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Antiradical Methods in Evaluating Antioxidant Potential of Preparations of Plant Origin

Anna Gramza-Michalowska

The Agricultural University of Poznan, Department of Food Technology and Human Nutrition, Wojska Polskiego 31, 60-624 Poznan, Poland

Correspondence: **angramza@au.poznan.pl**

ABSTRACT

The formula for antioxidants is very difficult to define, although they had been used in food for centuries. Antioxidants are compounds used to delay the accumulation of free radicals and strengthen the oxidative stability of food. According to the activity mechanism of antioxidants, they can act by means of many different processes. The properties of antioxidants are also the result of many factors, like mixing ability and activity in different reaction environment systems, stability in processing time, as well as research methods used. However, there exist limitations concerning the use of these substances. Research reflects much interest on the influence of radicals generated by different environmental factors on human health and food stability, but there is still a need to find the substances that have wide antiradical properties. There are many methods for evaluating antiradical activity. This manuscript discusses the most popular and adequate methods. Widely used parameters of free radical scavenging ability as well as the evaluation of antioxidant potential are reactions with $ABTS^{+\bullet}$ and $DPPH^{\bullet}$ radicals, the luminol chemiluminescence method, ORAC, FRAP, TRAP and many other assays. The aim of the comparison of these methods and this review is to qualify the effectiveness of different natural substances as scavengers possessing antiradical activity.

Keywords: free radicals

Abbreviations: ESR, Electron Spin Resonance Spectroscopy; **FRAP**, ferric reducing antioxidant power; **ORAC**, Oxygen radical absorbance capacity; **TEAC**, Trolox Equivalent Antioxidant Capacity; **TAA**, Total Antioxidant Activity; **DPPH**, 1,1-diphenylo-2-picrylhydrazyl; **ABTS**, 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid); **$ABTS^{+\bullet}$**, radical cation; **ABAP**, 2,2′-azo-bis(2-amidinopropane); **AAPH**, 2,2′-azobis(2-amidinpropane) dihydro chloride; **DMPO**, 5,5-dimethyl-1-pyrroline N-oxide, **DMSO**, dimethylsulfoxide; **DMF**, *N*,*N*-dimethylformamide; **Trolox**, 2-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

CONTENTS

INTRODUCTION

Antioxidants and their activity are very interesting and still an undiscovered field of much research. The antioxidative properties of constituents of plant origin have been well known for centuries, but had never been of so much interest to scientists. Today, there is much scientific research aimed at proving plant origin of constituents with antioxidant activity (Brahmchari 2006). There is much evidence about the key role of oxidative stress in aging and in different diseases. What is the most important for consumers is that food rich in plant products offers protection against many degenerative diseases, a result of their free radical activity (Ames *et al.* 1993; Halliwell 1994).

One of the fastest developing fields of research consists of examining the role of radicals in the activity of biological systems and their influence and etiology of many diseases (Larson 1988; Gordon 1996). Research of antioxidant activity of plant components uses radicals, defined as substance possessing one or more unpaired electrons on its orbital (Brand-Williams *et al*. 1995).

Increasing attention to the community has been directed into food intake habits, resulting in higher care for health and nutritional aspects. There are numerous antiradical methods that can be used for antioxidant activity evaluation of many components, especially of plant origin (Miller and Rice-Evans 1994; Re *et al*. 1999; Zieliński and Kozłowska 1999; Schwarz *et al*. 2001; Gramza-Michalowska and Bajerska-Jarzebowska 2007). Plant extracts are rich in components with radical scavenging ability. One of those groups

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Invited Mini-Review

are the polyphenols (Acker *et al.* 1996; Mensor *et al*. 2001). Research on the antioxidative activity of plant extracts in model systems is very important, especially when considering their further use in food stabilization. Scavenging of free radicals is one of the generally accepted mechanisms of delaying the lipid oxidation process (Frankel 1998; Squadriato and Peyor 1998). This process is based on reactions involving characteristic radicals with specific substances, and resulting in the formation of products easy to identify and to quantify.

There are many opposing theories for the antiradical activity of analyzed samples. Methods to measure antioxidative activity differ with the manner in which free radicals are generated, the kind and accompanying conditions, making difficult to compare the results achieved by different authors (Arnao 2000; Alonso *et al*. 2002; Huang *et al*. 2005; Zhang *et al*. 2006).

Assays for antiradical activity measurements suitable for simple and non-complex products could possibly fail in the presence of complex products, composed of a number of substances, possessing intensifying or masking features (Huang *et al*. 2005). That is why one fast, simple and reliable method is essential to obtain the univocal power of an antioxidant.

The evaluation of antioxidant potential in samples is conducted with the use of various tests based on: a) the inhibition of lipid oxidation in accelerated conditions, based on monitoring of the lipid oxidation process (Wasowicz *et al*. 2004; Singh *et al*. 2006); b) scavenging of radicals in which there is a simulation of basic mechanisms occurring during the oxidation process, measurement of the reduction of stabile radicals or radicals generated in different ways, such as radiolysis, photolysis or the Fenton reaction.

This paper reviews the most popular methods for determining total antioxidant capacity in different biological and chemical substances as a measure of free radical scavenging activity.

FREE RADICALS

Free radicals are very unstable molecules, reacting quickly with other compounds, capturing the needed electron to gain stability (Prior and Cao 1999; Sanchez-Moreno 2002; Huang *et al*. 2005). Highly reactive free radicals contain odd electrons with no formal charge. Free radicals attack the nearest stable molecule, taking its electron. A molecule with a lost electron becomes a free radical itself, beginning a chain reaction. Once the process is started, it becomes a chain reaction, finally resulting in the oxidation processes of different constituents and also the living cell.

Free radicals are normally generated in the body during metabolic processes. A healthy body can handle free radicals, although if free-radical generation becomes excessive and antioxidants are not available, damage could occur. Free radicals are considered important factors in biological aging (Ames *et al*. 1993), and could cause the cell's destruction. According to Harmon's free radical theory of aging, cells are continuously producing free radicals, further damaging or even killing the cells (Harmon 1956). Reactions of free radicals are catalyzed by heat, light, trace metals, other free radicals and the presence of peroxides. Inhibition of those reactions proceeds by the presence of antioxidants – the radical acceptors reacting with other free radicals (Frankel 1998). Usually free radicals are inactivated *in vivo* by a group of different antioxidants (Halliwell and Gutteridge 1990). However a relative deficiency of antioxidant defense in the body may lead to an increase of oxidative stress, and consequently the pathogenesis of various diseases (Emerit 1994; Rice-Evans and Burdon 1994; Frei 1995; Gutteridge 1995; Knight 1995). Antioxidant activity is mainly due to its redox properties, playing an important role in neutralizing free radicals, quenching singlet oxygen or decomposing hydroperoxides (Osawa 1994). The autoxidation process is mainly caused by radical chain reaction between certain substrates and oxygen. It was found that effective dietary antioxidants can scavenge reactive oxygen/nitrogen species (ROS/RNS) to stop the radical chain reaction, or inhibit the reactive oxidants from being formed (Wasowicz *et al*. 2004; Huang *et al*. 2005).

Consequently, there is increasing interest in the mechanisms of antioxidant protection against free-radical-induced injury and in identification of suitable indicators for measuring antioxidant capacity.

ANTIOXIDANTS

Although antioxidants had been used in food preparing and production for many centuries it is still difficult to define. Antioxidants are compounds used to delay the accumulation of free radicals and strengthen the oxidative stability of food (Halliwell and Guttherige 1995). According to the activity mechanism antioxidants divided into (Frankel 1998):

- primary antioxidants (donors), which action depends on peroxide inactivation by annexation to free radicals of fatty acid, interrupting the reaction sequence,
- secondary antioxidants (acceptors), protecting lipids by binding the oxygen in air or by delaying lipid oxidation as a result of different processes other than breaking of autoxidation chain reaction.

They can act by (Gordon 1990; Yanishlieva-Maslarowa 2001):

a. complexing metal ions that catalyze the autoxidation process, i.e. chelators;
b. primarily partially regenerating antioxidants;
c. creating a protective border between the oil and air surface;
d. oxygen scavenging;
e. decomposing peroxides and nonradical products;
f. absorbing UV radiation;
g. quenching singlet oxygen.

There is much research interest in the radicals generated by different environmental factors that influence human health and food stability, but there is still a need to find substances that have antiradical properties. There are many factors influencing antiradical activity e.g. mixing ability and activity in different solvents and stability in measuring time (Hooulihan and Ho 1985; Giese 1996; Gramza and Korczak 2005; Pokorny 2007).

Food products of plant origin are rich sources of natural antioxidative compounds (Niwa and Miyachi 1986; Yokozawa *et al*. 1998; Rababah *et al*. 2004). There are, however, a number of limitations, like carrying specific taste, scent and colour, temperature and light sensitivity, expensive production costs (Houlihan and Ho 1985; Pokorny 1991). For example average cost of green tea ethanol extract was nearly 40$ per 1 gram of the extract and rosemary extract nearly 20$ (Gramza-Michalowska, unpublished data). Extracts cleaning procedures resulted in extracts costs decrease for about 100%.

Today's world is in need for a search of new sources of antioxidant substances (Boskou 2006), accessible from the everyday diet, positively influencing unfavorable aging processes in the human body.

METHODS TO MEASURE RADICAL SCAVENGING

Over the past years a number of methods have been reported (**Table 1**). The appropriateness and correctness of a chosen method needs an interpretation on the basis of the source of the oxidative stress and the changes measured.

TEAC – TROLOX EQUIVALENT ANTIOXIDANT CAPACITY

The Trolox Equivalent Antioxidant Capacity (TEAC) assay was first proposed by Miller and Rice-Evans (1994). It is based on the spectrophotometric measurement of cation-radical $ABTS^{+\bullet}$ (2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) concentration changes in comparison to radical scavenging by Trolox. The oxidant $ABTS^{+\bullet}$, is generated by

Table 1 Comparison of different radicals scavenging activity measurements.

Method	Determination value	References
DPPH$^{\bullet}$	scavenging effect [%]	Yen and Chen 1995; Satoh *et al*. 2005; Katalinic *et al*. 2006;
	inhibition effect [%]	Aoshima and Ayabe 2007; Chen *et al*. 2007; Druzynska *et al*. 2007; Gramza-Michalowska *et al*. 2007; Liu and Yao 2007
	IC_{50} [mg/mL]	Trouillas *et al*. 2003
	inhibitory concentration of sample necessary to reduce the absorbance of DPPH by 50%	Sokmen *et al*. 2004
	AEAC [mg/100 g] (Ascorbic Acid Equivalent Antioxidant Capacity)	Chan *et al*. 2007
	AE (Antiradical Efficiency) standard equivalents (quercetin, Trolox)	Sanchez-Moreno *et al*. 1998; Sun and Ho 2001; Atoui *et al*. 2005; Gramza *et al*. 2005
	concentration EC_{50} needed to reach the steady state in TEC_{50} time [μM DPPH scavenged]	Soler-Rivas *et al*. 2000
ABTS$^{+\bullet}$	TAA or TEAC [mM Trolox equivalents] (Total Antioxidant Activity or Trolox Equivalent Antioxidant Activity)	Majchrzak *et al*. 2004; Ivanova *et al*. 2005; Stewart *et al*. 2005
	scavenging of ABTS radical [%] inhibition of radical [%]	Katalinic *et al*. 2006; Druzynska *et al*. 2007
	EC_{50} [mg Trolox/mg ABTS] concentration needed to reach the steady state in TEC_{50} time	Atoui *et al*. 2005
CL *	[μM Trolox/g]	Triantis *et al*. 2005
	IC_{50} [μg/ml] inhibitory concentration of sample necessary to reduce the chemiluminecence by 50%	Atoui *et al*. 2005
FRAP	[μM eq Trolox/100 mL]	Bravo *et al*. 2007
	[mg eq Gallic acid/g]	Chan *et al*. 2007
	[mg eq Catechin/g]	Agbor *et al*. 2005
	[μM Fe^2/L]	Jin-Weia *et al*. 2005; Katalinic *et al*. 2006
	[μM Fe^2/g]	
ORAC	[μM Trolox eq/g]	Zheng and Wang 2001; Henning *et al*. 2003; Price *et al*. 2006; Wojcikowski *et al*. 2007

* CL - chemilumiescence

persulfate oxidation of $ABTS^{2-}$. Incubation of ABTS with peroxidase (metmioglobine) and hydrogen peroxide generates stabile, blue-green cation-radical $ABTS^{+\bullet}$, which absorbance reduction is measurable at λ=734 nm (Miller and Rice-Evans 1996; Lemanska *et al*. 2001; Bompadre *et al*. 2004). Antioxidant activity is defined by Trolox concentration [mM], possessing antioxidant activity equal to 1.0 mM/L of examined substance. Antioxidant activity is defined similarly by two different expressions: TEAC, a value expressed for pure substances and TAA, or Total Antioxidant Activity, a value expressed for extracts.

A commercialized $ABTS^{+\bullet}$ radical cation method is the Randox test - Total Antioxidant Status. It consist of an antioxidant power evaluation kit (Randox®) based on $ABTS^{+\bullet}$ radical scavenging ability (Alonso *et al*. 2002). Measurement is conducted during the reaction of ddH_2O, Trolox and sample with chromogene inside (metmioglobin + ABTS), at a 0 and 3 min period, at 37°C. It is very useful tool for rapid screening the antioxidative capacity of examined materials.

TEAC assay with ABTS and metmyoglobin is based on oxidation of ABTS in presence of H_2O_2 and metmyoglobin to the radical cation $ABTS^{\bullet+}$, measured photometrically at 734 nm (Miller *et al.* 1993). A modification of the above method proposed by Miller *et al.* (1996), where the $ABTS^{\bullet+}$ radical cation was filtered by manganese dioxide powder, than diluted with phosphate buffer saline. Solutions of $ABTS^{\bullet+}$ and sample were centrifuged and the absorbance of the lower phase was measured at 734 nm. Results were expressed as % of antioxidant activity. Third modification of ABTS assay was published by Re *et al*. (1999). The ABTS stock solution was mixed with potassium persulfate and left for 12 h, until the reaction is complete. For measurements $ABTS^{\bullet+}$ solution was diluted with water (hydrophilic assay) or ethanol (lipophilic assay) and measured at 734 nm. This method does not require a controlled temperature, nevertheless it leads to reliable and easy to repeat results.

DPPH

A generally used method for the measurement of free radicals scavenging by plant extracts. DPPH method is based on spectrophotometric measurement of stabile radical DPPH$^{\bullet}$ changes (1,1-diphenylo-2-picrylhydrazyl). An ethanol or methanolic solution of DPPH is violet in colour, which disappears in the presence of antioxidants (AH), resulting in a decrease in absorbance (Brand-Williams *et al*. 1995).

$$DPPH^{\bullet} + AH > DPPH\text{-}H + A^{\bullet}$$

The decrease in absorbance is a result of radical scavenging by antioxidants, and the faster the decrease in absorbance, the stronger the antioxidant, having stronger hydrogen donation ability (Yen and Duh 1994; Yen and Chen 1995). The concentration of residual DPPH$^{\bullet}$ radical is evaluated on its starting concentration in the system and its concentration after *t*-time. Antiradical activity is measured using two possibilities: a) after 30 min of incubation or different periods the absorbance is measured (λ = 517 nm), and evaluated as percentage of antioxidant activity; b) antioxidant activity may also be evaluated by absorbance measurements until a plateau phase is reached (λ = 517 nm).

The range of antioxidants' activity is termed the AE parameter (Antiradical Efficiency) (Sanchez-Moreno *et al*. 1998; Gramza *et al*. 2005). Absorbance decrease of different extracts concentrations in ethanol solution of DPPH$^{\bullet}$ are measured continuously with data capturing at 1 min intervals on spectrophotometer, until reaction reached plateau. The remaining DPPH$^{\bullet}$ (%) is plotted to obtain the amount of antioxidant needed to decrease the initial radicals concentration by 50%. Time needed to reach the steady state to EC_{50} concentration (T_{EC50}) is calculated graphically. Higher the antioxidant ability of studied sample, lower the EC_{50} value. The absorbance decrease is a result of the antioxidants contained in the studied extracts radical scavenging ability. The faster the absorption decreases, the stronger antioxidant, possessing higher ability of hydrogen donation (Yen and Duh 1994). Taking into account that, EC_{50} and T_{EC50}, affect samples antiradical capacity, it was defined as:

$$AE = 1/ EC_{50} * T_{EC50}$$

Antiradical efficiency parameter allowed the extracts differentiation into antiradical activity groups:

$AE = 1 * 10^{-3}$	low antiradical activity
$1 * 10^{-3} < AE = 5 * 10^{-3}$	medium antiradical activity
$5 * 10^{-3} < AE = 10 * 10^{-3}$	high antiradical activity
$AE > 10 * 10^{-3}$	very high antiradical activity

CHEMILUMINESCENCE

One of free radicals and excitated particles measurements method is chemiluminescence (CL). Reactive oxygen species emit light, possible to be monitored by a luminometer. Emission of the light can be amplified by luminol (L^-), reacting with hydroxyl peroxide functions (Miyazawa *et al*. 1994). Cobalt ($CoCl_2 \cdot 6H_2O$) complexed with EDTA catalyses the hydroxyl radical's formation via Fenton reaction:

$L^- + OH \rightarrow$ light (430 nm)
$Co^{2+} + H_2O_2 \rightarrow Co^{3+} + HO^{\bullet} + HO^-$
$O_2^{\bullet -} + Co^{3+} \rightarrow O_2 + Co^{2+}$

On this basis Parejo *et al*. (2000) proposed method which advantages are that chemiluminescence intensity reaches plateau were they remain stable for 30s maximum, being recorded without integrator. The addition of free radical scavenger reduces chemiluminescence intensity, antioxidant results in light emission suppression that lasts until the antioxidant is consumed. Chemiluminescence is very sensitive and could be used for the detection of radicals like: $RO^{\bullet}$, $ROO^{\bullet}$ or $HO^{\bullet}$ (Parejo *et al*. 2000; Triantis *et al*. 2005).

According to method proposed by Triantis *et al*. (2005) it is expected that antioxidants in reaction with *O*-benzylacridine would scavenge the superoxide radical anion, leading to reduced light signals, from which antioxidants activity could be estimated. The blanks light reaction starts with adding saturated potassium superoxide solution in DMSO into a mixture of 9-benzylacridine in DMF. Obtained light intensity was compared with light intensities of analyzed samples containing 9-benzylacridine in DMF, sample in DMF and saturated potassium superoxide. The duration of light emission lasts only for few seconds. Antioxidant activity expressed as micromoles of Trolox equivalents per gram of fresh weight. Other authors used lucygenin and alkaline hydrogen peroxide to start the chemiluminescence's reaction (Parejo *et al*. 2000; Papadopoulos *et al*. 2003). A method published by Atanassova *et al*. (2005) proposed using the boric acid buffer, Co (II), EDTA and luminol mixed with H_2O_2 as the reaction mixture to measure the chemiluminescence intensity. For calibration the caffeic acid was used. Atoui *et al*. (2005) have used Trolox as a calibration agent. The antioxidant activity was expressed as antioxidant efficiency (AE), in quercetin or Trolox equivalents by comparing IC_{50} of the herbal extracts with IC_{50} of the standards.

A less popular method for evaluation of antioxidative activity is use of the Photochem (Analytic Jena AG). Measurement is based on light and photosensitizer presence generated anion-radicals reaction with cheminoluminogenic substance. The intensity of emitted light is a measure of free radicals in the examined system (Popov and Lewin 1999). Radical quenchers reduce photochemiluminescence intensity, proportionally to examined antioxidant concentration and activity. Reaction is based on optical excitation of a photosensitizer S which results in the generation of the superoxide radical $O_2^{\bullet -}$.

$S + h\nu + O_2 \rightarrow [S^{\bullet} O_2] \rightarrow S^{\bullet +} + O_2^{\bullet -}$

Free radicals are visualized with luminol, a chemiluminescent detection reagent (Schlesier *et al.* 2002). Operating principles are based on a standardized volume of photosensitizer substance added to the assay medium. The photosensitizer is optically excited to produce superoxide anion radical, partially scavenged by the sample (antioxidative compounds). Remaining radicals are quantified by luminescence generation; antioxidants are quantified by comparison with a standard: Trolox or Ascorbic acid. Results of the above experiments are expressed as mg equivalents of Trolox or Ascorbic acid per 1 gram (1 mL) of sample.

Using the Photochem® fast, easy and reliable determination of antioxidative capacity in water-soluble and lipid-soluble samples is measured (Zhai *et al*. 2003).

Chemiluminescence methods are difficult when the quantity of antioxidative activity must be evaluated. It is resulted because of radical's short time lifetime, it is also not known what kind of radicals are eliminated by the antioxidant (Ogawa *et al*. 1999).

ESR - ELECTRON SPIN RESONANCE SPECTROSCOPY

Electron Spin Resonance Spectroscopy is based on hydroxyl radical scavenging ability, generated during the Fenton reaction, in the presence of DMPO (5,5-dimethyl-1-pyrroline N-oxide). The antioxidant ability to scavenge Fremy's salt (potassium nitrosodisulfonate), a partially stable radical, affects hydrogen donors (Gardner *et al*. 1999). The procedure consists of a homoarginine solution with DMPO and an aqueous solution of the examined antioxidant, after which the ES spectrum is measured and further compared to homoarginine, as the standard spectrum. It was stated that although the detection of single antioxidant radical species is possible by electron spin resonance (ESR) spin-trapping technique, the evaluation of radical species in complex systems like biological and food samples is usually impossible by ESR (Ogawa *et al*. 1999). Guo *et al*. (1999) examined the correlations between ESR and DPPH radicals scavenging activity and chemical structure of tea catechins and found that there were significant differences. The differences between catechins sterical structures played a more important role in their abilities to scavenge large free radicals ($O_2^{\bullet -}$;$^1O_2$), than $DPPH^{\bullet}$. In other research Yoshiki *et al*. (2004) have examined potential relationship between photon emission and radical scavenging activity. Photon intensity measured with ESR showed a high correlation with chemopreventive activity against DPPH radical.

FRAP - FERRIC REDUCING ANTIOXIDANT POWER

The method of ferric reducing antioxidant power is based on the ability to measure the reduction of the TPTZ complex (Fe(III)-2,4,6-tri(2-pirydyl)-s-triazine) to an Fe(II)-TPTZ form, by reductant (antioxidant). The intensity of the generated blue colored complex is measured colorimetrically (λ = 593 nm) and is positively correlated with the reducer-antioxidant concentration (Benzie and Strain 1999). The FRAP assay is carried under acidic conditions (pH 3.6) and it involves the following procedure: TPTZ in HCl, acetate buffer and $FeCl_3 * H_2O$ mixed, and named FRAP reagent. The oxidant is not only $Fe(III)(TPTZ)_2$, it also contains other Fe(II) species, that could lead to problems as many metal chelators in extract could bind Fe(III) and for capable of reacting with antioxidants complexes (Huang *et al*. 2005). Freshly prepared FRAP reagent is warmed to 37°C and reagent blank is measured at λ = 593 nm, then the sample and water are added. Unfortunately the presented method does not take into account substances that do not possess the ability to reduce Fe(III). It is a fast, easy and reproducible method to evaluate antiradical activity of examined single antioxidants in pure solution and antioxidants mixtures (Benzie and Strain 1999). FRAP needs basic equipment of chemical laboratory and rather inexpensive, procedure reagents.

Table 2 Antioxidant activity of tea extracts measured by several radical scavenging methods.

Method		Green tea	Black tea
DPPH$^{\bullet}$	Scavenging [%]	49.7 c; 90.8 k; 90.9 m	38.02 c; 81.7 k; 52.8 m
	IC_{50} [μg/mL]	0.030 d; 4.14 g	27.02 g
	EC_{50} [μg/mL]	120-320 e	230-750 e
	AE (antiradical efficiency)	6.65 f; 349.16 p	5.57 f; 390.12 p
ABTS	TEAC or TAA [mM]	5.91 a; 21.6 n	3.92 a; 17.6 n
ORAC	[μM Trolox/g]	627.14 i; 1477 h	811 h
FRAP	[μM Trolox/100 mL]	1430.1 b	697.4 b
	[mg/g]	54.55 d	-
TRAP	[μM Trolox/mL]	14 j	-
	AC [mM]	17.85 o	3.54 o
CL	IC_{50}	0.17 f	0.18 f

According to: a - Ivanowa *et al.* 2005; b - Bravo *et al.* 2007; c - Chen *et al.* 2007; d - Chan *et al.* 2007; e - du Toit *et al.* 2001; f - Atoui *et al.* 2005; g - Yokozawa *et al.* 1998; h - Henning *et al.* 2003; i - Wojcikowski *et al.* 2007; j - Gil *et al.* 1996; k - von Gadow *et al.* 1997; m - Aoshima and Ayabe 2007; n - Majchrzak *et al.* 2004; o - Serafini *et al.* 1996; p - Gramza *et al.* 2005.

TRAP – TOTAL RADICAL TRAPPING POTENTIAL

A method developed by Wayner *et al*. (1985) is based on the measurement of induction time in the oxidation of lipids exposed to a free radical source in aerobic conditions. As the free radical source is the pyrolitic decomposition of ABAP (2,2'-azo-bis(2-amidinopropane) in an aqueous phase, the consumption of oxygen is linked as an endpoint. There are many modifications, employing different radicals or monitoring techniques (Whitehead *et al*. 1992; Lissi *et al*. 1999).

Other research examined the fluorescence of R-Phycoerythrin, which is quenched by ABAP (2,2'-azo-bis(2-amidinopropane)hydrochloride) as radical generator at 37°C. Antioxidants inhibit oxidation of R-Phycoerythrin, thus delay the fluorescence decrease. The antioxidative activity was expressed as the lag phase (Wayner *et al*. 1985).

ORAC - OXYGEN RADICAL ABSORBANCE CAPACITY

Oxygen radical absorbance capacity method uses the ability of the protein β-PE (beta-phycoerythrin) to fluorescence (λ = 540 nm). Thermal decomposition of AAPH (2,2'-azo-bis(2-amidinpropane) dihydrochloride) leads to the generation of peroxide radicals, which, by destroying the protein structure results in reduced fluorescence, proportional to radical concentration (Cao *et al*. 1993). An examined extract or any other constituent can protect the protein by radical scavenging, according to its antioxidant activity. Results are expressed as ORAC units, where one unit equals the net protection of 1 μM Trolox. Measurements in the change in fluorescence help to evaluate oxygen radical scavenging. ORAC is a simple, sensitive and reliable method.

CONCLUDING REMARKS

Most antiradical activity evaluation methods rely on reactions with radicals and their inactivation degree by the addition of pure antioxidative substances or mixtures. According to different methods free radicals are generated by an enzymatic, photochemical or chemical reaction, and to measure antioxidants effectivity spectrophotometric, chemiluminescence and electron paramagnetic resonance are used. Very often authors use the same methods, but do not use this same initial radical concentrations and reaction times (**Table 2**). Most of the assays, suitable for non-complex mixtures or chemical compounds, could partially fail to obtain good reproducibility when more complex biological samples are used, some of which possess masking features like proteins or pigments (Keogh *et al*. 2007).

Presented methods are inadequate for fast measurements of different substances. Antiradical activity measurement methods differ with the manner and kind of radical generation, resulting in differences in comparisons with other authors' results. An extremely important factor is also the different criteria to evaluate the degree to which a radical's reaction advances. Investigating the differences between presented methods it inevitably conduces to almost impossible comparisons of very often inconsistent results. The major problem is a lack of a validated measurement method that would reliably evaluate the antiradical capacity of different samples. Unfortunately also the differences between antioxidant activities of plant extracts may be a result of plants varietals, antioxidants quantity and quality in scavenging ability of different radicals or reactive oxygen species. Antioxidant analytical methodologies are developing rapidly, giving new methods every year. That is why the total antiradical activity needs to be interpreted according to oxidative stress source and its impact measurement.

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