DETERMINATION OF SYNTHETIC PHENOLIC ANTIOXIDANTS IN FOOD ITEMS USING HPLC AND TOTAL ANTIOXIDANTS USING FIA APPROACHES

by

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
ABTS$^{\cdot+}$	2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
ACN	Acetonitrile
ADI	Acceptable daily intake
AP	Ascorbyl palmitate
BCB	β-carotene
BHA (E320)	Butylated hydroxyanisole
BHT (E321)	Butylated hydroxytoluene
bw	Body weight
CBAs	Chain-breaking antioxidants
CD	Conjugated dienes
CE	Capillary electrophoresis
DG	Dodecyl gallate
DPPH$^{\cdot}$	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylene diamine tetra acetic acid/ disodium
ESI	Electronspray ionisation
FCR	Folin-Ciocalteu reagent
FI	Flow injection
FIA	Flow injection analysis
FTC	Ferric thiocyanate
GLC	Gas liquid chromatography
GSH	Reduced glutathione
HPLC	High-performance liquid chromatography

ICP-AES	Inductively coupled plasma-atomic emission spectrometry
ICP-MS	Inductively coupled plasma-mass spectrometry
Ionox-100	3, 5-di-tert-butyl-4-hydroxymethylphenol.
IR	Infrared spectrometry
JECFA	Joint FAO/WHO Expert Committee on Food Additive
LLE	Liquid-liquid extraction
LOD	Limit of detection
MeOH	Methanol
NDGA	Nordihydroguaiaretic acid
NMR	Nuclear magnetic resonance
$O_2^{\cdot -}$	Superoxide
OG	Octyl gallate
$OH^{\cdot}$	Hydroxyl radical
ORAC	Oxygen radical absorbing capacity
PFA	Prevention of Food Adulteration
PG	Propyl gallate
Phen	1,10-phenanthroline
PUFAs	Polyunsaturated fatty acids
$R^{\cdot}$	Lipid radical
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSD	Relative standard deviation
SH	Synergist
SPAs	Synthetic phenolic antioxidants

SPE	Solid phase extraction
TAA	Total antioxidant activity
TBA	Thiobarbituric acid
TBARS	2-thiobarbituric acid-reactive substances
TBHQ	Tert-butyl hydroquinone
THBP	2,4,5-trihydroxybutyrophenone
TLC	Thin-layer chromatography
UV-Vis	Ultraviolet spectrophotometry

PENENTUAN ANTIOKSIDAN FENOLIK SINTETIK DI DALAM MAKANAN MENGGUNAKAN HPLC DAN JUMLAH ANTIOKSIDAN MENGGUNAKAN PENDEKATAN FIA

ABSTRAK

Antioksidan fenolik sintetik (SPAs) merupakan bahan tambahan makanan yang ditambah ke dalam makanan untuk memanjangkan tempoh penyimpanannya. Sungguhpun terdapat tren semakin meningkat ke arah penggunaan antioksidan semula jadi, penggunaan antioksidan sintetik masih digunakan secara meluas. Penggunaan sintetik antioksidan dikawal oleh Akta Makanan dan Pengawalan Malaysia yang menyatakan nilai maksimum yang dibenarkan bagi kombinasi antioksidan sintetik di dalam beberapa jenis makanan adalah 200 ppm. Disebabkan oleh ketoksikan SPAs, kaedah analisis diperlukan untuk penentuannya.

Dua kaedah analisis dalam penentuan: (i) SPAs (propil gallat (PG), tert-butil hidrokuinon (TBHQ), butil hidroksianisol (BHA) and butil hidroksitoluena (BHT)), dan (ii) jumlah antioksidan dalam sampel minyak masak, mentega dan marjerin serta keju telah dikaji.

Pengestrakan cecair-cecair telah digunakan untuk memencilkan SPAs daripada makanan. Kesan pelarut pengestrakan dan keadaan pengestrakan telah dikaji untuk perolehan semula dengan menggunakan HPLC. Dalam keadaan HPLC yang optimum, keempat-empat SPAs dapat dipisah dalam tempoh kurang

daripada 8 minit dan perolehan semula antara 93.0-108.0% untuk PG dan TBHQ, sementara 96.0-101.0% dan 74.0-94.0% untuk BHA and BHT telah diperolehi apabila 50 ppm dan 200 ppm SPAs ditambah ke dalam minyak masak, mentega dan marjerin serta keju. Jumlah SPAs di dalam makanan yang dikaji adalah kurang daripada nilai had yang sepatutnya.

Dua pendekatan analisis suntikan aliran (FIA) telah dibangunkan bagi penentuan jumlah antioksidan berasaskan pengesanan spetrofotometri. Dalam kaedah pertama, sistem Fe(III)-phen yang berdasarkan penurunan Fe(III) kepada Fe(II) oleh antioksidan fenolik diikuti dengan penambahan 1,10-fenantrolina (phen) untuk membentuk kompleks merah-oren Fe(II)-$(phen)_3$ dibangunkan. Kaedah kedua (penyingkiran radikal ABTS) adalah berdasarkan pelunturan warna hijau larutan $ABTS^{\cdot+}$ dengan kehadiran antioksidan. Keadaan FIA yang optimum adalah linear dari 1.0-80.0 ppm dan 1.0-50.0 ppm bagi sistem Fe(III)-phen dan $ABTS^{\cdot+}$ masing-masing dengan had pengesanan 0.2 dan 0.5 ppm. Kadar pensampelan 30-40 sampel j^{-1} telah dicapai. Korelasi yang baik (r^2= 0.905) telah diperoleh di antara kaedah yang dicadangkan dengan kaedah manual spektrofotometri apabila diaplikasikan untuk penentuan jumlah antioksidan di dalam 38 jenis makanan.

DETERMINATION OF SYNTHETIC PHENOLIC ANTIOXIDANTS IN FOOD ITEMS USING HPLC AND TOTAL ANTIOXIDANTS USING FIA APPROACHES

ABSTRACT

Synthetic phenolic antioxidants (SPAs) are food additives that are added to food to extend their shelf life. While there is a growing trend towards using natural antioxidants, synthetic antioxidants continue to be widely used. The use of synthetic antioxidants is regulated by the Food Act and Regulations of Malaysia, which stipulates that the maximum level permitted for the combination of the synthetic antioxidants in a few food items is 200 ppm. Due to the toxicity of these SPAs, analytical techniques for their determination is required.

Two analytical methods for the determination of: (i) SPAs (propyl gallate (PG), tert-butyl hydroquinone (TBHQ), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), and (ii) total antioxidants in cooking oil, bread spread and cheese samples are described.

Liquid-liquid extraction was used to isolate the SPAs from the food items. The effect of extracting solvents and extraction conditions were investigated for their recoveries using HPLC. Under the optimized HPLC conditions, baseline separation of the four SPAs in less than 8 minutes was achieved, and recoveries in the range of 93.0-108.0% for PG and TBHQ meanwhile 96.0-101.0% and 74.0-94.0% for BHA and BHT, respectively when spiked with 50

ppm and 200 ppm SPAs to cooking oil, bread spread and cheese were found. The levels of SPAs in all food items analysed were below the legal limits.

Two approaches were developed for the flow injection analysis (FIA) determination of total antioxidants based on spectrophotometric detection. In the first assay, the Fe(III)-phen system that was based on the reduction of Fe(III) to Fe(II) by the phenolic antioxidant followed by addition of 1,10-phenanthroline (phen) to form a red-orange Fe(II)-$(phen)_3$ complex was developed. The second method (ABTS radical scavenging system) was based on the bleaching of the green coloured ABTS radical ($ABTS^{\cdot+}$) in the presence of antioxidants. The optimized FIA procedure is linear over 1.0-80.0 ppm and 1.0-50.0 ppm for Fe(III)-phen and ABTS radical system, respectively with detection limits of 0.2 and 0.5 ppm. Sampling rate of 30-40 samples h^{-1} was achieved. Good correlation (r^2= 0.905) between the proposed methods and the manual spectrophotometric methods were found when applied to the determination of total antioxidants in 38 food items.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Radicals and Oxidation

Radicals can be defined as unstable oxygen molecule that possesses an unpaired electron. The reactivity of free radicals varies from relatively low, as in the case of the oxygen molecule itself, to very high, as in the case of the short-lived and highly reactive hydroxyl radical ($OH^{\cdot}$) (Wettasinghe & Shahidi, 2000).

Stressful living is also one of the ways free radicals are formed. Reactive oxygen species (ROS), which include free radicals such as superoxide anion radicals ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$) and non free-radical species such as H_2O_2 and singlet oxygen ($1O_2$), are various forms of activated oxygen (Gulcin *et al*., 2003). Such oxygen-containing radicals play a substantial role in initiating tissue damage, causing breaks in DNA (and hence the risk of cancers), impairing the immune system and also enhance in oxidizing polyunsaturated fatty acids (Osawa, 1999) as well as reactive nitrogen species (RNS). These free radicals have been recognized over a half-century ago and a convenient summary of the sequence of events involved in free-radical induced cell damage have been provided (Figure 1.1) (Baskin & Salem, 1997).

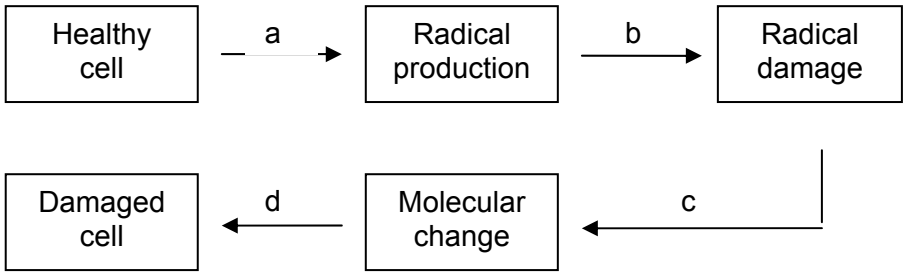


Figure 1.1 Diminishing radical-induced cell damage: a = radical formation prevention; b = radical scavenging; c = radical repair; d = biochemical repair (Baskin & Salem, 1997).

The reactions common to many radicals are (a) abstraction of a hydrogen atom from a nearby molecule (Equation 1.1) and (b) addition to molecular oxygen to form a peroxyl radical (Equation 1.2). Both of these require the presence of a second reactant.

(a) $CH_3^{\cdot} + R\text{-}H \longrightarrow CH_4 + R^{\cdot}$ hydrogen abstraction (1.1)

(b) $CH_3^{\cdot} + O_2 \longrightarrow CH_3\text{-}O\text{-}O^{\cdot}$ reaction with oxygen (1.2)

R-H, organic materials such as lipids.

Processes shown in Figure 1.2 have in vivo analogs that play an important role in the peroxidation of lipids. The two major reactive species that can initiate lipid peroxidation by abstraction of a proton from free polyunsaturated fatty acids (PUFAs) molecule (Wettasinghe & Shahidi, 2000) are $OH^{\cdot}$ and ONO_2^- (H_2O_2, $O_2^{\cdot -}$ and singlet oxygen are thought to play only minor roles). Lipid oxidation may also be augmented by light, heat, presence of trace metal ions (e.g., Cu, Fe and Co) and salt (Ramanathan & Das, 1993). These uncontrolled free radical generation is associated to rancidity of foods, especially for lipid and lipid-soluble substances in foods, leading to the formation of off-flavors and

undesirable chemical compounds (aldehydes, ketones and organic acids), as well as destructive of human body cells, by interfering in metabolic reactions (Tu & Maga, 1994; Louli *et al*., 2004).

Thus, unwanted or excessive oxidation (radical) reactions need to be limited in order for organisms to survive and for the stability of foods. Moreover, control of radical reactions is viewed as a potential route for prevention/intervention in certain diseased states such as inflammation.

1.2 Antioxidants

Antioxidants are regarded as the foundation of health and have been used for many years in the protection of biological and food system from the harmful effects of oxidative processes (Cuvelier *et al*., 1994). Common antioxidants are the vitamins C, A, and E. However, these “low molecular-mass molecules” or chain-breaking antioxidants (CBAs) are just a few in a whole multiplicity of natural defenses used by the body to combat ROS, reactive oxygen species and RNS, reactive nitrogen species attack. According to Rule 58 of PFA Rules (Prevention of Food Adulteration) in 1955, an antioxidant has been defined as a substance which when added to food retards or prevents oxidative deterioration of food and this does not include sugar, cereal, oil, flours, herbs and spices. In biological systems, an antioxidant have been defined as “any substance that, when present at low concentrations compared to those of an oxidisable substrate (e.g., lipids, proteins and DNA), significantly delays or prevents oxidation of that substrate and acts as “free radical scavenger” (Benzie & Strain, 1996; Albu *et al*., 2004). This definition therefore includes not only an array of

antioxidants of CBAs (e.g., ascorbic acid, tocopherol, uric acid, reduced glutathione (GSH), bilirubin and flavanoids) and non-enzymatic antioxidants which include antioxidants of high molecular weight, such as albumin, ceruloplasmin and ferritin but also enzymatic systems (e.g., superoxide dismutase, catalase, glutathione peroxidase) and protein used to sequester metals capable of $OH^{\cdot}$ production (e.g., transferrin, ferritin, hemopexin and albumin)(Baskin & Salem, 1997; Prior & Cao, 2000).

Supplementation with only a few antioxidants though, provides much less protection, than utilizing a complete array of antioxidants. This is due to the fact that the antioxidant defense system works as a team and they will increase the antioxidant power, due to synergism (Capitan, *et al*., 2004). As a result, maximum protection requires the complete array of established antioxidants in nutritionally meaningful amounts.

1.2.1. Classification of Antioxidants

Generally, antioxidants can be classified as natural and synthetic antioxidants. Based on their functions, antioxidants are further classified as primary or chain-breaking antioxidants and synergists or secondary antioxidants. Antioxidants containing a phenol group play a prominent role in biological and food system (Shui & Leong, 2004).

1.2.1.1 Natural and Synthetic Antioxidants

Natural antioxidants constitute a broad range of compounds including phenolic or nitrogen species and carotenoids (Hart & Scott, 1995; Aehle *et al.*, 2004).

These compounds are particularly rich in higher plants (vegetables, fruits and tea) (Weisburger, 1999) where they may function as reducing agents, free radical or active oxygen scavengers (Duh, 1998), or complexants of pro-oxidant transition metals. They suppress the levels of reactive oxygen intermediates and thus play an important role in the defense mechanisms of plants (Gulcin *et al.*, 2003; Aehle *et al.*, 2004). Natural antioxidants can also protect the human body from free radicals and retard the progress of many chronic diseases as well as lipid oxidation in foods.

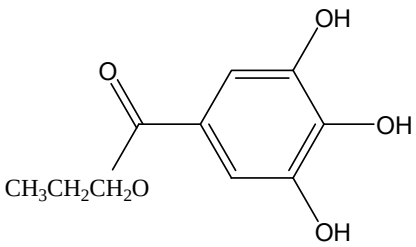
Common foods of plant origin contain a variety of hydroxylated flavonoids and other phenolics in amounts ranging from traces to several grams per kilogram (Lesage-Meessen *et al*., 2001). Literature studies have shown that grapes (Bonilla *et al*., 1999; Baydar *et al*., 2004) and wines contain large amounts of phenolic compounds, mostly flavonoids at high concentration of 1000-8000 mg L^{-1} (Lopez *et al*., 2001) and 1000-5000 mg L^{-1} total phenolics for young red wines (Costin *et al*., 2003). Quercetin is one of the most abundant flavonoids and lycopene is a carotenoid that imparts the red pigment in some fruits and vegetables (Davis *et al.*, 2003). The quercetin is mainly present as glycosides, such as quercetin-4'-glucoside in onion, quercetin-3-rutinoside (rutin) in tomato, and quercetin-3-galactoside in apple (Ishii *et al*., 2003).

The most important natural antioxidants commercially exploited are tocopherols, ascorbic acid and recently plant extracts such as from rosemary (Tena *et al.*, 1997), sage (Djarmati *et al*., 1991), green tea (Wang *et al*., 2000), spinach (Aehle *et al*., 2004), grape (Baydar *et al*, 2004) and marigold (Cetkovic *et al*.,

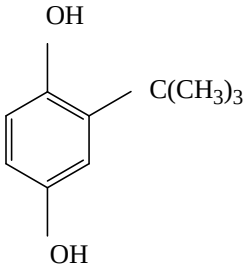
2004). These extracts contain mainly phenolic compounds (e.g. flavonoids, phenolic acids), and they are well known for their antioxidant (Wang *et al*., 2000; Lopez *et al*., 2001; Gulcin *et al.*, 2003), anti-mutagenic, anti-inflammatory (Caillet *et al*., 2005), anti-ulcer, anti-carcinogen (Wang *et al*., 2000) and anti-microbial (Gulcin *et al.*, 2003) properties, as well as for reducing the risk of cardiovascular diseases (Louli *et al*., 2004; Cetkovic *et al.*, 2004). Nevertheless natural antioxidants are usually of poor stability because these are often lost during processing or storage, which justify the need for the addition of exogenous antioxidants (Pinho *et al*., 2000).

Many synthetic antioxidants, which are characterized by a better antioxidant activity than natural antioxidants and are more easily available, have been used in a wide variety of food products. These synthetic or chemical antioxidants include propyl, octyl and dodecyl gallate (PG, OG, and DG), tert-butyl hydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and nordihydroguaiaretic acid (NDGA). They contain mainly phenolic compounds whose structure allows them to form low-energy radicals through stable resonance hybrids and will not further propagate the oxidation reaction (Karovicova & Simko, 2000). Figure 1.3 shows examples of common antioxidants.

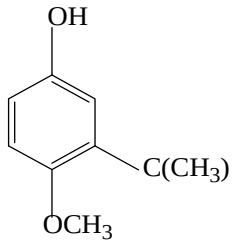
(a) Synthetic antioxidants



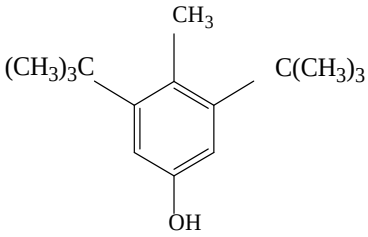
PG



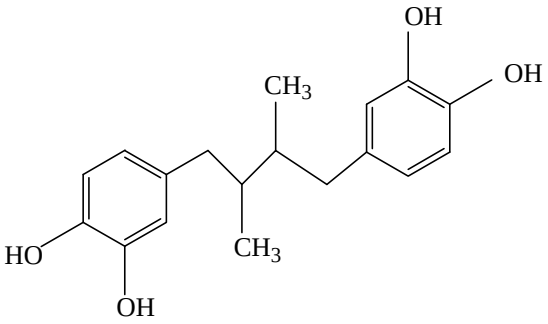
TBHQ



BHA

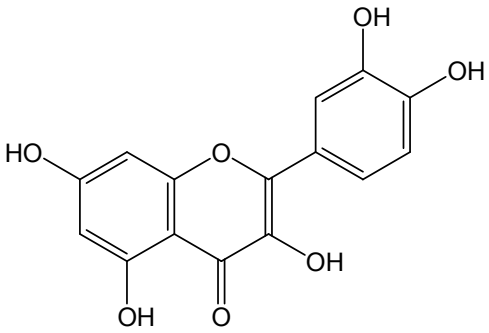


BHT

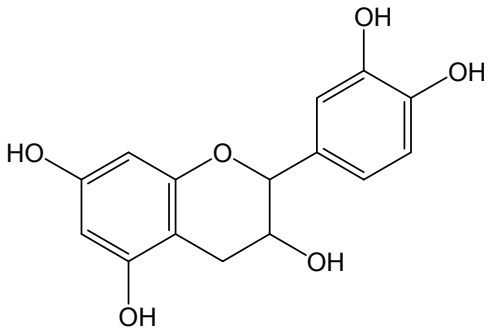


NDGA

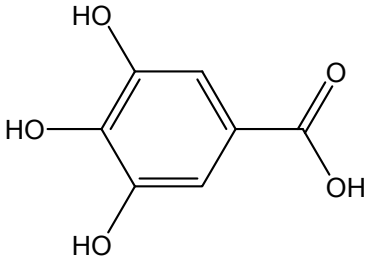
(b) Natural antioxidants



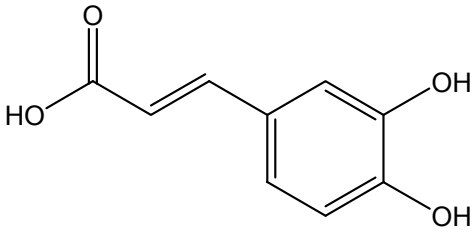
quercetin



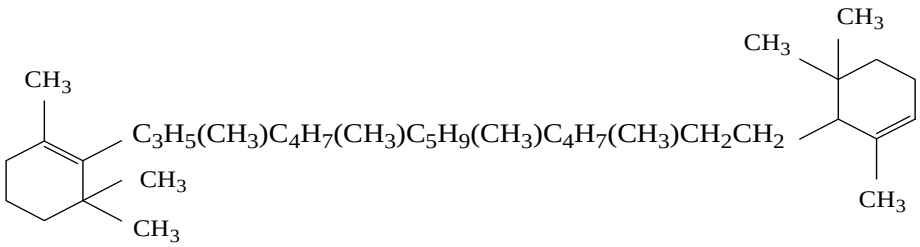
(-)epicatechin



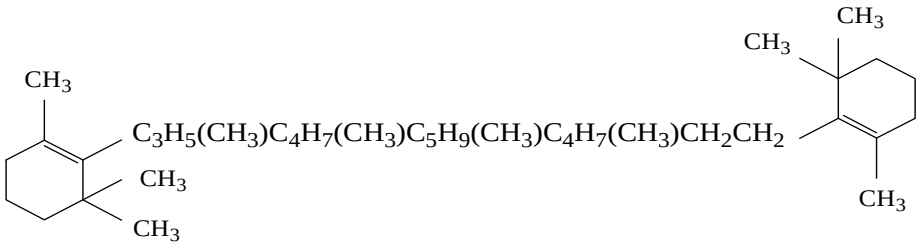
gallic acid



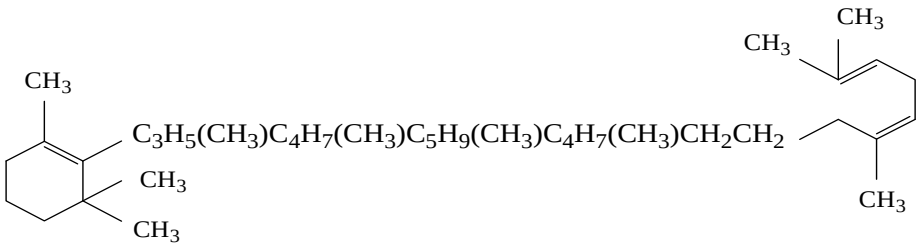
caffeic acid



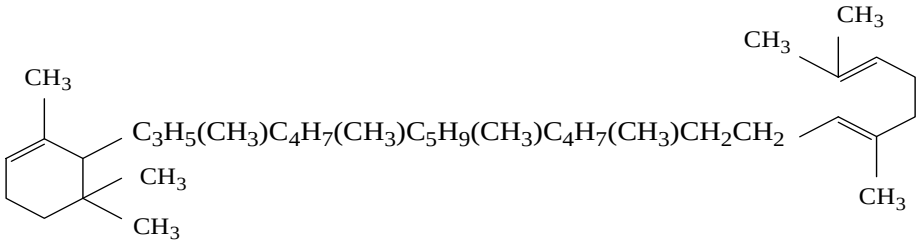
α- carotene



β- carotene



γ- carotene



δ- carotene

Figure 1.2 Some examples of (a) synthetic, and (b) natural antioxidants.

1.2.1.2 Primary and Secondary Antioxidants

Antioxidants can also be divided into primary and secondary antioxidants based on their antioxidant mechanisms (Clason, 1976). All the primary antioxidants commonly used in foods, have either two -OH groups or one -OR group in the ortho or para positions (Hudson, 1990; Peterson *et al*., 2002). They are effective at extremely low concentrations of 0.01% or less and for some of them the effectiveness decreases as concentration is increased. At high concentrations they may become pro-oxidant due to their involvement in the initiation reactions (Cillard *et al*., 1980; Bartosz *et al*., 1997). Phenolic (primary) antioxidants, whether naturally occurring, e.g. tocopherols or flavanoids or permitted synthetic compounds, such as hindered phenolic (e.g., BHT, BHA, TBHQ) and polyhydroxy phenolic (e.g., gallates), inhibit chain reactions by acting as hydrogen donors or free radical acceptors, resulting in the formation of more stable products. They interfere directly with the free radical propagation process and they block the chain reaction. The reaction mechanisms of a primary antioxidant, AH (Antunes *et al*., 1999) and secondary antioxidant BH, is shown below,

(a) Reaction of primary antioxidant, AH with lipid radical.

$$AH + ROO^{\cdot} \longrightarrow ROOH + A^{\cdot} \quad (1.3)$$

$$RH + A^{\cdot} \longrightarrow AH + R^{\cdot} \quad (1.4)$$

$$AH + ROO^{\cdot} \longrightarrow [ROO^{\cdot}AH] \text{ Complex} \quad (1.5)$$

(b) Termination reaction.

$$[ROO^{\cdot}AH] \longrightarrow \text{non-radical product} \quad (1.6)$$

$$A^{\cdot} + A^{\cdot} \longrightarrow AA \quad (1.7)$$

$$A^{\cdot} + R^{\cdot} \longrightarrow RA \quad (1.8)$$

$$A^{\cdot} + ROO^{\cdot} \longrightarrow ROOA \quad (1.9)$$

(c) Regeneration of primary antioxidant.

$$A^{\cdot} + BH \longrightarrow AH + B^{\cdot} \quad (1.10)$$

Figure 1.3 Reaction mechanism of primary antioxidant with free radical. AH, antioxidant; $ROO^{\cdot}$, lipid peroxyl radical; ROOH, hydroperoxide; $A^{\cdot}$, antioxidant free radical; RH, unsaturated lipid; $R^{\cdot}$, lipid radical; $ROO^{\cdot}$ AH, stable compound (non-radical product); BH, secondary hydrogen donor; $B^{\cdot}$, secondary antioxidant free radical.

The inhibitory reactions (1.3) to (1.5) influence the overall inhibition rate, and reaction (1.3) is more important than others. The stable resonance hybrid of antioxidant free radical $A^{\cdot}$, and the non-radical reaction (1.6) to (1.9) products thus produced are capable of inhibition the propagation of the chain reactions.

Secondary antioxidants or synergist (SH) can be accounted for (i) metal chelator (Khokhar & Owusu Apenten, 2003; Andrade Jr. *et al*., 2005), (ii) phenolic types where antioxidant is not destroyed so rapidly by free radicals

generated by peroxide decomposition, and thus remains effective for a long period. They have little direct effect on the autoxidation of lipids but are able to enhance considerably the action of primary antioxidants. Chelating agents and sequestering agents like citric acid and isopropyl citrate, amino acids, phosphoric acid, tartaric acid, ascorbic acid (AA) and ascorbyl palmitate (AP), ethylenediaminetetraacetic acid (EDTA) (Strlic *et al*., 2001), which chelate metallic ions such as copper and iron, promote lipid oxidation through a catalytic action. The chelators are referred to as synergists since they greatly enhance the action of phenolic antioxidants. It is suggested that the synergist (SH) regenerates the primary antioxidant according to the reaction.

$$SH + A\cdot \longrightarrow AH + S\cdot \qquad (1.11)$$

As an example, ascorbic acid can regenerate phenolic antioxidants by supplying hydrogen atoms to the phenoxy radicals that formed when the phenolic antioxidants yield hydrogen atoms to the lipid oxidation chain reaction. To achieve this action in lipid, ascorbic acid is made less polar by esterification to fatty acids to form compounds such as ascorbyl palmitate, so that it will dissolve in fat. Thus, when one of these substances is added to a fat in combination with a phenolic antioxidant, it is found that the antioxidant effect of the combination is greater than the sum of the effect obtained when component is used alone. This combination created a synergist effect due to the presence of the secondary antioxidant. Moreover, a synergist-like combining one or more of the phenolic antioxidants is also possible. Classification of food antioxidants is summarized in Figure 1.4.

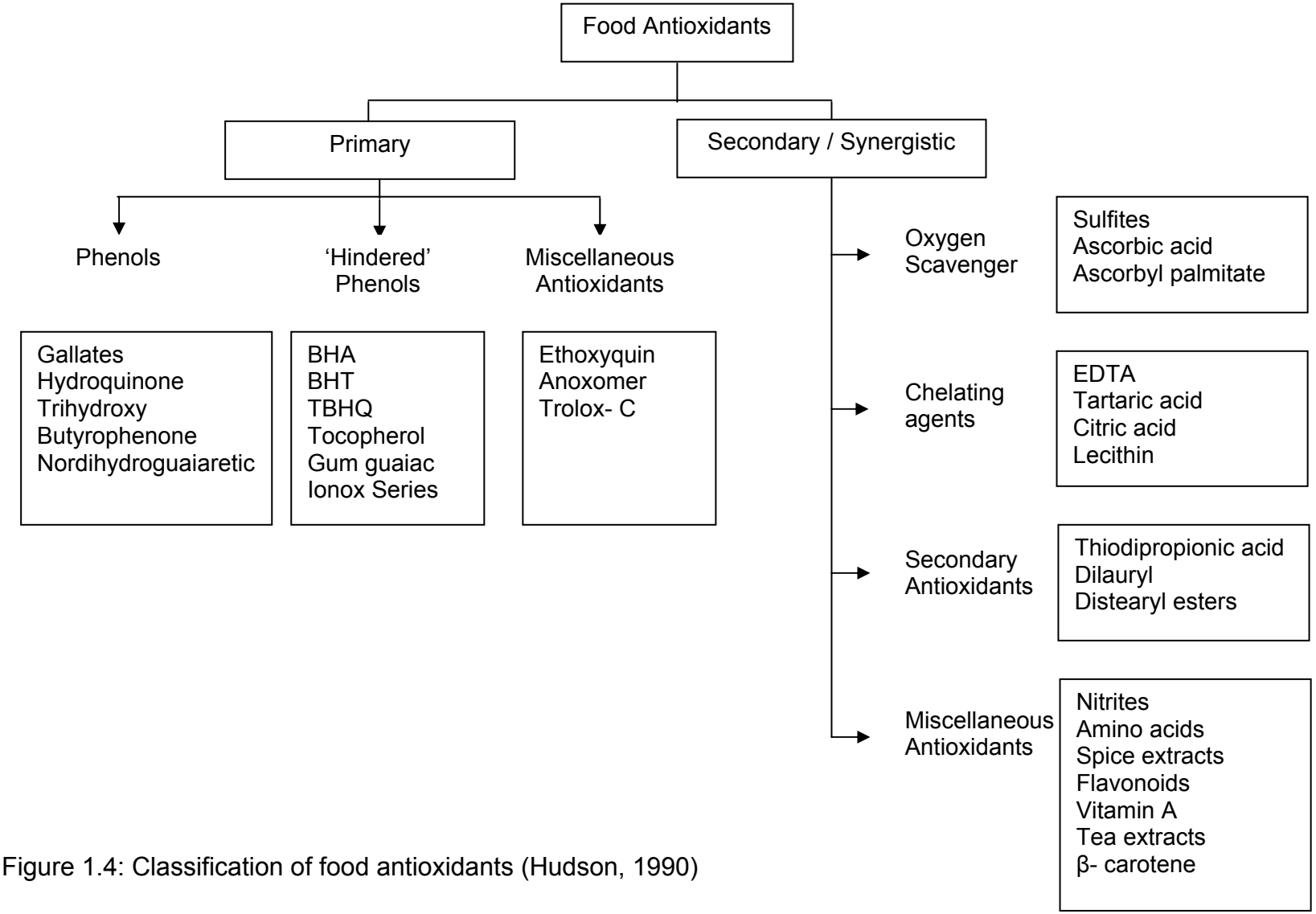


Figure 1.4: Classification of food antioxidants (Hudson, 1990)

1.2.2 Characteristics of Effective Antioxidants

The antioxidant activity of phenolic compounds is correlated to some structure-activity relationships, such as redox properties and the number and arrangement of the hydroxyl groups (Cotelle *et al*., 1996). Therefore the requisite characteristics for effective antioxidant molecules include a number of structure features.

i. The presence of hydrogen or electron donating substituents with appropriate reduction potentials, in relation to those of the redox couples of the radicals to be scavenged. As a result, it appears that the polarity of phenolic compounds is a determinant of free radical-scavenging capacity. It is known that the polyhydroxylated phenolic compounds have a higher polarity than those of the other phenols. Consistent with most polyphenolic antioxidants, both the configuration and the total number of hydroxyl groups substantially influence several mechanisms of antiradical activity (Skerget *et al*., 2004; Kulisic *et al*., 2004; Caillet *et al*., 2005). As an example, several researchers have suggested that a 3', 4'-diphenolic group on ring B is required for flavonoids to be effective free radical scavengers.

ii. Phenol itself does not act as an antioxidant, but substitution of bulky alkyl groups into 2-, 4- and 6- positions increase the electron density on the hydroxyl group by an inductive effect and thus increase hydrogen donation ability (Khan & Shahidi, 2001), such as BHA. The effective antioxidant activity of BHA is due to the strong electron donating potency of its methoxy substituent. However, methylation of the hydroxyl groups

eliminated the antioxidant activity effects, indicating that the antioxidantive effect is correlated to the hydroxyl groups.

iii. The ability to delocalize the resulting radical (Bors *et al*., 1990), whether a phenoxyl radical (e.g. those derived from α-tocopherol or butylated hydroxytoluene), a aryloxyl radical (e.g. those derived from flavonoids), a polyunsaturated hydrocarbon chain radical (e.g. β-carotene), or a thiyl radical (e.g. dihydrolipoic acid). The stability of the phenolic antioxidant free radical is explained on the basis of the resonance of the phenoxy system (Clason, 1976), as follow:

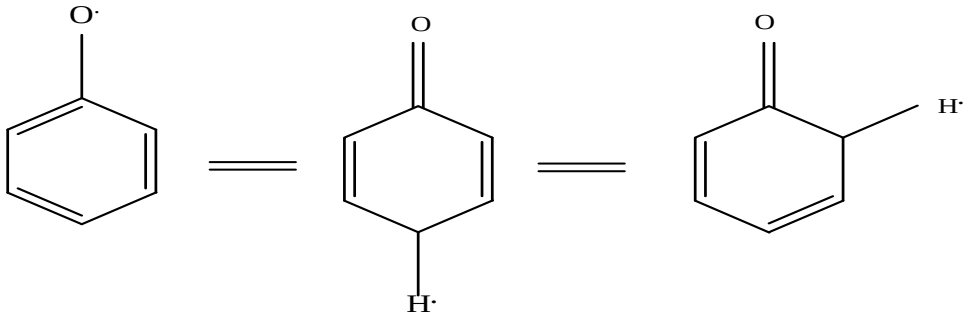


Figure 1.5 Basis resonance of the phenoxy system (Clason, 1976).

iv. The presence of bulky branched groups, (e.g. in BHA and BHT), increase the stability of phenoxy radicals. The phenoxy radical formed is stabilized by delocalization of the unpaired electron around the aromatic ring. The stability of the phenoxy radicals reduces the rate of propagation and further reaction and thus increases the oxidative stability of lipids (Hudson, 1990).

v. The transition metal-chelating potential (Thompson *et al*., 1976; Yoshino & Murakami, 1998) based on the nature of the functional groups and their arrangement within the molecule. The general chelating ability of

phenolics is probably related to high nucleophilic character of the aromatic rings rather than to specific chelating groups within the molecule (Shon *et al.*, 2003). It was suggested that flavonoid compounds (with *o*-diphenolic groups in the 3, 4-dihydroxy position in ring B and the ketol structure, 4-*oxo*, 3-OH or 4-*oxo*, 5-OH in the C ring of the flavonols) and phenolics acids (with *o*-dihydroxyl groups) might be exerting their protective effects through chelation of metal ions in the course of the Fenton reaction, or by altering the iron redox chemistry (Cetkovic *et al*., 2004).

Transition metals such as iron and copper can participate in the generation of reactive oxygen species, which are associated with many pathological conditions. Thus, chelating transition metal by polyphenolics, suppress the initiation of hydroxyl radical formation during catalytic oxidation of lipids. Recent studies showed that the antioxidant effect of polyphenolics using iron redox reaction, and classified natural polyphenolics into two groups: flavonoids enhance autooxidation of ferrous to ferric ion, and nonflavonoid polyphenolics reduce iron and form Fe^{2+}-polyphenol complexes (Yoshino & Murakami, 1998; Khokhar & Owusu Apenten, 2003). This metal-chelating activity had been considered as a minor mechanism in the antioxidant action.

These characteristics have been attributed to various mechanisms, among which is prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction,

reductive capacity, radical scavenging as well as oxygen scavenging and stimulating the antioxidative defense enzyme activities.

1.2.3 Applications of Antioxidants

The applications of natural and synthetic antioxidants have been growing steadily in preventing or delaying oxidative rancidity processes, improving the safety and appearance of the products. The applications of antioxidants are widespread in the food industry (Formanek *et al*., 2001; Zhang *et al*., 2004) and are used in preventing polymers from oxidative degradation, lubricant from sludge formation, rubber and plastic from losing strength, gasoline from autoxidation, synthetic and natural pigments from discoloration and as additives to cosmetics, foodstuffs (especially oils and fats as well as oil-containing food products) (Denis page & Charbonneau, 1989; Gonzalez *et al*., 1999; Karovicova & Simko, 2000), feedstuffs (McCarthy *et al*., 2001), beverages (Yamaguchi *et al*., 1998) and baking products (Rafecas *et al*., 1998) as well as dietary supplements (Prior & Cao, 2000).

For the last 50 years ago, food manufacturers use food-grade commercial antioxidants such as PG, TBHQ, BHA and BHT as food preservatives to prevent deterioration of products and to maintain their nutritional value. The effectiveness of antioxidants varies depending on the food and conditions of processing and storage. PG is very effective in animal fats, vegetables oils, meat products, spices and snacks (Tu & Maga, 1995). TBHQ is known to be a very effective antioxidant for vegetable oils and fried foods (Gordon & kourimska, 1995; Tu & Maga, 1995) and it is not an allowed additive in Europe.

BHA has good stability and is an effective antioxidant in fats and oils, fat-containing foods, confectionary, essential oils, food-coating materials, and waxes (Tu & Maga, 1995). BHT is very effective in animal fats, low-fat food, fish products, packaging materials, paraffin, and mineral oils but is less effective in vegetable oils and may be lost during frying because of its steam volatility (Gordon & Kourimska, 1995; Tu & Maga, 1995).

Some of these synthetic antioxidants, however, are suspected to be carcinogenic. Therefore, consumers may prefer to use natural antioxidants. Many studies are focused on utilizing more effective antioxidants from natural sources, such as α-tocopherol (vitamin E) is best well known as one of the most efficient naturally occurring lipid-soluble antioxidants (Mallet *et al*., 1994; McCarthy *et al*., 2001). Extracts rich in antioxidative compounds from natural sources such as rapeseed oil by-products extracts (Thiyam *et al*., 2004)), rice bran (Iqbal *et al*., 2005) and red grape marc extracts (Bonilla *et al*., 1999) have been reported to be used as endogenous antioxidants to stabilize refined oils instead of commercial antioxidants. Spices including cloves, cinnamon, black pepper, turmeric, ginger, garlic and onion are used widely and exhibit antioxidative activities in a variety of food systems (Ramanathan & Das, 1993). The blending of antioxidants in the manufacture of some fatty dairy-like products based on vegetable oils is also common (Al-Neshawy & Al-Eid, 2000).

In recent years there has been an increasing interest in the application of antioxidants to the medical field as information is constantly gathered linking the

development of human diseases to oxidative stress. The generally accepted hypothesis is that in any biological system, an important balance must be maintained between the formation of ROS and RNS and their removal. These reactive radicals are all products of normal pathways of the human organs, but under certain conditions, when in excess they can exert harmful compounds. Superoxide ($O_2^{\cdot -}$), the most important source of initiating radicals *in vivo*, is produced in mitochondria during electron chain transfers and it regularly leaks outside of the mitochondria. To maintain an oxido/redox balance, organs protect themselves from the toxicity of excess ROS/RNS in different ways, including the use of endogenous and exogenous antioxidants.

The use of antioxidants as therapeutic intervention in cancer treatment, radiation and chemotherapy is also a rapidly evolving area. Numerous studies showed that antioxidant treatment in combination with chemotherapy and irradiation help to reduce the adverse effects of chemotherapy and prolonged the survival time of patients compared to without the composite oral therapy. Antioxidants also can be used as a tool for improvement of psoralen photochemotheraphy. It was found that antioxidants (e.g. α-tocopherol, butylated hydroxytoluence) selectively inhibited the photochemical stage of erythema and hyperpigmentation but had no impact on the post-irradiation stages of these processes.

1.2.4 Toxicological Aspect and Regulations of Synthetic Antioxidants

From the legal point of view, antioxidants are substances which prolong the shelf-life of foodstuffs by protecting them against deterioration caused by

oxidation, such as fat rancidity, color change and loss of nutrient value. Numerous benefits are gained by the both food processors and consumers that result from antioxidant usage. However, there are a number of controversies surrounding the use of synthetic antioxidants. Since food additives are subjected to the most stringent toxicological testing procedures, only a few synthetic antioxidants have been used in foods for any length of time. Table 1.1 presents the most common antioxidants permitted for use in food products.

Table1.1 Antioxidants conventionally permitted for use in foods (Watson, 2000).

Ascorbic acid, sodium, calcium salts	Gylcine
Ascorbyl palmitate and stearate	Gum guaiac
Anoxomer	Lecithin
Butylated hydroxyanisole (BHA)	Ionox－100
Butylated hydroxytoluene (BHT)	Polyphosphates
Tert- butyl hydroquinone (TBHQ)[a]	Propyl, octyl and dodecyl gallates
Citric acid, stearyl and isopropyl esters	Tartaric acid
Erythorbic acid and sodium salt	Thiodipropionic acid, dilauryl and distearyl esters
Ethoxyquin	Tocopherols
EDTA and calcium disodium salt	Trihydroxy butyrophenone

[a] Not permitted for use in European Economic Community countries

Since the toxicity of some synthetic antioxidants is not easily assessed, and as a result, a chemical may be considered safe by a country, tolerated in another

country and forbidden in a third one. For example, TBHQ is authorized as an antioxidant in the US while it is forbidden in the European Union countries. For BHA, in United States, only 200 mg kg^{-1} in fats, oils and chewing-gum and 50 mg kg^{-1} in breakfast cereals or dehydrated soups are permitted (Cruces-Blanco *et al*., 1999). Normally, up to 100-200 µg g^{-1} of several synthetic antioxidants especially PG, OG, TBHQ, BHA and BHT are used in oils and other fats, whether singly or in combinations, are allowed in many countries (Denis & Claudette, 1989; Gonzalez *et al.*, 1998; Noguera-Orti *et al*., 1999 (a); Noguera-Orti *et al*., 1999 (b); Viplava *et al*., 1999; Fuente *et al*,, 1999). The carcinogenicity of BHA and BHT in experimental animals has been reported (Hocman, 1998; Williams, 1986). Reports have shown that BHA has carcinogenic effects in non-rodents (pigs, monkeys) and causes lesion formation in the rat fore stomach whereas BHT has carcinogenic effects in the liver of rats and mice (Botterweck *et al*., 2000; Pinho *et a*l., 2000).

Thus, toxicological studies are crucial in determining the safety of an antioxidant and also in determining the acceptable daily intake (ADI) levels. ADIs for widely used antioxidants such as TBHQ, BHA, BHT and gallates have changed over the years mainly because of their toxicological effects in various species. Table 1.2 presents the ADIs allocated by Joint FAO/WHO Expert Committee on Food Additives (JECFA).

Table 1.2 ADIs of some antioxidants permitted in foods (Watson, 2000).

Antioxidant	ADI (mg/kg bw)*
Propyl gallate (PG)	0-2.5
Butylated hydroxyanisole, BHA (E320)	0-0.5
Butylated hydroxytoluene, BHT (E321)	0-0.3
Tert-butyhydroqiunone, TBHQ	0-0.2
Tocopherols	0.15-2.0
Gum guaiac	0-2.5
Ethoxyquin	0-0.06
Phosphates	0-70.0
EDTA	2.5
Tartaric acid	0-30.0
Citric acid	not limited
Lecithin	not limited
Ascorbic acid	not limited
Sulphites (as sulphur dioxide)	0-0.7
Ascorbyl palmitate or ascorbyl strearate (or the sum of both)	0-1.25

*ADI, acceptable daily intake; bw, body weight; E number refers to food additives.

1.3 Analytical Determination of Antioxidants

The use of antioxidants is subject to regulations that establish permitted compounds and their concentration limits (Gonzalez *et al.*, 1999). Therefore, a lot of research has been conducted to determine the presence and quantitate

antioxidants especially the synthetic phenolic antioxidants (SPAs) in foods. Common techniques previously described for the determination of specific antioxidants include UV-Vis spectrophotometry, thin-layer chromatography (TLC) (Ragazzi & Veronese, 1973), gas liquid chromatography (GLC) (Gonzalez *et al*., 1999; Fries & Puttmann, 2002), high-performance liquid chromatography (HPLC) (Guillou *et al*., 1993; AOAC Official method 983.15, 1995; Hart & Scott, 1995; Razali *et al*., 1997; Rafecas *et al*., 1998; Yamaguchi *et al*., 1998; Karovicova & Simko, 2000; Shui & Leong, 2004), capillary electropherosis (CE) (Guan *et al*., 2005) and differential-pulse voltammetry (Guanghan *et al*., 1994).

The clean-up procedure is an important step to HPLC applications to remove interfering matrix elements and particulates as well as to concentrate analytes to enhance sensitivity in chromatographic analysis. There are some options for sample preparations, the most common ones being liquid-liquid extraction (LLE) and solid phase extraction (SPE).

Apart from determining a particular antioxidant, there are several model oxidation systems (Dapkevicius *et al*., 2001) and strategies (Arnao *et al*., 1996) that are used to assess the antioxidant activity of those compounds. These include:

i. β-carotene bleaching,
ii. methyl linoleate peroxidation (Hamid *et al*., 2002),
iii. luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) chemiluminescence inhibition,

iv. 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) bleaching (Kovatcheva *et al*., 2001; Ukeda *et al*., 2002),

v. 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) bleaching, (Collins *et al*., 1998; Re *et al.*, 1999; Berg *et al*., 1999; Berg *et al*., 2000; Javanmardi *et al*., 2003) and

vi. inhibition of ferric thiocyanate formation (Mackeen *et al*., 2000; Habsah *et al*., 2000; Zainol *et al*., 2003).

Generally, methods to examine the antioxidant activity of a sample can be divided into two major categories:

a) Measuring its ability to donate an electron (or hydrogen atom) to a specific ROS or to any electron acceptor.
b) Testing its ability to remove any source of oxidative initiation, e.g., inhibition enzymes, chelating of transition metal ions and absorption of UV radiation.

As an example, strategies used for studying the antioxidant activity using ABTS (Arnao *et al*., 1996) are:

i. Decolouration assay, in which the reaction between ABTS and hydrogen peroxide is allowed to proceed until a mixture with a stable color is produced by the generation of a stable ABTS radical. An aliquot of the sample is next added and the diminished colour or the colour remaining can be used as an index of total antioxidant activity (TAA).

ii. An inhibition assay, measuring the parameters at a predetermined time; ABTS, sample, and hydrogen peroxide are added to the mixture and the reaction is started by adding metmyoglobin. After a fixed time, the

absorbance is read and the percentage of inhibition is determined by comparison with a blank assay.

iii. An inhibition assay in which the reaction rates are measured. All the reagents are added together, and the reaction is started by the addition of hydrogen peroxide. Comparison is made using the reaction rates rather than absorbance at a fixed time.

iv. Lag time measurement. All the reagents are mixed together at time 0 and the time taken for colour to develop to equilibrium is monitored. The length of the lag time before reaching the steady state in the reaction rate is proportional to the concentration of antioxidant in the sample.

In summary, the antioxidant activity is attributed to varied mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Yen & Hung, 2000; Gulcin *et al*., 2003; Shon *et al*., 2003; Kulisic *et al*., 2004). The strategies for the determination of antioxidants are summarized in Table 1.3.