Selection of markers for fatty acid profile change is essential for determination of the degree of oxidation and antioxidant activity. While some methods use the ratio of different fatty acid groups, others monitor the change of a particular fatty acid. Kim, Yeo, Kim, Kim, and Lee (2013) presented antioxidant activity of sesamol in thermally treated lard and soybean oil by measuring changes in the ratio of total saturated fatty acids over total unsaturated fatty acids as well as the concentration of a specific fatty acid, caprylic acid in particular. Chung, Lee, and Choe (2006) used linolenic acid level changes in combination with conjugated dienes and *p*-anisidine value of fried products as indicators of lipid oxidation during frying in soybean oil and reported improved oxidative stability by addition of sesame oil in the frying oil. Decrease in linoleic acid and increase in oleic acid contents were correlated with oxidation of pine nut oil and antioxidant activity of tocopherols and carotene; the content of saturated fatty acids was not altered significantly during oxidation (Kim, Rhee, & Cheigh, 1995).

3.1.3. *Measurement of oxidation products*

Oxidation of fats/oils, triacylglycerols and free fatty acids or their alkyl esters leads to continuous formation of primary and secondary oxidation products. Antioxidants suppress oxidation reaction and inhibit the formation of oxidation products, which can be measured by different methods using a variety of oxidation markers, which are summarized in Table 2.

The peroxide value (PV) is an indicator of the initial stages of oxidative change, and inhibition of formation and/or action of these unstable species by antioxidants can be used for assessing antioxidant activity. A number of methods have been developed for determination of PV, among which the iodometric titration, ferric ion complex measurement spectrophotometry, and infrared spectroscopy are most frequently used (Yildiz, Wehling, & Cuppett, 2003). Iodometric titration assay, which is based on the oxidation of the iodide ion (I^-) to iodine by hydroperoxides, is the basis of current standard methods for PV determination. The liberated iodine (I_2) is titrated against a standardized solution of sodium thiosulphate and starch as an end point indicator. The PV is calculated and reported as milliequivalents of oxygen per kilogram of sample (meq/kg). Although iodometric titration is the most commonly practised method for PV measurement, it is time consuming and labour intensive and introduces difficulties in the titration end point determination. Furthermore, result interpretation may be confounded by possible absorption of iodine across unsaturated bonds and oxidation of iodide by dissolved oxygen (Shahidi & Wanasundara, 2002). Modifications such as colourimetric determination at 560 nm (Asakawa & Matsushita, 1978), potentiometric end point determination (Hara & Totani, 1988), and spectrophotometric determination of the I^{3-} chromophore at 290 or 360 nm (Gebicki & Guille, 1989; Hicks & Gebicki, 1979)

Table 2 – Antioxidant activity assays measuring oxidation products.

Name of assay	Oxidation marker	Reagent	Detection	Antioxidant activity expression
Peroxide value	Hydroperoxide	Potassium iodide	Titration	% inhibition
		Thiocyanate or xylenol orange	Spectrophotometry	% inhibition
		Triphenylphosphine	FTIR	% inhibition
Conjugated dienes	Conjugated dienes		Spectrophotometry	% inhibition
TBARS	MDA equivalent	Thiobarbituric acid	Spectrophotometry	% inhibition
p-anisidine value	2-alkenals and 2,4-alkadienals	Anisidine	Spectrophotometry	% inhibition
Total carbonyls	Carbonyl compounds	2,4-Dinitrophenylhydrazine	Spectrophotometry	% inhibition
Headspace volatile	Volatile compounds, e.g. hexanal, propanal		Headspace GC	% inhibition
Oil stability index (OSI)	Volatile organic acids		Conductivity measurement	Induction period, protection factor
Free radical measurement	Free radicals	α-Phenyl-*tert*-butylnitrone (PBN) spin trap	EPR	% inhibition

have been reported. An electrochemical technique has been proposed as an alternative to the titration step in order to increase the sensitivity for determination of low PV by reduction of released iodine at a platinum electrode maintained at a constant potential (Shahidi & Wanasundara, 2002).

Alternatively, PV can be determined spectrophotometrically by measuring the ability of lipid hydroperoxides to oxidize ferrous to ferric ions, which are complexed by either thiocyanate or xylenol orange to form red-violet or blue-purple chromophores, respectively (Eymard & Genot, 2003; Jiang, Woollard, & Wolff, 1991). The spectrophotometric methods are rapid, simple and more sensitive than iodometric titration method. However, because many factors such as the amount of oil sample, solvent used and source of reagents such as xylenol orange may affect the absorption coefficient, knowledge of the nature of hydroperoxides present in the oil sample and careful control of the conditions used are required for accurate measurements (Dobarganes & Velasco, 2002).

Lipid hydroperoxides can also be quantitatively determined by IR spectroscopy via measurement of their characteristic O—H stretching absorption band (Sedman, van de Voort, & Ismail, 1997). An absorption band at about 2.93 μm indicates the generation of hydroperoxides, whereas the replacement of a hydrogen atom on a double bond or polymerization accounts for the disappearance of a band at 3.20 μm. A rapid Fourier transform infrared (FTIR) spectroscopy method based on the stoichiometric reaction of triphenylphosphine (TPP) with hydroperoxides has been developed and successfully applied for PV determination (Dong, Ma, van de Voort, & Ismail, 1997). The hydroperoxides present in oil samples react stoichiometrically with TPP to produce triphenylphosphine oxide (TPPO), which has an intense absorption band at 542 cm^{-1} in the mid-IR spectrum (Riuz, Ayora-Canada, & Lendl, 2001). The band intensity is measured and converted to peroxide value. By using tert-butylhydroperoxide spiked oil standards and evaluation of the band formed at 542 cm^{-1} a linear calibration graph covering the range of 1–100 PV was obtained (Riuz et al., 2001). The FTIR method can also be coupled with flow injection analysis, which offers exact and highly reproducible timing of sample manipulation and reaction as well as a closed environment with oxygen and light being easily excluded (Riuz et al., 2001). The FTIR spectroscopy is a simple, rapid and highly precise method. It shows excellent correlation with the iodometric titration method and avoids the solvent and reagent disposal problems associated with the standard wet chemical method (Dong et al., 1997; Riuz et al., 2001). It provides an automated, efficient and low-cost means of evaluating oxidative state of lipid and efficiency of antioxidants in the model oil system undergoing accelerated oxidation induced by thermal stress (Innawong, Mallikarjunan, Irudayaraj, & Marcy, 2004; Kiritsakis, Kanavouras, & Kiritsakis, 2002). However, there is a need to characterize the spectral changes, assign wavelengths to more common molecular species produced and access potential spectral cross interferences (Kiritsakis et al., 2002). An improved FTIR attenuated total reflectance (FTR-ATR) method using the whole FTIR spectral data instead of particular wavenumbers has been proposed (Innawong et al., 2004).

In addition to the hydroperoxide marker, conjugated dienes and trienes can be used as indicators of the degree of oxidation and inhibition by antioxidant. Due to the fact that polyunsaturated fats and oils are generally methylene-interrupted in nature, conjugated dienes and trienes are formed upon oxidation, which can be measured spectrophotometrically at 234 and 268 nm in the ultraviolet (UV) region, respectively (Shahidi & Wanasundara, 2002). An increase in UV absorption theoretically reflects the formation of primary oxidation products in fats and oils. Therefore, antioxidant activity can be assessed by lag phase measurements and percentage inhibition in the presence of a certain antioxidant (Antolovich et al., 2002). Good correlations between conjugated dienes and peroxide value have been reported (Wanasundara, Shahidi, & Jablonski, 1995). Ultraviolet detection of conjugated dienes is simple, fast, and requires no chemical reagents and only small amounts of samples are needed. However, this method has less specificity and sensitivity than PV measurement. Furthermore, the result may be affected by the presence of compounds absorbing in the same region, such as carotenoids (Shahidi & Wanasundara, 2002). To avoid these interferences, an alternate spectroscopic method measuring conjugable oxidation products (COPs) has been proposed. In this method, hydroperoxides and some decomposition products are converted to more conjugated chromophores by reduction and dehydration. The concentrations of the resultant conjugated trienes and tetraenes are determined from their respective absorption at 268 and 301 nm and expressed as COP values (Gordon, 2001; Shahidi & Wanasundara, 2002).

The PV and conjugated dienes are primary oxidation products. They are unstable and decompose to more stable secondary oxidation products, such as a variety of carbonyl compounds, including aldehydes and ketones. Hence, primary oxidation products alone are less evidential and are usually reported in combination with secondary oxidation products measurement for a more comprehensive picture of the oxidation process. Among the secondary oxidation products produced from breakdown of hydroperoxides, TBARS (thiobarbituric acid reactive substances) are a well-known marker of oxidation process both for the early appearance as oxidation occurs and for the sensitivity of the analytical method (Cesa, 2004). TBARS are reported as malonaldehyde or malondialdehyde (MDA) equivalents. MDA, a degradation product of polyunsaturated fatty acids, reacts with TBA (thiobarbituric acid) and forms a pink MDA–TBA complex that has maximum absorption at 530–535 nm. Since certain oxidation products other than MDA also react with TBA and contribute to the colour intensity, the term TBARS is used to include an array of substances mainly carbonyl compounds that react with TBA in similar mechanism as that of MDA. These substances include alkanals, 2-alkenals, 2,4-alkdienals, ketones, ketosteroids, acids and esters, among others. Despite the lack of specificity and sensitivity (de las Heras, Schoch, Gibis, & Fischer, 2003), TBARS is among the most frequently used tests for assessing oxidation in fats and oils. In more complicated food matrix system, interference may arise from other non-lipid components such as proteins, sucrose, urea, pyridines, and pyrimidines, among others (Guillen-Sans & Guzman-Chozas, 1998; Jardine, Antolovich, Prenzler, & Robards, 2002). For improved specificity and sensitivity, TBARS test has been modified by using FTIR technology for direct detection of MDA and HPLC for separation and characterization of individual species of TBARS (Jardine et al., 2002). For oil systems containing antioxidants, a decrease in the amount of total TBARS

or a specific species can be quantified for oxidation inhibition and in other words efficiency of the antioxidants.

The *p*-anisidine value (*p*-AnV) is another measure of the content of aldehydic oxidation products (principally 2-alkenals and 2,4-alkadienals) generated during decomposition of hydroperoxides. It is based upon the colour reaction of *p*-methoxyaniline (anisidine) and the aldehydic compounds under acidic conditions which yields yellowish products that absorb at 350 nm (Doleschall, Kemeny, Recseg, & Kovari, 2002). The *p*-AnV is a reliable indicator of oxidative rancidity in fats and oils and thus a good approach to measure inhibition of oxidation by antioxidants. A highly significant correlation between *p*-AnV and PV has been found, although its significance may depend on a number of factors such as storage time and temperature, among others (List, Evans, Kwolek, Warner, & Boundy, 1974). Nevertheless, the same oil model system should be used when evaluating antioxidant activity by *p*-AnV method, since the initial AnV varies among oil sources, being higher for unsaturated oils than for saturated oils, due to stronger absorption of coloured products from unsaturated aldehydes than that from saturated aldehydes (White, 1995) at this wavelength.

The analysis of total carbonyl compounds provides an alternative approach to measure the extent of lipid oxidation in fats and oils and hence antioxidant activity in fat/oil model systems. This method is based on the absorbance of the carbonyl derivatives through a colourimetric 2,4-dinitrophenylhydrazone formation reaction. The carbonyl compounds formed during lipid oxidation are reacted with 2,4-dinitrophenylhydrazine (DNPH) followed by reaction of the resulting hydrazones with alkali. The final coloured products are then analysed spectrophotometrically (Melton, 1983). Many variations of this method using an alternate solvent, reagent, wavelength, or workup have been reported. The determination of total content of carbonyls has been used in different oxidative stability studies. However, it is worth noting that in food model systems other than fats and oils, carbonyls produced from oxidation of other components such as proteins may also contribute to the colourimetric reaction giving rise to higher total carbonyl values than expected. Meanwhile, high temperature must be avoided because some short-chain carbonyls may be removed and hence affecting the total carbonyls.

Some of the secondary oxidation products are volatile with varying threshold values, which contribute to the off-flavour or aroma of oxidized oils through different routes and mechanisms depending on nature of the oil and oxidation conditions. Measurement of headspace volatile compounds as total volatiles or specific compounds, usually volatile aldehydes, has been an effective tool for assessment of lipid oxidation and antioxidants in lipid-containing model systems. For oils rich in omega-6 fatty acids such as majority of vegetable oils, hexanal is the major volatile compound formed upon oxidation, while marine oils rich in omega-3 fatty acids produce mainly propanal (Zhong, Lall, & Shahidi, 2007). Gas chromatography (GC) equipped with a headspace sampler is used in determining secondary volatile oxidation products. More recently, headspace solid-phase microextraction (HS-SPME) technique has been used for analysis of volatile oxidation products. In this technique, the analytes are directly adsorbed from an aqueous or gaseous phase onto the fibre coating and later thermally desorbed onto the GC injector and transferred to the capillary column (Gioti, Fiamegos, Skalkos, & Stalikas, 2007). HS-SPME has been used for measurement of oxidation and antioxidant performance in model oil systems. Gioti et al. (2007) evaluated antioxidant activity of St. John's wort extract in a sunflower oil model system by simultaneous detection of MDA, pentanal and hexanal using HS-SPME technique. Lee, Kim, Chang, and Lee (2007) suggested the use of HS-SPME to determine antioxidant activity in a mixed free fatty acid model system using total volatiles or 2-heptenal as oxidation markers.

In addition to volatile aldehydes, volatile organic acids, mainly formic and acetic acids, are produced as secondary volatile oxidation products at high temperatures, simultaneously with hydroperoxides (Kiritsakis et al., 2002; Schwarz et al., 2001). Furthermore, other secondary products including alcohols and carbonyl compounds can be further oxidized to carboxylic acids (Kiritsakis et al., 2002). The formation of volatile acids can be measured by monitoring the change in electrical conductivity when effluent from oxidizing oils is passed through water (Gordon, 2001). This method provides the oil stability index (OSI) value, which is defined as the point of maximal change of oxidation rate, attributed to conductivity increase by the formation of volatile organic acids during lipid oxidation (Miura, Kikuzaki, & Nakatani, 2002). Two commercially available equipment, the Rancimat (Metrohm Ltd.) and the Oxidative Stability Instrument (Omnion Inc.) are employed for determining the OSI value. This method requires a somewhat higher level of oxidation (PV > 100) to obtain measurable results than other methods in which hydroperoxides are the most important products formed and detected (De la Presa-Owens, Lopez-Sabater, & Rivero-Urgell, 1995). Therefore, for some oils that are stable under normal conditions, the oxidation process is accelerated by exposing them to elevated temperatures in the presence of excess air or oxygen (Canizares-Macias, Garcia-Mesa, & Luque de Castro, 2004; Tan, Che-Man, Selamat, & Yusoff, 2002). In the OSI assay, a flow of air is bubbled through a heated oil, usually at 100 °C or above. For marine oils temperatures as low as 80 °C are often used. The OSI assay enables continuous monitoring of the oxidation process, and excellent correlation between Rancimat and conjugated dienes has been reported (Canizares-Macias et al., 2004). The OSI assay has been practised as industrial standard for oxidative stability of oils and effectiveness of antioxidant products. However, its application for evaluation of thermosensitive antioxidant compounds is questionable. Furthermore, it is not recommended for assessment of volatile antioxidants which may be swept out of the oil by the air flow under test conditions (Gordon, 2001).

Oxidation level of fats and oils in the absence and presence of antioxidants can also be measured directly by detecting the formation of radicals by using EPR spectroscopy. However, the short life times and low steady state concentration of the highly reactive lipid-derived radicals make it difficult to detect these radicals at concentrations lower than the 10^{-9} M minimum detectable concentration (Andersen & Skibsted, 2002). To overcome this problem, various approaches have been used, including pulse radiolysis and UV photolysis, continuous flow systems and spin trapping, among which spin trapping has been the most widely employed procedure (Antolovich et al., 2002). Spin trapping technique allows the accumulation of detectable concentrations of longer-lived radicals by addition to samples of a spin trapping agent, which reacts with free

radicals to form more stable spin-adducts (Andersen & Skibsted, 2002; Antolovich et al., 2002; Velasco, Anderson, & Skibsted, 2004). Nitroso compounds and nitrones are the most common spin traps, both leading to nitroxyl type spin-adducts such as α-phenyl-*tert*-butylnitrone (PBN)-adducts (Andersen & Skibsted, 2002). EPR spectroscopy is of great value for the study of the early stages of lipid oxidation and prediction of oxidative stability of fats and oils as well as evaluation of antioxidant activity. It has high sensitivity, and allows mild conditions by applying low temperatures and requiring little sample preparation. Nevertheless, spin traps used in the EPR assay have been reported to exhibit widely differing trapping efficiencies for different radicals and show both prooxidant and antioxidant effects (Halliwell & Gutteridge, 1999; Lee, 1994), thus may give erroneous results of antioxidant activity.

3.1.4. *Measurement of system change during oxidation process*

While a majority of methods measure the formation of oxidation products or consumption of reactants (substrates and oxygen), a few methods monitor the change of the whole system during the oxidation process without targeting particular species. These include NMR spectroscopy and DSC.

High-resolution ^{1}H NMR spectroscopy has been used to evaluate oxidative deterioration of fats and oils (Shahidi & Wanasundara, 2002). The principle of NMR is that hydrogen atoms in a strong magnetic field absorb radiation energy in the radiofrequency range, depending on their molecular environment, which changes during the oxidation process (Shahidi & Wanasundara, 1999). These changes may be monitored by NMR spectroscopy as a reflection of oxidation level of the oil or fatty acids. More specifically, the hydrogen atoms (proton, ^{1}H) located at different positions in the triacylglycerol or free fatty acid molecules can be calculated from the NMR spectra, and ratios of aliphatic to olefinic protons (Rao) and aliphatic to diallylmethylene protons (Rad) can be obtained (Shahidi & Wanasundara, 1999). Both ratios increase steadily during lipid oxidation, as the allylic and bisallylic positions are the reactive sites for oxidation. Attenuation of this ratio increment suggests antioxidant effectiveness in the oil or fatty acid model systems. NMR spectroscopy has been used by many researchers, and the changes in Rao and Rad measured by NMR correlated well with both conjugated dienes and TBA values (Senanayake & Shahidi, 1999).

In addition to ^{1}H NMR, ^{13}C NMR and ^{31}P NMR are also powerful tools to predict oxidative stability of oils. ^{13}C NMR enables direct observation of carbon atoms. The selectivity and dispersion of ^{13}C NMR spectra are very high (Diehl, 1998). ^{13}C NMR assesses lipid oxidation by monitoring the changes of carbon chains in triacylglycerol or fatty acid molecules, revealing the specific sites where oxidative degradation occurs (Medina, Sacchi, Giudicianni, & Aubourg, 1998). However, because the abundance of ^{13}C isotope is only 1.12% of that of ^{12}C, the sensitivity of ^{13}C NMR is usually much lower than that of ^{1}H NMR (Diehl, 1998).

NMR spectroscopy is a rapid, non-destructive and reliable technique for assessing lipid oxidation. It simultaneously measures both the primary and the secondary oxidative changes in the oil or fatty acid model systems, and provides specific information on oxidative regions in the substrate molecules. Using NMR for antioxidant test, a reduction in oxidation of the model system relative to a control sample is used as an index of antioxidant activity.

DSC is another tool to monitor the oxidation process in terms of change in energy. During lipid oxidation, fats and oils reveal a number of thermally-induced transitions such as the transfer of oxygen molecules to unsaturated fatty acids (exothermic process) (Tan & Che Man, 2002). Therefore, thermal analysis can be used in accelerated oxidation tests. The DSC technique, which is based on thermal release of oxidation reactions, has the potential as a non-chemical method for assessing oxidative stability of fats and oil model systems as affected by antioxidants (Velasco et al., 2004). It provides unique energy profile information, which specifically measures the temperature and heat flows associated with lipid oxidation as a function of time and temperature (Tan & Che Man, 1999). The method uses isothermal or non-isothermal conditions and a flow of oxygen as purge gas, with a calorimeter measuring the heat flow into (endothermic) or out of (exothermic) an oil model system undergoing oxidation changes (Tan & Che Man, 2002; Velasco et al., 2004). The oxidation curves of the sample are obtained with different heating time, and a dramatic increase for the evolved heat can be observed with the appearance of a sharp exothermic curve during initiation of oxidation. The end point is taken at the time where a rapid exothermic reaction between oil and oxygen occurs, and induction period (IP) determined automatically by intersection of extrapolated baseline and tangent line (leading edge) of the exotherm. The DSC also measures oxidation onset temperature, the temperature at maximum reaction, and the ending temperature (Tan & Che Man, 2002). The isothermal and non-isothermal DSC show good agreement, suggesting that both isothermal and non-isothermal DSC are suitable for oxidative stability studies of oils (Litwinienko, Daniluk, & Kasprzycka-Guttman, 1999). Addition of antioxidants can improve the oxidative stability of the model system. This improvement can be determined by DSC method and used to indicate the antioxidant activity. The DSC technique has been reviewed by Tan and Che Man (2002).

3.2. *Measurement of antioxidant activity in model emulsion systems*

Antioxidants are found to behave differently when used in different media. Hence, their efficacy in bulk oil may not necessarily reflect that in oil/water emulsions. Moreover, it has been recognized that high surface-to-volume ratio of emulsions are the natural conditions, while low surface-to-volume bulk lipid is more like an artifact that is less common in foods and biological systems. Many food lipids exist as oil-in-water emulsions (e.g. milk, mayonnaise, dressings, dips, sauces, beverages, ice cream, etc.), which are often more susceptible to oxidation than bulk oils due to their higher surface areas that promote interaction of the oil with prooxidants in the aqueous phase. Therefore, it is important to also include information on effectiveness of an antioxidant in oil/water emulsion systems for a more comprehensive assessment of antioxidant activity.

The β-carotene bleaching assay is one of the oldest and commonly practiced methods of estimating antioxidant activity in oil-in-water emulsion. In this method, the oxidative state of an emulsion composed of β-carotene, linoleic acid and water

is measured spectrophotometrically in the absence and presence of the antioxidant. Free radicals generated from oxidation of linoleic acid in the emulsion lead to discolouration (bleaching) of β-carotene, which can be monitored with time at 470 nm. The presence of antioxidants can reduce the loss and hence discolouration of β-carotene during the coupled oxidation of linoleic acid and β-carotene in the emulsified aqueous system. Usually, a kinetic curve of absorbance is obtained, which suggests how a certain antioxidant can attenuate the decrease of absorbance. The β-carotene bleaching assay in different versions has been applied in the assessment of various synthetic and natural antioxidant extracts. It was recently suggested that standardization of this method is necessary as the results can be significantly influenced by a number of factors, such as type and amount of solvent in which the antioxidants are dissolved, and presence of hydrogen and metal ions, among others (Dawidowicz & Olszowy, 2010).

In addition to the well-established β-carotene bleaching assay, there are many other emulsion model systems for antioxidant evaluation tailored for specific need. Such model systems include vegetable oil-in-water emulsion (e.g. sunflower oil, or soybean oil-in-water emulsions) (Abdalla & Roozen, 1999), fish oil-in-water emulsion (Farvin et al., 2014), or fatty acids/water emulsions (Wardhani, Fucinos, Vazquez, & Pandiella, 2013). Most oxidation markers discussed above for bulk oil model systems are also generally suitable for oil/water emulsions. Typical tests include measurement of PV, TBARS, and volatile hexanal or propanal, among others. Some emulsion model systems involve use of emulsifiers to stabilize the emulsion against lipid/water phase separation. However, emulsifier introduced in the system may affect antioxidant effectiveness and cause false interpretation of results. The quantity and type (cationic, anionic and non-ionic) of emulsifier in a heterogeneous system can drastically change the antioxidant distribution in the emulsified medium and in turn the antioxidant activity. Moreover, emulsifiers in high quantities (above critical micelle concentration) form micelles, which may trap antioxidants in these self-assembled structures and carry them to the water phase. Therefore, mass transfer of antioxidants in emulsions between micelles and the oil–water interface must be taken into consideration (Shahidi & Zhong, 2011).

3.3. *Measurement of antioxidant activity in muscle food model system*

Lipids are not only important components of bulk oils such as cooking oil and emulsions such as salad dressings and margarines, they are also incorporated into processed whole tissue foods such as in many fast foods and are responsible, to a large extent, for their quality deterioration. The lipids in processed whole tissue foods are characterized by a high surface-to-volume ratio and are usually considered a very polar medium (Porter, 1993). These foods generally require a larger amount and different types of antioxidants due to their predisposition to oxidation during precooking and long storage time under high surface-to-volume conditions (Porter, 1993). Cooked ground muscle foods provide an excellent model for assessing effectiveness of antioxidants in thermally processed whole or modified tissue foods, where heating causes rapid oxidation of lipid and meat flavour deterioration as well as development of "warmed-over flavour" (Rubin & Shahidi, 1988).

Lipid oxidation in muscle foods proceeds by different mechanisms and interaction with other components such as protein, haem, metal and salt as well as processing and storage conditions play important roles in their oxidative stability (Ladikos & Lougovois, 1990). For example, disruption of the muscle membrane by homogenization allows oxygen to penetrate inside the tissue and, in turn, accelerate lipid peroxidation (Pikul, 1992). Similarly, cooking causes breakdown of haem constituents and the release of iron. Non-haem iron is an active catalyst responsible for rapid oxidation of lipids in heated meat (Stodolak, Starzynska, Czyszczon, & Zyla, 2007). Proteins in meat also undergo oxidation during thermal treatment, and produce carbonyl compounds leading to quality deterioration. Addition of antioxidants can inhibit oxidation of both lipid and proteins, and the overall effect is considered as their efficiency in quality preservation.

Muscle food model systems used for antioxidant assessment vary depending on the ultimate application of the antioxidants. More general antioxidant screening typically utilizes ground pork, beef, poultry meat or fish, ground or fillet, in fresh, refrigerated, or cooked forms. Antioxidants may be added in different processing steps and their efficiency is evaluated by measuring the difference in levels of oxidative markers with and without antioxidants. The per cent inhibition in oxidation by antioxidants compared to a control without antioxidants can be calculated and reported as antioxidant efficiency. As already noted for other system, TBARS and headspace volatiles (hexanal, propanal and others) are common oxidative markers used for muscle foods. Measurement of TBARS in muscle foods differs slightly from that of oil, owing to the matrix structure that entraps the carbonyl lipid and protein oxidation products. The TBA reagent reacts directly with the meat sample, followed by extraction of the TBA-MDA and related adducts prior to the spectrophotometric analysis. Alternatively, the MDA equivalent TBARS are extracted either by steam distillation or solvent extraction and the extract is then reacted with the TBA reagent for subsequent absorbance measurement of the coloured product. Tarladgis, Watts, Younathan, and Dugan (1960) reported that the distillation of an acidified sample was essential to liberate MDA from precursor or bound forms, to produce maximal colour development and, especially, to separate TBARS from the food matrix (de las Heras et al., 2003). However, distillation method is generally considered to lack accuracy and reproducibility, and hence methods involving direct reaction of TBA reagent with food samples are becoming more popular for determining TBARS in meat. Like in oil model systems, TBARS in meat can also be detected by techniques other than spectrophotometry. Papastergiadis, Mubiru, van Langenhove, and de Meulenaer (2012) detected TBARS in pork sausage and cooked fish by spectrophotometric and HPLC methods and concluded that spectrophotometric method tended to overestimate the content of MDA and related products.

Headspace hexanal for meat and poultry and propanal for fish are frequently used oxidation marker and indicator for antioxidant activity (Hammond & Skonberg, 2012; Vuorela et al., 2005). Headspace volatile measurement is simple, rapid, and accurate with minimum sample treatment requirement; muscle tissue can be directly measured for the headspace volatile

without extraction or colourimetric reaction. However, special attention needs to be paid to possible loss of volatiles during meat processing and storage or conversion into tertiary oxidation products. It is recommended that the headspace hexanal/propanal method be used in combination with other oxidation tests for a full picture of antioxidant effectiveness.

4. Measurement of antioxidant activity in biological model systems

In addition to the use in food products as additives for quality preservation, antioxidants are of great interest to health professionals for their protective roles in the body against oxidative stress and the associated diseases and health disorders. *In vitro*, *ex vivo* and *in vivo* biological model systems are employed for assessment of antioxidants in fighting oxidative stress. Similar to the food model systems, biological systems used differ in the type of substrate, oxidation initiators, reaction conditions and oxidation markers. Typical biological systems for antioxidant potential evaluation are discussed in this section (section 4) and summarized in Table 3.

4.1. *LDL-cholesterol oxidation inhibition assay*

Oxidative modification of LDL-cholesterol plays a key role in the pathogenesis of atherosclerosis and heart disease (Sajilata, Bajaj, & Singhai, 2008). Oxidized LDL containing both lipid oxidation products and oxidized apoprotein particles contribute to all stages of the atherosclerosis process, thus promoting the atherogenic effects (Shahidi, Zhong, & Chandrasekara, 2012). Antioxidants such as many polyphenols can inhibit LDL oxidation by scavenging free radicals and other ROS, chelating prooxidant metals, and binding with the apo-lipoprotein B, which promotes the access of antioxidant to the lipids and prevents interaction with prooxidants (Satue-Gracia, Heinonen, & Frankel, 1997). Antioxidants are potential anti-atherogenic agents in cardiovascular diseases risk reduction, and their potencies can be assessed by the LDL-cholesterol oxidation inhibition assay.

In the LDL-cholesterol assay, oxidation of LDL-cholesterol can be induced by transition metals such as copper ion (Cu^{2+}) or peroxyl radical generated from thermal decomposition of AAPH. AAPH-generated peroxyl radical is considered a preferred oxidation initiator due to its biological relevance over the copper ion (Magalhaes et al., 2008). The oxidation substrate (LDL-cholesterol), initiator metal ion or peroxyl radical and antioxidants are incubated at physiological temperature of 37 °C, and formation of conjugated dienes is monitored spectrophotometrically at 234 nm periodically or at a certain end point. Antioxidant activity is reported as per cent inhibition of conjugated dienes formation as compared to a control without antioxidants. Alternatively, oxidative state of LDL-cholesterol can be measured by the formation of TBARS as MDA equivalents, which is more time-consuming and labour intensive. This assay is generally carried out in aqueous buffer but also occasionally in organic solvents and micelles. Reaction in micelles requires special sample treatment since direct spectrophotometry is not suitable (Karadag et al., 2009). The LDL-cholesterol oxidation inhibition assay has been used by many researchers for antioxidant activity and potential anti-atherogenic effect of various antioxidant compounds and extracts (Chandrasekara & Shahidi, 2011; Madhujith & Shahidi, 2007; Zhong & Shahidi, 2012b).

Similar to conjugated dienes method for antioxidants in oil model systems, food components that also absorb at 234 nm will interfere with the antioxidant activity assessment in the LDL-cholesterol model system. This interference may be minimized or eliminated by including a blank without the LDL substrate. Another drawback of this assay is that isolation and purification of LDL-cholesterol is a lengthy procedure and may introduce inconsistency which leads to compromised reproducibility of the assay.

4.2. *Inhibition of DNA oxidation and nicking*

Oxidative stress in cells also leads to DNA damage, which is implicated in mutagenesis and carcinogenesis, and other pathological processes as well as ageing. Oxidative damage to the DNA may occur at both the phosphate backbone and the nucleotide bases, resulting in a wide variety of modifications, including strand scission, sister chromatid exchange, DNA–DNA and DNA–protein cross-links as well as base modification (Davies, 1995). Antioxidant activity of many polyphenol compounds and extracts has been evaluated in DNA model systems for their potential as antimutagenic agents in cancer prevention and/or treatment (Chandrasekara & Shahidi, 2011; Zhong & Shahidi, 2012b).

Effect of antioxidants on free radical induced DNA strand scission has been determined in a supercoiled plasmid pBR322 DNA model system, where DNA strand breaking is induced by hydroxyl or peroxyl radicals (Chandrasekara & Shahidi, 2011; Zhong & Shahidi, 2012b). Hydroxyl and peroxyl radicals are selected because they are a major source of biologically relevant ROS responsible for oxidative damage of DNA, especially the

Table 3 – Evaluation of antioxidant activity in biological model systems.

Model system (substrate)	Oxidant	Oxidation marker	Detection
LDL-cholesterol	Cu^{2+} or AAPH-generated peroxyl radical	Conjugated dienes	Spectrophotometry
Supercoiled DNA	Fe^{2+}/hydrogen peroxide-generated hydroxyl radical, or AAPH-generated peroxyl radical	Open circular form of DNA, or linear form DNA	Gel electrophoresis
Red blood cells	Hydrogen peroxide, or AAPH-generated peroxyl radical	Haemolysis	Spectrophotometry, or turbidity measurement
HepG2 cells	ABAP-generated peroxyl radical	2′,7′-dichlorofluorescin	Fluorometry

AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; ABAP, 2,2′-azobis(2-amidinopropane) hydrochloride.

mitochondrial DNA (Perron, Hodges, Jenkins, & Brumaghim, 2008). Hydroxyl radical can be generated by the Fenton reaction between ferrous ion and hydrogen peroxide, while peroxyl radical is usually generated by AAPH. After incubation with radicals and antioxidants at 37 °C, the DNA fractions are separated by gel electrophoresis and bands identified after visualization. Both open circular form of the DNA from single strand cleavage and linear form from double strand cleavage may be observed as results of DNA oxidation, with the open circular form being dominant. The concentration of the native (supercoiled) and nicked DNA fractions is obtained from densitometer as indicated by the intensity or density of the corresponding bands. Antioxidants inhibit DNA scission possibly through a combination of radical scavenging and ferrous ion chelation mechanisms, and their inhibition efficiency can be calculated as DNA retention (per cent of DNA retained un-oxidized and supercoiled).

The DNA oxidation inhibition assay provides valuable information on the efficacy of antioxidants in protecting DNA from oxidative stress, which may suggest their anti-mutagenic and anti-carcinogenic potentials.

4.3. *Cellular assays for evaluation of antioxidant activity*

Development of the *in vitro* chemical assays and use of biologically relevant macromolecule model systems have enabled evaluation of intrinsic antioxidant activity of a great number of synthetic bioactive compounds and natural extracts. However, these tests fail to predict the antioxidant activity *in vivo* in many circumstances, due to the cellular absorption, metabolism and antioxidant behaviour in the more complicated cellular environment that varies from one substance to another. Since it is not always an easy approach to measure antioxidant effectiveness directly in animal or human subjects for initial antioxidant screening, it is suggested that cellular models be used for a more comprehensive understanding of antioxidant activity at cellular level and better correlation with their performance *in vivo*. Cell culture models provide cost-effective and relatively fast approach that addresses the issues of uptake, distribution, and metabolism of antioxidant compounds (Wolfe & Liu, 2007).

4.3.1. *Haemolysis inhibition assay*

Plasma serves as an excellent substrate of oxidation and model system for antioxidant evaluation. It is biologically relevant and suitable for both hydrophilic and lipophilic antioxidants (Omata, Yoshida, & Niki, 2010). The erythrocytes (red blood cells, RBC) are constantly exposed to oxidative stress owing to their specific role as oxygen carrier in the blood. The RBC, lacking nucleus, mitochondrial and other typical cellular organelles as well as sufficient self-repair mechanisms, provide a simplified, yet sensitive, model for antioxidant assessment (Giao et al., 2010). The ROS, especially hydrogen peroxide as a normal cell metabolite released from enzymatic and nonenzymatic reactions, can initiate oxidation of protein (haemoglobin) and lipid (mainly cholesteryl ester), leading to destruction of the cell shape and membrane structure and ultimately haemolysis (Giao et al., 2010).

Capability of antioxidants to inhibit haemolysis can be measured for evaluation of antioxidant activity. Hydrogen peroxide and AAPH generated peroxyl radical have been used as common oxidation initiators, among others. After incubation with the oxidation initiator and antioxidant at 37 °C, the degree of haemolysis can be determined periodically by centrifugation and spectrophotometrically measuring the concentration of released haemoglobin in the supernatant solution at 524 nm (Giao et al., 2010; Tai, Sawano, Yazama, & Ito, 2011). Inhibition of haemolysis by antioxidants can then be calculated by comparing with a control containing no antioxidant. The haemolysis inhibition assay has been used to evaluate efficacy of a number of antioxidant compounds and extracts. The original spectrophotometric determination of the haemolysis is time-consuming and labour intensive, and has recently been modified using the automated microplate reader technology which allows simultaneous measurement of a large number of antioxidant samples. In this modified method, the degree of haemolysis is determined by turbidity (through light scattering detection) of the erythrocyte suspension without centrifugation (Takebayashi, Iwahashi, Ishimi, & Tai, 2012).

4.3.2. *Cellular antioxidant assay (CAA)*

Cellular antioxidant assay (CAA) was recently developed to quantitatively measure capability of antioxidants in inhibiting oxidation in cells (Song et al., 2010; Wolfe et al., 2008; Wolfe & Liu, 2007). Different from haemolysis inhibition assay, CAA takes advantage of both cell culture models and chemical assays by introducing a special fluorescent probe into the cells. In this method, the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) probe is taken up by the cultured HepG2 human hepatocarcinoma cells, where the absorbed DCFH-DA is activated to DCFH through cellular esterases-catalysed deacetylation. DCFH is sensitive to ROS such as hydrogen peroxide and peroxyl radical and RNS (reactive nitrogen species) such as nitric oxide and peroxynitrite, and is converted to its oxidation product DCF which is fluorescent upon excitation. The CAA specifically uses ABAP as peroxyl radical generator to initiate oxidation of DCFH to DCF. The fluorescence intensity measured is proportional to the level of oxidation. Antioxidants absorbed into the cells can scavenge the peroxyl radical resulting in lower degree of oxidation observed as attenuated fluorescence increase. Per cent inhibition of oxidation by antioxidants can be calculated as indicator of antioxidant activity. In-depth description of this method and its use to assess different types of antioxidants is available (Wolfe & Liu, 2007).

The CAA assay is believed to offer many advantages for antioxidant assessment in biological model systems. These include use of biologically relevant substrate under physiological conditions, low level of oxidants (ABAP concentration), cellular uptake of antioxidants being considered that correlates with bioavailability *in vivo*, among others (Wolfe & Liu, 2007). Like most chemical assays utilizing fluorescence probes, CAA is automated with microplate reader technology and net area under the curve (AUC) integration calculation, allowing kinetic study of all oxidation reaction stages including lag time.

The CAA has been used to evaluate efficacy of a variety of antioxidant compounds and extracts, including extracts of common fruits, vegetables, and dietary supplements (Song et al., 2010; Wolfe et al., 2008; Wolfe & Liu, 2007). It was proposed as a standardized method for measuring cellular antioxidant activity in order to minimize cross laboratory variations caused

by difference in types of cell lines and oxidants, cell treatment procedure and quantification method for oxidation and antioxidant activity, etc. The CAA is attracting more attention from researchers as a result of growing bioavailability issue with many bioactive phytochemicals. However, stability of the cell line and other issues have raised concern about reproducibility of results and opportunity to compare results from different laboratories with one another, among other concerns. It should also be noted that proper choice of cell lines may well address this shortcoming, e.g. use of Caco2 or other cells instead of HepG2 cells.

5. Summary

Antioxidants are of growing interest to food scientists, health professionals and the general public owing to their protective roles in food products against oxidative deterioration and in the body against oxidative stress-mediated pathological processes. Natural and potent antioxidants are in demand for food preservatives and nutraceuticals/pharmaceuticals. Effective search for sources of naturally occurring antioxidants and design of novel antioxidant compounds require reliable methods of antioxidant activity evaluation. A number of chemical assays, food and biological model systems have been developed to measure at molecular or cellular level the radical scavenging capacity, reducing power, and other specific attributes of antioxidants as well as overall oxidation inhibition in more complex food and biological systems. These methods vary in terms of antioxidant mechanism, substrate type, oxidation initiator, result expression and ease of operation. Proper method selection or combination of methods is important for valid assessment of antioxidant activity and ultimately potential of the antioxidant as food preservative or health promoting agents.

REFERENCES

Abdalla, A. E., & Roozen, J. P. (1999). Effect of plant extracts on the oxidative stability of sunflower oil and emulsion. *Food Chemistry*, *64*, 323–329.

Abdullin, I. F., Turova, E. N., Budnikov, G. K., Ziyatdinova, G. K., & Gajsina, G. K. (2002). Electrogenerated bromine – Reagent for determination of antioxidant capacity of juices and extracts. *Zavodskaya Laboratoriya Diagnostika Materialov*, *68*, 12–15.

Amarowicz, R., Karamac, M., & Shahidi, F. (1999). Synergistic activity of capelin protein hydrolysates with synthetic antioxidants in a model system. *Journal of Food Lipids*, *6*, 271–275.

Amarowicz, R., Naczk, M., & Shahidi, F. (2000). Antioxidant activity of crude tannins of canola and rapeseed hulls. *American Oil Chemists' Society*, *77*, 957–961.

Amatatongchai, M., Laosing, S., Chailapakul, O., & Nacapricha, D. (2012). Simple flow injection for screening of total antioxidant capacity by amperometric detection of DPPH radical on carbon nanotube modified-glassy carbon electrode. *Talanta*, *97*, 267–272.

Andersen, M. L., & Skibsted, L. H. (2002). Detection of early events in lipid oxidation by electron spin resonance spectroscopy. *European Journal of Lipid Science and Technology*, *104*, 65–68.

Andreu-Navarro, A., Fernandez-Romero, J. M., & Gomez-Hens, A. (2011). Determination of antioxidant additives in foodstuffs by direct measurement of gold nanoparticle formation using resonance light scattering detection. *Analytica Chimica Acta*, *695*, 11–17.

Antolovich, M., Prenzler, P. D., Patsalides, E., McDonald, S., & Robards, K. (2002). Methods for testing antioxidant activity. *The Analyst*, *127*, 183–198.

Apak, R., Gorinstein, S., Böhm, V., Schaich, K. M., Özyürek, M., & Güçlü, K. (2013). Methods of measurement and evaluation of natural antioxidant capacity/activity. *Pure and Applied Chemistry*, *85*, 957–998.

Apak, R., Güçlü, K., Özyürek, M., & Karademir, S. E. (2004). Novel total antioxidant capacity index for dietary polyphenols, vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. *Journal of Agricultural and Food Chemistry*, *52*, 7970–7981.

Arnao, M. B. (2000). Some methodological problems in the determination of antioxidant activity using chromogen radicals: A practical case. *Trends in Food Science and Technology*, *11*, 419–421.

Asakawa, T., & Matsushita, S. (1978). Colorimetric determination of peroxide value with potassium iodide-silica gel reagent. *American Oil Chemists' Society*, *55*, 619–620.

Athukorala, Y., Lee, K. W., Song, C., Ahn, C. B., Shin, T. S., Cha, Y. J., Shahidi, F., & Jeon, Y. J. (2003). Potential antioxidant activity of marine red alga Grateloupia filicina extracts. *Journal of Food Lipids*, *10*, 251–263.

Barahona, T., Chandia, N. P., Encinas, M. V., Matsuhiro, B., & Zuniga, E. A. (2011). Antioxidant capacity of sulfated polysaccharides from seaweeds. A kinetic approach. *Food Hydrocolloids*, *25*, 529–535.

Becker, E. M., Nissen, L. R., & Skibsted, L. H. (2004). Antioxidant evaluation protocols. Food quality or health effects. *European Food Research and Technology*, *219*, 561–571.

Beckman, K. B., & Ames, B. N. (1998). The free radical theory of aging matures. *Physiological Reviews*, *78*, 547–581.

Bener, M., Őzyürek, M., Güçlü, K., & Apak, R. (2010). Development of a low-cost optical sensor for cupric reducing antioxidant capacity measurement of food extracts. *Analytical Chemistry*, *82*, 4252–4258.

Bener, M., Őzyürek, M., Güçlü, K., & Apak, R. (2013). Novel optical fiber reflectometric CUPRAC sensor for total antioxidant capacity measurement of food extracts and biological samples. *Journal of Agricultural and Food Chemistry*, *61*, 8381–8388.

Benzie, I. F. F., & Strain, J. J. (1999). Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods in Enzymology*, *299*, 15–27.

Berker, K. I., Demirata, B., & Apak, R. (2012). Determination of total antioxidant capacity of lipophilic and hydrophilic antioxidants in the same solution by using ferric-ferricyanide assay. *Food Analytical Methods*, *5*, 1150–1158.

Berker, K. I., Gueclue, K., Tor, I., Demirata, B., & Apak, R. (2010). Total antioxidant capacity assay using optimized ferricyanide/Prussian blue method. *Food Analytical Methods*, *3*, 154–168.

Berker, K. I., Olgun, F. A. O., Ozyurt, D., Demirata, B., & Apak, R. (2013). Modified Folin-Ciocalteu antioxidant capacity assay for measuring lipophilic antioxidants. *Journal of Agricultural and Food Chemistry*, *61*, 4783–4791.

Bezzi, S., Loupassaki, S., Petrakis, C., Kefalas, P., & Calokerinos, A. (2008). Evaluation of peroxide value of olive oil and antioxidant activity by luminol chemiluminescence. *Talanta*, *77*, 642–646.

Blasco, A. J., Rogerio, M. C., Gonzalez, M. C., & Escarpa, A. (2005). Electrochemical index as a screening method to determine total polyphenolics in foods: A proposal. *Analytica Chimica Acta*, *539*, 237–244.

Blois, M. S. (1958). Antioxidant determination by use of a stable freeradical. *Nature*, *181*, 1199–1200.
Bonanni, A., Campanella, L., Gatta, T., Gregori, E., & Tomassetti, M. (2007). Evaluation of the antioxidant and prooxidant properties of several commercial dry spices by different analytical methods. *Food Chemistry*, *102*, 751–758.
Brainina, K. Z., Varzakova, D. P., & Gerasimova, E. L. (2012). A chronoamperometric method for determining total antioxidant activity. *Journal of Analytical Chemistry*, *67*, 364–369.
Brcanovic, J. M., Pavlovic, A. N., Mitic, S. S., Stojanovic, G. S., Manojlovic, D. D., Kalicanin, B. M., & Veljkovic, J. N. (2013). Cyclic voltammetric determination of antioxidant capacity of cocoa powder, dark chocolate and milk chocolate samples: Correlation with spectrophotometric assays and individual phenolic compounds. *Food Technology and Biotechnology*, *51*, 460–470.
Canizares-Macias, M. P., Garcia-Mesa, J. A., & Luque de Castro, M. D. (2004). Determination of the oxidative stability of olive oil, using focused-microwave energy to accelerate the oxidation process. *Analytical and Bioanalytical Chemistry*, *378*, 479–483.
Cerretani, L., Giuliani, A., Maggio, R. M., Bendini, A., Gallina Toschi, T., & Cichelli, A. (2010). Rapid FTIR determination of water, phenolics and antioxidant activity of olive oil. *European Journal of Lipid Science and Technology*, *112*, 1150–1157.
Cesa, S. (2004). Malondialdehyde contents in infant milk formulas. *Journal of Agricultural and Food Chemistry*, *52*, 2119–2122.
Chan-Eam, S., Teerasong, S., Damwan, K., Nacapricha, D., & Chaisuksant, R. (2011). Sequential injection analysis with electrochemical detection as a tool for economic and rapid evaluation of total antioxidant capacity. *Talanta*, *84*, 1350–1354.
Chandrasekara, A., & Shahidi, F. (2010). Content of insoluble bound phenolics in millets and their contribution to antioxidant capacity. *Journal of Agricultural and Food Chemistry*, *58*, 6706–6714.
Chandrasekara, A., & Shahidi, F. (2011). Bioactivities and antiradical properties of millet grains and hulls. *Journal of Agricultural and Food Chemistry*, *59*, 9563–9571.
Christodouleas, D., Fotakis, C., Papadopoulos, K., Yannakopoulou, E., & Calokerinos, A. C. (2009). Development and validation of a chemiluminogenic method for the evaluation of antioxidant activity of hydrophilic and hydrophobic antioxidants. *Analytica Chimica Acta*, *652*, 295–302.
Chung, J., Lee, Y., & Choe, E. (2006). Effects of sesame oil addition to soybean oil during frying on the lipid oxidative stability and antioxidants contents of the fried products during storage in the dark. *Journal of Food Science*, *71*, C222–C226.
Collomb, M., & Spahni, M. (1996). Review of the methods for determining lipid oxidation products, in particular lipids from milk products. *Schweizerische Milchwirtschaftliche Forschung*, *25*, 3–24.
Cumby, N., Zhong, Y., Naczk, M., & Shahidi, F. (2008). Antioxidant activity and water-holding capacity of canola protein hydrolysates. *Food Chemistry*, *109*, 144–148.
de las Heras, A., Schoch, A., Gibis, M., & Fischer, A. (2003). Comparison of methods for determining malondialdehyde in dry sausage by HPLC and the classic TBA test. *European Food Research and Technology*, *217*, 180–184.
Dalton, T. P., Shertzer, H. G., & Puga, A. (1999). Regulation of gene expression by reactive oxygen. *Annual Review of Pharmacology and Toxicology*, *39*, 67–101.
Davies, K. J. (2000). Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. *IUBMB Life*, *50*, 279–289.
Davies, K. J. A. (1995). Oxidative stress: The paradox of aerobic life. In C. Rice-Evans, B. Halliwell, & G. G. Lunt (Eds.), *Free radical and oxidative stress: Environments, drugs and food additives* (pp. 1–31). London, UK: Portland Press Ltd.
Dawidowicz, A. L., & Olszowy, M. (2010). Influence of some experimental variables and matrix components in the determination of antioxidant properties by β-carotene bleaching assay: Experiments with BHT used as standard antioxidant. *European Food Research and Technology*, *231*, 835–840.
Dawidowicz, A. L., Wianowska, D., & Olszowy, M. (2012). On practical problems in estimation of antioxidant activity of compounds by DPPH method (problems in estimation of antioxidant activity). *Food Chemistry*, *131*, 1037–1043.
De la Presa-Owens, S., Lopez-Sabater, M. C., & Rivero-Urgell, M. (1995). Shelf-life prediction of an infant formula using an accelerated stability test (Rancimat). *Journal of Agricultural and Food Chemistry*, *43*, 2879–2882.
Decker, E. A., Warner, K., Richards, M. P., & Shahidi, F. (2005). Measuring antioxidant effectiveness in Foods. *Journal of Agricultural and Food Chemistry*, *53*, 4303–4310.
Diehl, B. W. L. (1998). Multinuclear high-resolution nuclear magnetic resonance spectroscopy. In R. J. Hamilton (Ed.), *Lipid analysis in oils and fats* (pp. 87–135). London: Blackie Academic & Professional.
Dobarganes, M. C., & Velasco, J. (2002). Analysis of lipid hydroperoxides. *European Journal of Lipid Science and Technology*, *104*, 420–428.
Doleschall, F., Kemeny, Z., Recseg, K., & Kovari, K. (2002). A new analytical method to monitor lipid peroxidation during bleaching. *European Journal of Lipid Science and Technology*, *104*, 14–18.
Dong, J., Ma, K., van de Voort, F. R., & Ismail, A. A. (1997). Stoichiometric determination of hydroperoxides in oils by Fourier transform near infrared spectroscopy. *Journal of AOAC International*, *80*, 345–352.
Durmaz, G. (2012). Freeze-dried $ABTS^{\bullet+}$ method: A ready-to-use radical powder to assess antioxidant capacity of vegetable oils. *Food Chemistry*, *133*, 1658–1663.
El-Magoli, S. B., Karel, M., & Yong, S. (1980). Acceleration of lipid oxidation by volatile products of hydroperoxide decomposition. *Journal of Food Biochemistry*, *3*, 111–124.
Escarpa, A., & González, M. C. (2001). An overview of analytical chemistry of phenolic compounds in foods. *Critical Reviews in Analytical Chemistry*, *31*, 57–139.
Eymard, S., & Genot, C. (2003). A modified xylenol orange method to evaluate formation of lipid hydroperoxides during storage and processing of small pelagic fish. *European Journal of Lipid Science and Technology*, *105*, 497–501.
Farvin, K. H. S., Andersen, L. L., Nielsen, H. H., Jacobsen, C., Jakobsen, G., Johansson, I., & Jessen, F. (2014). Antioxidant activity of Cod (Gadus morhua) protein hydrolysates: In vitro assays and evaluation in 5% fish oil-in-water emulsion. *Food Chemistry*, *149*, 326–334.
Fassoula, E., Economou, A., & Calokerinos, A. (2011). Development and validation of a sequential-injection method with chemiluminescence detection for the high throughput assay of the total antioxidant capacity of wines. *Talanta*, *85*, 1412–1418.
Floyd, R. A., & Hensley, K. (2002). Oxidative stress in brain aging. Implications for therapeutics of neurodegenerative diseases. *Neurobiology of Aging*, *23*, 795–807.
Folin, O., & Ciocalteu, V. (1927). On tyrosine and tryptophane determinations in proteins. *Journal of Biological Chemistry*, *73*, 627–650.
Gardner, P. T., McPhail, D. B., & Duthie, G. G. (1998). Electron spin resonance spectroscopic assessment of the antioxidant potential of teas in aqueous and organic media. *Journal of the Science of Food and Agriculture*, *76*, 257–262.
Gebicki, J. M., & Guille, J. (1989). Spectrophotometric and high performance chromatographic assays of hydroperoxides by the iodometric technique. *Analytical Biochemistry*, *176*, 360–364.

Genot, C., Kansci, G., & Laroche, M. (1994). Measurement of phospholipid oxidation in model membranes by determination of oxygen consumption with a semi-automic polarographic method. *Sciences Des Aliments*, *14*, 673–682.

Giao, M. S., Leitao, I., Pereira, A., Borges, A. B., Guedes, C. J., Fernandes, J. C., Belo, L., Santos-Silva, A., Hogg, T. A., Pintado, M. E., & Malcata, F. X. (2010). Plant aqueous extracts: Antioxidant capacity via haemolysis and bacteriophage P22 protection. *Food Control*, *21*, 633–638.

Giokas, D. L., Vlessidis, A. G., & Evmiridis, N. P. (2007). On-line selective detection of antioxidants free-radical scavenging activity based on Co(II)/EDTA-induced luminol chemiluminescence by flow injection analysis. *Analytica Chimica Acta*, *589*, 59–65.

Gioti, E. M., Fiamegos, Y. C., Skalkos, D. C., & Stalikas, C. D. (2007). Improved method for the in vitro assessment of antioxidant activity of plant extracts by headspace solid-phase microextraction and gas chromatography-electron capture detection. *Journal of Chromatography. A*, *1152*, 150–155.

Godoy-Navajas, J., Aguilar-Caballos, M. P., & Gomez-Hens, A. (2011). Long-wavelength fluorimetric determination of food antioxidant capacity using Nile blue as reagent. *Journal of Agricultural and Food Chemistry*, *59*, 2235–2240.

Gong, Y., Plander, S., Xu, H., Simandi, B., & Gao, Y. (2011). Supercritical CO_2 extraction of oleoresin from marigold (Tagetes erecta L.) flowers and determination of its antioxidant components with online HPLC-$ABTS^+$ assay. *Journal of the Science of Food and Agriculture*, *91*, 2875–2881.

Gordon, M. (2001). Measuring antioxidant activity. In J. Pokorny, N. Yanishlieva, & M. Gordon (Eds.), *Antioxidants in food: Practical applications* (pp. 71–84). Cambridge, England: Woodhead Publishing Ltd.

Guclu, K., Kibrislioglu, G., Ozyurek, M., & Apak, R. (2014). Development of a fluorescent probe for measurement of peroxyl radical scavenging activity in biological samples. *Journal of Agricultural and Food Chemistry*, *62*, 1839–1845.

Guillen-Sans, R., & Guzman-Chozas, M. (1998). The thiobarbituric acid (TBA) reaction in foods: A review. *Critical Reviews in Food Science and Nutrition*, *38*, 315–330.

Gulaboski, R., Mirceski, V., & Mitrev, S. (2013). Development of a rapid and simple voltammetric method to determine total antioxidative capacity of edible oils. *Food Chemistry*, *138*, 116–121.

Halliwell, B., & Gutteridge, J. M. C. (1999). *Free radicals in biology and medicine*. New York, NY: Oxford University Press.

Hammond, M. D., & Skonberg, D. I. (2012). Antioxidant properties of chitosan coatings on frozen Atlantic salmon fillet portions. *Journal of Aquatic Food Product Technology*, *21*, 351–361.

Hara, S., & Totani, Y. (1988). A highly sensitive method for the micro-determination of lipid hydroperoxides by potentiometry. *Journal of the American Oil Chemists' Society*, *65*, 1948–1950.

Hegerman, A. E., Riedl, K. M., Jones, G., Sovik, K. N., Rechard, N. T., Hartzfeld, P. W., & Reichel, T. L. (1998). High molecular weight plant polyphenolics (tannins) as biological antioxidants. *Journal of Agricultural and Food Chemistry*, *46*, 1887–1892.

Hicks, M., & Gebicki, J. M. (1979). Spectrophotometric method for the determination of lipid hydroperoxides. *Analytical Biochemistry*, *99*, 249–253.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A., & Deemer, E. K. (2002). Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated β-cyclodextrin as the solubility enhancer. *Journal of Agricultural and Food Chemistry*, *50*, 1815–1821.

Huang, D., Ou, B., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, *53*, 1841–1856.

Innawong, B., Mallikarjunan, P., Irudayaraj, J., & Marcy, J. E. (2004). The determination of frying oil quality using Fourier transform infrared attenuated total reflectance. *Lebensmittel Wissenschaft Und Technologie*, *37*, 23–28.

Isik, E., Sahin, S., & Demir, C. (2013). Development of a new chromium reducing antioxidant capacity (CHROMAC) assay for plants and fruits. *Talanta*, *111*, 119–124.

Jardine, D., Antolovich, M., Prenzler, P. D., & Robards, K. (2002). Liquid chromatography-mass spectrometry (LC-MS) investigation of the thiobarbituric acid reactive substances (TBARS) reaction. *Journal of Agricultural and Food Chemistry*, *50*, 1720–1724.

Jiang, Z. Y., Woollard, A. C. S., & Wolff, S. P. (1991). Lipid hydroperoxide measurement by oxidation of Fe2+ in the presence of xylenol orange. Comparison with the TBA assay and an iodometric method. *Lipids*, *26*, 853–856.

Karadag, A., Ozcelik, B., & Saner, S. (2009). Review of methods to determine antioxidant capacities. *Food Analytical Methods*, *2*, 41–60.

Karawita, R., Siriwardhana, N., Lee, K. W., Heo, M. S., Yeo, I. K., Lee, Y. D., & Jeon, Y. J. (2005). Reactive oxygen species scavenging, metal chelation, reducing power and lipid peroxidation inhibition properties of different solvent fractions from Hizikia fusiformis. *European Food Research and Technology*, *220*, 363–371.

Kedare, S. B., & Singh, R. P. (2011). Genesis and development of DPPH method of antioxidant assay. *Journal of Food Science and Technology*, *48*, 412–422.

Keyrouz, R., Abasq, M. L., le Bourvellec, C., Blanc, N., Audibert, L., ArGall, E., & Hauchard, D. (2011). Total phenolic contents, radical scavenging and cyclic voltammetry of seaweeds from Brittany. *Food Chemistry*, *126*, 831–836.

Khan, M. A., & Shahidi, F. (2000). Oxidative stability of stripped and non-stripped borage and evening primrose oils and their emulsions in water. *Journal of the American Oil Chemists' Society*, *77*, 963–968.

Kim, M., Rhee, S. K., & Cheigh, H. S. (1995). Effect of tocopherols and carotene on the oxidation of purified pinenut oil in the model system. *Journal of the Korean Society of Food Science and Nutrition*, *24*, 60–66.

Kim, T. S., Yeo, J. D., Kim, J. Y., Kim, M. J., & Lee, J. H. (2013). Determination of the degree of oxidation in highly-oxidised lipids using profile changes of fatty acids. *Food Chemistry*, *138*, 1792–1799.

Kiritsakis, A., Kanavouras, A., & Kiritsakis, K. (2002). Chemical analysis, quality control and packaging issues of olive oil. *European Journal of Lipid Science and Technology*, *104*, 628–638.

Kruglov, A. G., Nikiforova, A. B., Shatalin, Y. V., Shubina, V. V., Fisyuk, A. S., & Akatov, V. S. (2010). Sulfur-containing compounds quench 3,7- dihydro-2-methyl-6-(4-methoxyphenyl) imidazole [1,2-a] pyrazine-3-one chemiluminescence: Discrimination between true antioxidants and quenchers using xanthine oxidase. *Analytical Biochemistry*, *406*, 230–232.

Kruidenier, L., & Verspaget, H. W. (2012). Review article: Oxidative stress as a pathogenic factor in inflammatory bowel disease – Radicals or ridiculous? *Alimentary Pharmacology and Therapeutics*, *16*, 1997–2015.

Ladikos, D., & Lougovois, V. (1990). Lipid oxidation in muscle foods: A review. *Food Chemistry*, *35*, 295–314.

Lee, D. M. (1994). α-Phenyl *N*-*tert*-butylnitrone as antioxidant for low density lipoproteins. *Methods in Enzymology*, *234*, 513–523.

Lee, J. M., Kim, D. H., Chang, P. S., & Lee, J. H. (2007). Headspace-solid phase microextraction (HS-SPME) analysis of oxidized volatiles from free fatty acids (FFA) and application for measuring hydrogen donating antioxidant activity. *Food Chemistry*, *105*, 414–420.

List, G. R., Evans, C. D., Kwolek, W. F., Warner, K., & Boundy, B. K. (1974). Oxidation and quality of soybean oil: A preliminary study of the anisidine test. *Journal of the American Oil Chemists' Society*, *51*, 17–21.

Litwinienko, G., Daniluk, A., & Kasprzycka-Guttman, T. (1999). A differential scanning calorimetry study on the oxidation of C_{12}-C_{18} saturated fatty acids and their esters. *Journal of the American Oil Chemists' Society*, *76*, 655–657.

Liyana-Pathirana, C., Dexter, J., & Shahidi, F. (2006). Antioxidant properties of wheat as affected by pearling. *Journal of Agricultural and Food Chemistry*, *54*, 6177–6184.

Lucas-Abellan, C., Mercader-Ros, M. T., Zafrilla, M. P., Gabaldon, J. A., & Nunez-Delicado, E. (2011). Comparative study of different methods to measure antioxidant activity of resveratrol in the presence of cyclodextrins. *Food and Chemical Toxicology*, *49*, 1255–1260.

Lyras, L., Cairns, N. J., Jenner, A., & Jenner, P. (1997). An assessment of oxidative damage to proteins, lipids, and DNA in brain from patients with Alzheimer's disease. *Neurochemistry*, *68*, 2061–2069.

Madhujith, T., & Shahidi, F. (2007). Antioxidative and antiproliferative properties of selected barley (Hordeum vulgarae L.) cultivars and their potential for inhibition of low-density lipoprotein (LDL) cholesterol oxidation. *Journal of Agricultural and Food Chemistry*, 55, 5018–5024.

Magalhaes, L. M., Segundo, M. A., Reis, S., & Lima, J. L. F. C. (2008). Methodological aspects about in vitro evaluation of antioxidant properties. *Analytica Chimica Acta*, *613*, 1–19.

Medina, I., Sacchi, R., Giudicianni, I., & Aubourg, S. (1998). Oxidation in fish lipids during thermal stress as studied by ^{13}C nuclear magnetic resonance spectroscopy. *Journal of the American Oil Chemists' Society*, *75*, 147–154.

Melton, S. L. (1983). Methodology for following lipid oxidation in muscle foods. *Food Technology*, *37*, 105–111.

Milardovic, S., Kerekovic, I., & Rumenjak, V. (2007). A flow injection biamperometric method for determination of total antioxidant capacity of alcoholic beverages using bienzymatically produced ABTS. *Food Chemistry*, *105*, 1688–1694.

Miller, N. J., Diplock, A. T., Rice-Evans, C., Davies, M. J., Gopinathan, V., & Milner, A. (1993). A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Science*, *84*, 407–412.

Min, B., & Ahn, D. U. (2005). Mechanism of lipid peroxidation in meat and meat products—A review. *Food Science and Biotechnology*, *14*, 152–163.

Minioti, K. S., & Georgiou, C. A. (2008). High throughput flow injection bioluminometric method for olive oil antioxidant capacity. *Food Chemistry*, *109*, 455–461.

Miura, K., Kikuzaki, H., & Nakatani, N. (2002). Antioxidant activity of chemical components from sage (Salvia officinalis L.) and thyme (Thymus vulgaris L.) measured by the oil stability index method. *Journal of Agricultural and Food Chemistry*, 50, 1845–1851.

Molina-Diaz, A., Ortega-Carmona, I., & Pascual-Reguera, M. I. (1998). Indirect spectrophotometric determination of ascorbic acid with ferrozine by flow-injection analysis. *Talanta*, *47*, 531–536.

Musa, K. H., Abdullah, A., Kuswandi, B., & Hidayat, M. A. (2013). A novel high throughput method based on the DPPH dry reagent array for determination of antioxidant activity. *Food Chemistry*, *141*, 4102–4106.

Nawar, W. W. (1996). Lipids. In R. O. Fennema (Ed.), *Food chemistry* (pp. 225–320). New York, NY: Marcel Dekker, Inc.

Nikokavoura, A., Christodouleas, D., Yannakopoulou, E., Papadopoulos, K., & Calokerinos, A. C. (2011). Evaluation of antioxidant activity of hydrophilic and lipophilic compounds in edible oils by a novel fluorimetric method. *Talanta*, *84*, 874–880.

Nkhili, E., & Brat, P. (2011). Reexamination of the ORAC assay: Effect of metal ions. *Analytical and Bioanalytical Chemistry*, *400*, 1451–1458.

Nuengchamnong, N., de Jong, C. F., Bruyneel, B., Niessen, W. M. A., Irth, H., & Ingkanina, K. (2005). HPLC coupled on-line to ESI-MS and a DPPH-based assay for the rapid identification of antioxidants in Butea superba. *Phytochemical Analysis*, *16*, 422–428.

Omata, Y., Yoshida, Y., & Niki, E. (2010). Assessment of the antioxidant capacity of natural fruit extracts by inhibition of probe decay and plasma lipid peroxidation. *Bioscience, Biotechnology, and Biochemistry*, *74*, 531–535.

Ortiz, R., Antilen, M., Speisky, H., Aliaga, M. E., Lopez-Alarcon, C., & Baugh, S. (2012). Application of a microplate-based ORAC-pyrogallol red assay for the estimation of an antioxidant capacity: First action 2012.03. *Journal of AOAC International*, *9*, 1558–1561.

Ozyurt, D., Demirata, B., & Apak, R. (2010). Modified cerium(IV)-based antioxidant capacity (CERAC) assay with selectivity over citric acid and simple sugars. *Journal of Food Composition and Analysis*, *23*, 282–288.

Őzyürek, M., Bektaşoğğlu, B., Güçlü, K., Güngör, N., & Apak, R. (2008). Simultaneous total antioxidant capacity assay of lipophilic and hydrophilic antioxidants in the same acetone–water solution containing 2% methyl-b-cyclodextrin using the cupric reducing antioxidant capacity (CUPRAC) method. *Analytica Chimica Acta*, *630*, 28–39.

Őzyürek, M., Güngör, N., Baki, S., Güçlü, K., & Apak, R. (2012). Development of a silver nanoparticle-based method for the antioxidant capacity measurement of polyphenols. *Analytical Chemistry*, *84*, 8052–8059.

Padmarajaiah, N., Anandamurthy, S., Narayana, A., Anantharaman, S., Krishnegowda, A., & Honnur, K. (2012). Quantification of antioxidants in medicinal plants and foodstuffs using Ce(IV) with indigo carmine as chromogenic probe. *Food Analytical Methods*, 5, 909–919.

Papadopoulos, K., Triantis, T., Yannakopoulou, E., Nikokavoura, A., & Dimotikali, D. (2003). Comparative studies on the antioxidant activity of aqueous extracts of olive oils and seed oils using chemiluminescence. *Analytica Chimica Acta*, *494*, 41–47.

Papastergiadis, A., Mubiru, E., van Langenhove, H., & de Meulenaer, B. (2012). Malondialdehyde measurement in oxidized foods: Evaluation of the spectrophotometric thiobarbituric acid reactive substances (TBARS) test in various foods. *Journal of Agricultural and Food Chemistry*, *60*, 9589–9594.

Perkins, E. G. (1992). Effect of lipid oxidation on oil and food quality in deep frying. In A. J. St. Angelo (Ed.), *Lipid oxidation in food* (pp. 310–321). Washington, DC: American Chemical Society.

Perron, N. R., Hodges, J. N., Jenkins, M., & Brumaghim, J. L. (2008). Predicting how polyphenol antioxidants prevent DNA damage by binding to iron. *Inorganic Chemistry*, *47*, 6153–6161.

Pikul, J. (1992). Lipid oxidation and warmed-over flavour formation in heated and stored meat. Part I. *Gosp Miêsna*, *7*, 20–23, (in Polish).

Plank, D. W., Szpylka, J., Sapirstein, H., Woollard, D., Zapf, C. M., Lee, V., Chen, C. Y., Liu, R. H., Tsao, R., Düsterloh, A., & Baugh, S. (2012). Determination of antioxidant activity in foods and beverages by reaction with 2,2′-diphenyl-1-picrylhydrazyl (DPPH): Collaborative study First Action 2012.04. *Journal of AOAC International*, *95*, 1562–1569.

Porter, W. L. (1993). Paradoxical behavior of antioxidants in food and biological systems. In G. M. Williams (Ed.), *Antioxidants: Chemical, physiological, nutritional and toxicological aspects* (pp. 93–122). Princeton, NJ: Princeton Scientific.

Povilaityte, V., Cuvelier, M. E., & Berset, C. (2001). Antioxidant properties of Moldavian dragonhead (Dracocephalum moldavica L.). *Journal of Food Lipids*, *8*, 45–64.

Prior, R. L., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L., Hampsch-Woodill, M., Huang, D., Ou, B., & Jacob, R. (2003). Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity ($ORAC_{FL}$) of plasma and other biological and food samples. *Journal of Agricultural and Food Chemistry*, *51*, 3273–3279.

Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, *53*, 4290–4302.

Pulido, R., Bravo, L., & Saura-Calixto, F. (2000). Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing antioxidant power assay. *Journal of Agricultural and Food Chemistry*, *48*, 3396–3402.

Qiu, J., Chen, L., Zhu, Q., Wang, D., Wang, W., Sun, X., Liu, X., & Du, F. (2012). Screening natural antioxidants in peanut shell using DPPH-HPLC-DAD-TOF/MS methods. *Food Chemistry*, *135*, 2366–2371.

Quaranta, M., Nugroho Prasetyo, E., Koren, K., Nyanhongo, G. S., Murkovic, M., Klimant, I., & Guebitz, G. M. (2013). Enzyme-based online monitoring and measurement of antioxidant activity using an optical oxygen sensor coupled to an HPLC system. *Analytical and Bioanalytical Chemistry*, *405*, 2371–2377.

Ragubeer, N., Beukes, D. R., & Limson, J. L. (2010). Critical assessment of voltammetry for rapid screening of antioxidants in marine algae. *Food Chemistry*, *121*, 227–232.

Raudonis, R., Bumblauskiene, L., Jakstas, V., Pukalskas, A., & Janulis, V. (2010). Optimization and validation of post-column assay for screening of radical scavengers in herbal raw materials and herbal preparations. *Journal of Chromatography. A*, *1217*, 7690–7698.

Riuz, A., Ayora-Canada, M. J., & Lendl, B. (2001). A rapid method for peroxide value determination in edible oils based on flow analysis with Fourier transform infrared spectroscopic detection. *The Analyst*, *126*, 242–246.

Roginsky, V., & Liss, E. A. (2005). Review of methods to determine chain-breaking antioxidant activity in food. *Food Chemistry*, *92*, 235–254.

Rubin, L. J., & Shahidi, F. (1988). Lipid oxidation and the flavor of meat products. Proc. 34th International Congress in Meat Science and Technology, ICMST, Brisbane, pp 295–301.

Sajilata, M. G., Bajaj, P. R., & Singhai, R. S. (2008). Tea polyphenols as nutraceuticals. *Comprehensive Reviews in Food Science and Food Safety*, *7*, 229–254.

Sariahmetoglu, M., Wheatley, R. A., Cakici, I., Kanzik, I., & Townshend, A. (2003). Flow injection analysis for monitoring antioxidant effects on luminol chemiluminescence of reactive oxygen species. *Analytical Letters*, *36*, 749–765.

Satue-Gracia, M. T., Heinonen, M., & Frankel, E. N. (1997). Anthocyanins as antioxidants on human low-density lipoprotein and lecithin-liposome systems. *Journal of Agricultural and Food Chemistry*, *45*, 3362–3367.

Schaich, K. M. (2005). Lipid oxidation: Theoretical aspects. In F. Shahidi (Ed.), *Bailey's industrial oil and fat products* (6th ed., Vol. 1, pp. 269–356). Hoboken, NJ: John Wiley & Sons, Inc.

Schwarz, K., Bertelsen, G., Nissen, L. R., Gardner, P. T., Heinonen, M. I., Hopia, A., Huynh-Ba, T., Lambelet, P., McPhail, D., Skibsted, L. H., & Tijburg, L. (2001). Investigation of plant extracts for the protection of processed foods against lipid oxidation. Comparison of antioxidant assays based on radical scavenging, lipid oxidation and analysis of the principal antioxidant compounds. *European Food Research and Technology*, *212*, 319–328.

Scott, G. (1997). *Antioxidants: In science, technology, medicine and nutrition* (pp. 36–79). Chichester, UK: Albion Publishing.

Sedman, J., van de Voort, F. R., & Ismail, A. A. (1997). Application of Fourier transform infrared spectroscopy in edible-oil analysis. In R. E. McDonald & M. M. Mossoba (Eds.), *New techniques and applications in lipid analysis* (pp. 283–324). Champaign, IL: AOCS Press.

Senanayake, S. P. J., & Shahidi, F. (1999). Oxidative deterioration of borage and evening primrose oils as assessed by NMR spectroscopy. *Journal of Food Lipids*, *6*, 195–203.

Shahidi, F. (2000). Antioxidants in foods and food antioxidants. *Die Nahrung*, *44*, 158–163.

Shahidi, F., Alasalvar, C., & Liyana-Pathirana, C. M. (2007). Antioxidant phytochemicals in hazelnut (*Corylus avellana* L.) and its by-products. *Journal of Agricultural and Food Chemistry*, *55*, 1212–1220.

Shahidi, F., & Amarowicz, R. (1996). Antioxidant activity of protein hydrolysates from aquatic species. *Journal of the American Oil Chemists' Society*, *73*, 1197–1199.

Shahidi, F., Desilva, C., & Amarowicz, R. (2003). Antioxidant activity of extracts of defatted seeds of niger (*Guizotia abyssinica*). *Journal of the American Oil Chemists' Society*, *80*, 443–450.

Shahidi, F., & Han, X. Q. (1993). Encapsulation of food ingredients. *Critical Reviews in Food Science and Nutrition*, *33*, 501.

Shahidi, F., & Wanasundara, U. N. (2002). Methods for measuring oxidative rancidity in fats and oils. In C. C. Akoh & D. B. Min (Eds.), *Food lipids: Chemistry, nutrition and biotechnology* (pp. 465–487). New York, NY: Marcel Dekker Inc.

Shahidi, F., & Zhong, Y. (2005). Lipid oxidation: Measurement methods. In F. Shahidi (Ed.), *Bailey's industrial oil and fat products* (6th ed., Vol. 1, pp. 357–386). Hoboken, NJ: John Wiley & Sons, Inc.

Shahidi, F., & Zhong, Y. (2007). Measurement of antioxidant activity in food and biological systems. In F. Shahidi & C. T. Ho (Eds.), *Antioxidant measurement and applications* (pp. 36–66). ACS symposium series 956. Washington, DC: American Chemical Society.

Shahidi, F., & Zhong, Y. (2010). Lipid oxidation and improving the oxidative stability. *Chemical Society Reviews*, *39*, 4067–4079.

Shahidi, F., & Zhong, Y. (2011). Revisiting the polar paradox theory: A critical overview. *Journal of Agricultural and Food Chemistry*, *59*, 3499–3504.

Shahidi, F., Zhong, Y., & Chandrasekara, G. A. (2012). Antioxidants and human health. In L. Yu, R. Tsao, & F. Shahidi (Eds.), *Cereals and pulses: Nutraceutical properties and health benefits* (1st ed., pp. 273–308). Chichester, UK: John Wiley & Sons, Inc.

Sherwin, E. R. (1990). Antioxidants. In L. Branen (Ed.), *Food additives* (pp. 139–193). New York: Marcel Dekker.

Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. *Methods in Enzymology*, *299*, 152–178.

Sireerat, I., & Schulte, A. (2012). Automated electrochemical free radical scavenger screening in dietary samples. *Analytical Chemistry*, *84*, 6767–6774.

Song, W., Derito, C. M., Liu, K. M., He, X., Dong, M., & Liu, R. H. (2010). Cellular antioxidant activity of common vegetables. *Journal of Agricultural and Food Chemistry*, *58*, 6621–6629.

Stepanyan, V., Arnous, A., Petrakis, C., Kefalas, P., & Calokerinos, A. (2005). Chemiluminescent evaluation of peroxide value in olive oil. *Talanta*, *65*, 1056–1058.

Stodolak, B., Starzynska, A., Czyszczon, M., & Zyla, K. (2007). The effect of phytic acid on oxidative stability of raw and cooked meat. *Food Chemistry*, *101*, 1041–1045.

Tai, A., Sawano, T., Yazama, F., & Ito, H. (2011). Evaluation of antioxidant activity of vanillin by using multiple antioxidant assays. *Biochimica et Biophysica Acta – General Subjects*, *1810*, 170–177.

Takebayashi, J., Iwahashi, N., Ishimi, Y., & Tai, A. (2012). Development of a simple 96-well plate method for evaluation of antioxidant activity based on the oxidative haemolysis inhibition assay (OxHLIA). *Food Chemistry*, *134*, 606–610.

Tan, C. P., & Che Man, Y. B. (1999). Differential scanning calorimetric analysis for monitoring the oxidation of heated oils. *Food Chemistry*, *67*, 177–184.

Tan, C. P., & Che Man, Y. B. (2002). Recent developments in differential scanning calorimetry for assessing oxidative deterioration of vegetable oils. *Trends in Food Science and Technology*, *13*, 312–318.

Tan, C. P., Che-Man, Y. B., Selamat, J., & Yusoff, M. S. A. (2002). Comparative studies of oxidative stability of edible oils by differential scanning calorimetry and oxidative stability index methods. *Food Chemistry*, *76*, 385–389.

Tarladgis, B. G., Watts, B. M., Younathan, M. T., & Dugan, L. (1960). A distillation method for the quantitative determination of malonaldehyde in rancid foods. *Journal of the American Oil Chemists' Society*, *37*, 44–48.

Tian, X., & Schaich, K. M. (2013). Effects of molecular structure on kinetics and dynamics of the trolox equivalent antioxidant capacity assay with ABTS+•. *Journal of Agricultural and Food Chemistry*, *61*, 5511–5519.

Tufan, A. N., Celik, S. E., Özyürek, M., Güçlü, K., & Apak, R. (2013). Direct measurement of total antioxidant capacity of cereals: QUENCHER-CUPRAC method. *Talanta*, *108*, 136–142.

USDA. (2010). Oxygen Radical Absorbance Capacity (ORAC) of Selected Foods, Release 2. <http://www.ars.usda.gov/News/docs.htm?docid=15866>.

Velasco, J., Anderson, M. L., & Skibsted, L. H. (2004). Evaluation of oxidative stability of vegetable oils by monitoring the tendency to radical formation. A comparison of electron spin resonance spectroscopy with the Rancimat method and differential scanning calorimetry. *Food Chemistry*, *85*, 623–632.

Velasco, J., & Dobarganes, C. (2002). Oxidative stability of virgin olive oil. *European Journal of Lipid Science and Technology*, *104*, 661–676.

Vercellotti, J. R., St. Angelo, A. J., & Spanier, A. M. (1992). Lipid oxidation in foods: An overview. In A. J. St. Angelo (Ed.), *Lipid oxidation in food* (pp. 1–11). Washington, DC: American Chemical Society.

Vuorela, S., Salminen, H., Makela, M., Kivikari, R., Karonen, M., & Heinonen, M. (2005). Effect of plant phenolics on protein and lipid oxidation in cooked pork meat patties. *Journal of Agricultural and Food Chemistry*, *53*, 8492–8497.

Wanasundara, U. N., Shahidi, F., & Jablonski, C. R. (1995). Comparison of standard and NMR methodologies for assessment of oxidative stability of canola and soybean oils. *Food Chemistry*, *52*, 249–253.

Wang, J., Zhou, N., Zhu, Z., Huang, J., & Li, G. (2007). Detection of flavonoids and assay for their antioxidant activity based on enlargement of gold nanoparticles. *Analytical and Bioanalytical Chemistry*, *388*, 1199–1205.

Wardhani, D. H., Fucinos, P., Vazquez, J. A., & Pandiella, S. S. (2013). Inhibition kinetics of lipid oxidation of model foods by using antioxidant extract of fermented soybeans. *Food Chemistry*, *139*, 837–844.

Wattenberg, L. W. (1986). Protective effects of 2(3)-tert-butyl 4-hydroxanisole on chemical carcinogenesis. *Food and Chemical Toxicology*, *24*, 1099–1102.

Wettasinghe, M., & Shahidi, F. (2002). Iron (II) chelation activity of extracts of borage and evening primrose meals. *Food Research International*, *35*, 65–71.

White, P. J. (1995). Conjugated diene, anisidine value and carbonyl value analyses. In K. Warner & N. A. M. Eskin (Eds.), *Methods to assess quality and stability of oils and fat-containing foods* (pp.159–178). Champaign, IL: AOCS Press.

Wijeratne, S. K., Abou-Zaid, M., & Shahidi, F. (2006). Antioxidant polyphenols in almond and its co-products. *Journal of Agricultural and Food Chemistry*, *54*, 312–318.

Winrow, V. R., Winyard, P. G., Morris, C. J., & Blake, D. R. (1993). Free radicals in inflammation: Second messengers and mediators of tissue destruction. *British Medical Bulletin*, *49*, 506–522.

Wolfe, K. L., Kang, X., He, X., Dong, M., Zhang, Q., & Liu, R. H. (2008). Cellular antioxidant activity of common fruits. *Journal of Agricultural and Food Chemistry*, *56*, 8418–8426.

Wolfe, K. L., & Liu, R. H. (2007). Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *Journal of Agricultural and Food Chemistry*, *55*, 8896–8907.

Xie, J., & Schaich, K. M. (2014). Re-evaluation of the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) assay for antioxidant activity. *Journal of Agricultural and Food Chemistry*, *62*, 4251–4260.

Yanishlieva, N. V., & Marinova, E. M. (2001). Stabilisation of edible oils with natural antioxidants. *European Journal of Lipid Science and Technology*, *103*, 752–767.

Yanishlieva-Maslarova, N. V. (2001). Inhibiting oxidation. In J. Pokorny, N. V. Yanishlieva, & M. Gordon (Eds.), *Antioxidants in food. Practical applications* (pp. 22–70). Cambridge, UK: Woodhead Publishing Ltd.

Yildiz, G., Wehling, R. L., & Cuppett, S. L. (2003). Comparison of four analytical methods for the determination of peroxide value in oxidized soybean oils. *Journal of the American Oil Chemists' Society*, *80*, 103–107.

Zargoosh, K., Ghayeb, Y., Aeineh, N., & Qandalee, M. (2014). Evaluation of antioxidant capacity of hydrophilic and hydrophobic antioxidants using peroxyoxalate chemiluminescence reaction of the novel furandicarboxylate derivative. *Food Analytical Methods*, *7*, 283–290.

Zhang, D., Chu, L., Liu, Y., Wang, A., Ji, B., Wu, W., Zhou, F., Wei, Y., Cheng, Q., Cai, S., Xie, L., & Jia, G. (2011). Analysis of the antioxidant capacities of flavonoids under different spectrophotometric assays using cyclic voltammetry and density functional theory. *Journal of Agricultural and Food Chemistry*, *59*, 10277–10285.

Zhang, Y., Li, Q., Xing, H., Lu, X., Zhao, L., Qu, K., & Bi, K. (2013). Evaluation of antioxidant activity of ten compounds in different tea samples by means of an on-line HPLC-DPPH assay. *Food Research International*, *53*, 847–856.

Zhong, Y., Lall, S. P., & Shahidi, F. (2007). Effects of oxidized dietary oil and vitamin E supplementation on lipid profile and oxidation of muscle and liver of juvenile Atlantic cod (*Gadus morhua*). *Journal of Agricultural and Food Chemistry*, *55*, 6379–6386.

Zhong, Y., Ma, C. M., & Shahidi, F. (2012). Antioxidant and antiviral activities of lipophilic epigallocatechin gallate (EGCG) derivatives. *Journal of Functional Foods*, *4*, 87–93.

Zhong, Y., & Shahidi, F. (2011). Lipophilized epigallocatechin gallate (EGCG) derivatives as novel antioxidants. *Journal of Agricultural and Food Chemistry*, *59*, 6526–6533.

Zhong, Y., & Shahidi, F. (2012a). Antioxidant behavior in bulk oil: Limitations of polar paradox theory. *Journal of Agricultural and Food Chemistry*, *60*, 4–6.

Zhong, Y., & Shahidi, F. (2012b). Lipophilized epigallocatechin gallate (EGCG) derivatives and their antioxidant potential in food and biological systems. *Food Chemistry*, *131*, 22–30.

Zielinski, H., Zielinska, D., & Kostyra, H. (2012). Antioxidant capacity of a new crispy type food products determined by updated analytical strategies. *Food Chemistry*, *130*, 1098–1104.
Ziyatdinova, G., Nizamova, A., & Budnikov, H. (2011). Novel coulometric approach to evaluation of total free polyphenols in tea and coffee beverages in presence of milk proteins. *Food Analytical Methods*, *4*, 334–340.
Ziyatdinova, G., Salikhova, I., & Budnikov, H. (2014). Coulometric titration with electrogenerated oxidants as a tool for evaluation of cognac and brandy antioxidant properties. *Food Chemistry*, *150*(2014), 80–86.