Review

The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants

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Abstract: The activity of antioxidants in foods and biological systems is dependent on a multitude of factors, including the colloidal properties of the substrates, the conditions and stages of oxidation and the localisation of antioxidants in different phases. When testing natural antioxidants *in vitro*, it is therefore important to consider the system composition, the type of oxidisable substrate, the mode of accelerating oxidation, the methods to assess oxidation and how to quantify antioxidant activity. Antioxidant effectiveness is also determined by the heterogeneity and heterophasic nature of the system, the type of lipid substrate, including its physicochemical state and degree of unsaturation, the types of initiators, notably transition metals, other components and their possible interaction. For this reason there cannot be a short-cut approach to determining antioxidant activity. Each evaluation should be carried out under various conditions of oxidation, using several methods to measure different products of oxidation. Because most natural antioxidants and phytochemicals are multifunctional, a reliable antioxidant protocol requires the measurement of more than one property relevant to either foods or biological systems. Several recent studies on natural phytochemical compounds produced conflicting results because non-specific one-dimensional methods were used to evaluate antioxidant activity. There is a great need to standardise antioxidant testing to minimise the present chaos in the methodologies used to evaluate antioxidants. Several methods that are more specific should be used to obtain chemical information that can be related directly to oxidative deterioration of food and biological systems.

Keywords: antioxidant tests; protocols; phytochemicals; interfacial phenomena; colloid systems; natural antioxidants; bioassay; foods; biological systems

INTRODUCTION

With the current upsurge in interest in the efficacy and function of natural antioxidants in foods and biological systems, the testing of antioxidant activity has received much attention. Although there is a great multiplicity of methods used for antioxidant testing, there are no approved, standardised methods. Several rapid test methods to screen for antioxidant activity have been published and many different *in vitro* antioxidant protocols are currently used to evaluate antioxidants of interest in food and nutrition, health and disease.[1–3] Obviously, the significance and relevance of antioxidant evaluations for food and biological systems depend strongly on the test method. Inconsistent results have been obtained for a number of recognised antioxidants depending on the methods used to test activity.

Model test systems used under conditions of accelerated oxidation require careful interpretation of antioxidant action. Many different substrates, system compositions and analytical methods are employed in screening tests to evaluate the effectiveness of antioxidants. For this reason the data obtained by different researchers are extremely difficult to compare and interpret. Several test procedures can be criticised for employing questionable methodologies to analyse oxidation products, for not targeting relevant substrates and for not reflecting gross characteristics of food or biological systems.[4–6] Particular problems arise from the use of rapid one-dimensional methods to evaluate natural antioxidants, which are generally multifunctional.

For the judicious choice of antioxidant protocols the

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following important questions should be addressed in food systems.

1 What are the true protective properties of antioxidants? What is the antioxidant protecting against?
2 What substrates are oxidised and what products are inhibited?
3 What is the location of the antioxidant in the system?
4 What is the effect of other interacting components?
5 What conditions are relevant to real-life applications?

Further questions to be addressed in biological systems include the following.

1 What is the true impact of biological oxidation?
2 What are real effects of antioxidants?
3 What is the mechanism of protection? What are the relative oxidation products of lipids, proteins, DNA and interaction products?
4 What is the accessibility of substrates to antioxidants and prooxidants?

Several approaches used to test antioxidants in foods and biological systems consist of oxidising a lipid or lipoprotein substrate under standard conditions and assessing the activity by various methods to determine how much oxidation is inhibited (Table 1).[7–25] Other protocols classified as free radical-trapping methods measure the ability of antioxidants to intercept free radicals (Table 2).[26–47] In the latter methods the target compound or free radical molecule is often selected so that its consumption can be measured directly. In some cases the activity is evaluated from a coupled reaction. The validity of some of the currently used antioxidant test methods (Tables 1 and 2) may be questioned because the assay models do not take into account the complexity of antioxidant actions. The data obtained with such diversity of methods may confuse the interpretation of antioxidant activity. Misleading data can be obtained in many of these test systems by neglecting important compositional and interfacial phenomena concerning charge and solubility of multiple components in real food or biological systems that strongly affect antioxidant performance.

Natural antioxidants are often multifunctional and the activity and mechanism dominating in a particular test system depend on the oxidation conditions, which affect both the kinetics of oxidation and the composition of the system.[48–54] The effectiveness of antioxidants in complex heterogenous foods and biological systems and in multiphase models is affected by many factors. Notable factors include the partitioning properties of the antioxidants between lipid and

Table 1. *In vitro* antioxidant assays with lipid substrates to determine activity of food and biological antioxidants[a]

Oxidising species	*Solvent*	*Conditions, inducers*	*Measurement and quantification*	*Antioxidants tested*	*References*
Tween-emulsified linoleic acid with β-carotene	Buffer pH 7.0	25 °C in cuvette, different inducers	Rate of β-carotene destruction (nmol min^{-1})	Several	7–9
Tween-emulsified linoleic acid	Buffer pH 7.0–7.4	37 °C, dark, 16 h, $FeSO_4$	TBARS assay, conjugated dienes (234 nm) or HPLC	Phenolics, anthocyanins	10
SDS-emulsified linoleic acid	Buffer pH 7.4	37 or 40 °C, minutes, AAPH	Conjugated dienes (234 nm), K_{inh}/K_p[b]	Several	11–13
Methyl linoleate	Hexane/2-propanol/ethanol	37 °C, AMVN	Methyl linoleate hydroperoxides by HPLC	Buckwheat compounds	14
Methyl linoleate	Dodecane	110 °C, O_2 bubbling	Residual methyl linoleate by GC	Phenolics, spices	15, 16
PC liposomes with phenylpropionic acid[c]	Buffer pH 7.0 with NaCl	23 °C in cuvette, ~20 min, $FeCl_2$	Decrease in relative fluorescence intensity	Tart cherry compounds	17
Various lipids	No solvent	98 °C air or 50 °C, up to 50 h	Peroxide value or reactivity with sesamol dimer	Phenolics	18
Human low-density lipoprotein (LDL)	PBS[d]	2 h–several hours, Cu^{2+}, metmyoglobin	Conjugated dienes (234 nm), hexanal, induction time, 50% oxidation, % inhibition	Several	19–22
LDL or membrane phospholipids with PnA incorporated[e]	PBS[d]	37 °C, 35 min–2 h, AAPH or AMVN or H_2O_2	Rate of PnA fluorescence decrease	Phenolics	23–25

[a] Abbreviations: TBARS, thiobarbituric acid reactive substances; SDS, sodium dodecyl sulphate; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); PC, phosphatidylcholine.

[b] K_{inh}, rate constant for reaction of the linoleic acid peroxyl radical with the antioxidant; K_p propagation rate for linoleic acid oxidation.

[c] Liposomes are a mixture of 1-stearoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine and 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl] phenylpropionic acid probe.

[d] PBS is phosphate-buffered (10 mM, pH 7.4) saline (100 mM).

[e] PnA, *cis*-parinaric acid, is a fluorescent 18-carbon conjugated tetraene fatty acid.

Table 2. Selected protocols used to evaluate free radical-scavenging properties of antioxidants[a]

Free radical reactant	*Solvent system*	*Conditions, inducers*	*Measurement and quantification*	*Antioxidants tested*	*References*
$DPPH^{\cdot}$	Methanol		$DPPH^{\cdot}$ decrease at 515 nm, $1/EC_{50}$[b] or $1/EC_{50} \times T_{EC50}$[c]	Several phenols, wines	26–30
Linoleic acid (TRAP assay)	PBS pH 7.4, plasma	37 °C in oxygen electrode cell, ABAP	Oxygen uptake, induction period of plasma, Trolox reference	Vitamin E, urate, ascorbate, –SH	31
Superoxide $O_2^{\cdot -}$[d]	Buffer pH 7.4	Phenazine methosulphate–NADH, NBT	NBT reduction, % inhibition or IC_{50}[d]	Phenolic extracts	32–34
$ABTS^{\cdot +}$ (TEAC assay)	PBS	H_2O_2 and metmyoglobin	$ABTS^{\cdot +}$ decay at 734 nm, Trolox reference	Numerous, plant extracts, juices and wines	35–38
β-PE (ORAC assay)	Buffer pH 7.0	37 °C in cuvette, AAPH	β-PE fluorescence decay, molar radical absorbance vs Trolox	Numerous, various extracts	39–43
$CCl_3O_2^{\cdot}$[e]	1% CCl_4–50% 2-propanol, phosphate buffer		Rate constants of reaction, $CCl_3O_2^{\cdot}$	Gallates, spice phenolics	44, 45
Fe^{3+}-TPTZ (FRAP assay)	Aqueous	4–8 min	ΔA 593 nm, amount of Fe^{3+} reduced	Various, fruit juices	46, 47

[a] Abbreviations: $DPPH^{\cdot}$, 2,2-diphenyl-1-picrylhydrazyl; ABAP, 2,2′-azobis(2-amidinopropane) hydrochloride; PBS, phosphate saline buffer; NBT, nitroblue tetrazolium; $ABTS^{\cdot +}$, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate); β-PE, β-phycoerythrin; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; TBTZ, tripyridyltriazine.

[b] EC_{50} is efficient concentration of antioxidant to decrease initial [$DPPH^{\cdot}$] by 50%.

[c] T_{EC50} is time needed to reach steady state at EC_{50}.

[d] Phenazine methosulphate and NADH are used to generate superoxide anions non-enzymatically; IC_{50} is the phenol concentration required to inhibit NBT reduction by 50%.

[e] $CCl_3O_2^{\cdot}$ is a reactive trichloromethyl peroxyl radical generated by radiolysis and oxidation of an aqueous mixture of CCl_4 and propan-2-ol.

aqueous phases,[55–58] the oxidation conditions and the physical state of the oxidisable substrate.[6,50,55,56,59] Clearly, the influence of all relevant parameters cannot be evaluated by using only a one-dimensional assay protocol. Of particular importance are the conditions used to accelerate oxidation by raising the temperature, by using transition metal catalysts or other types of initiators, by increasing surface and by exposing to light of varying intensity.[5] Finally, the results of evaluation tests are influenced by the specificity and methods employed to analyse the progress of oxidation, including the degree of oxidation chosen as endpoint for testing.[5,6] To understand and better predict how natural antioxidants such as phytochemical compounds may exert beneficial effects both in protecting foods and in preventing disease, these complicated issues need to be carefully considered in designing test protocols for evaluating antioxidant activity. In this review we summarise the principles of assay and antioxidant evaluation and critically assess potential drawbacks in some of the widely used one-dimensional protocols.

DEFINITIONS AND MECHANISMS OF ANTIOXIDANTS

In foods, antioxidants have been defined as 'substances that in small quantities are able to prevent or greatly retard the oxidation of easily oxidisable materials such as fats'.[60] In biological systems the definition for antioxidants has been extended to 'any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate'.[61] This latter definition covers all oxidisable substrates, ie lipids, proteins, DNA and carbohydrates. Neither of these definitions relates to the antioxidant mechanism, however. In turn, in biology, all compounds that can retard or prevent the effects of oxidation have been broadly considered as antioxidants, including eg compounds that either inhibit specific oxidising enzymes or react with oxidants before they damage critical biological molecules.[62] Biological antioxidants have currently assumed a broad definition to include repair systems such as iron transport proteins (eg transferrin, albumin, ferritin, caeruloplasmin), antioxidant enzymes, factors affecting vascular homeostasis, signal transduction and gene expression. This definition has become so broad and encompassing that it has lost its traditional meaning. Some of the biological 'antioxidant' activities attributed to various plant extracts and flavonoids (eg anti-allergic, -haemorrhagic, -mutagenic, -neoplastic, -platelet activities, immunomodulation, oral hygiene, interactions with specific receptors)[63] may in fact have little to do with antioxidant activity. Similar 'non-antioxidant' activities may be indicated for the effect of α-tocopherol on protein kinase C leading to changes in gene transcription and intracellular signalling, and control of smooth muscle cell proliferation.[64]

Mechanistic definitions of antioxidants comprise the radical acceptor term, as originally conceived by Uri[65] who defined antioxidants as 'compounds able to accept radicals'. Thus the implicit antioxidant mechanism to inhibit lipid oxidation is generally considered to be radical chain-breaking. The available evidence suggests that antioxidants (AH) break radical chains by donating hydrogen atoms to the chain carrier peroxyl radicals, $LOO^{\bullet}$, under atmospheric conditions (reaction (1)) and to lipid radicals, $L^{\bullet}$, under conditions of limited oxygen availability (reaction (2)):

$$AH + LOO^{\bullet} \rightarrow LOOH + A^{\bullet} \quad (1)$$

$$AH + L^{\bullet} \rightarrow LH + A^{\bullet} \quad (2)$$

Under atmospheric conditions, because of the extremely rapid reaction of $L^{\bullet}$ with air oxygen, approaching diffusion rates, the termination reaction (1) is considered most prevalent in food and biological systems.[6,66] Scott[62] defined antioxidants reacting with peroxyl radicals by reaction (1) as chain-breaking electron *donors*, and antioxidants reacting with free lipid radicals by reaction (3) as chain-breaking electron *acceptors*:

$$L^{\bullet} + A^{\bullet} \rightarrow LA \rightarrow L^{*} + AH \quad (3)$$

where L* indicates a lipid molecule containing a new double bond.

Hydroperoxides decompose homolytically by producing alkoxyl radicals ($LO^{\bullet}$), which undergo β cleavage (4) to form aldehydes and other breakdown products that contribute to oxidative and flavour deterioration of food lipids and cause damage in biological systems:

$$R_2 - \downarrow - CH(O^{\bullet}) - \downarrow - R_1 \rightarrow$$

$$\text{aldehydes} + \text{breakdown products} \quad (4)$$

Antioxidants can inhibit the decomposition reaction (4) by reacting with the alkoxyl radicals, either by hydrogen donation to form stable hydroxy compounds (reaction (5)) or by the termination reaction (6) with antioxidant radicals:

$$AH + LO^{\bullet} \rightarrow LOH + A^{\bullet} \quad (5)$$

$$A^{\bullet} + LO^{\bullet} \rightarrow LOA \quad (6)$$

Although the activity of antioxidants is generally measured by their effectiveness to inhibit hydroperoxide formation by reaction (1), their activity to inhibit aldehyde formation by reaction (5) is an important property that should also be measured in any judicious protocol.

Hindered phenols are the most common antioxidant compounds to readily scavenge lipid peroxyl radicals by donating hydrogen atoms.[62,67] The well-recognised structure–activity relationships of radical-scavenging phenolic antioxidants (tocopherols, gallic acid and gallic acid esters, polyphenolic flavanols, flavonoids and phenolic acids) involve the ability to donate a phenolic hydrogen as well as the stabilisation of the resulting antioxidant radical by electron delocalisation and/or intramolecular hydrogen bonding or by further oxidation.[6,68,69] For phenolic antioxidants the loss of a hydrogen may take place by donation of an electron followed by deprotonation. Recently, an evaluation of bond energies for hydrogens in catechins (C—H vs O—H bond dissociation enthalpies) has led to the hypothesis that donation of non-phenolic hydrogen atoms could explain the antioxidant mechanism of catechins.[70]

'Preventive antioxidants' are defined either as 'preventing introduction of initiating radicals'[62] or as reducing the rate at which new chains are started.[71] Metal chelators are preventive antioxidants by complexing with transition metal ions, thereby hindering metal-catalysed initiation reactions and decomposition of lipid hydroperoxides.[6] Other antioxidant mechanisms include singlet oxygen quenching, oxygen scavenging, and blocking the prooxidant effects by binding certain proteins containing catalytic metal sites. This last mechanism was suggested to play a role in the inhibitory action of flavonoids and grape phenolics on the *in vitro* copper-induced oxidation of human low-density lipoprotein (LDL).[54,72]

Antioxidant action becomes more complex in real foods and biological systems where a variety of mechanisms become effective, including free radical chain breaking, oxygen scavenging, singlet oxygen quenching, metal chelation, and inhibition of oxidative enzymes.[6,73,74] On the one hand, mechanistic definitions limited only to radical chain breaking may exclude important antioxidants in foods and biology. On the other hand, too broad a definition of antioxidants may include too many types of action that can limit proper understanding and interpretation of antioxidant mechanisms. Valid evaluation of antioxidant activity therefore requires the use of several different assay methods to include different mechanisms of inhibition of lipid oxidation. Meaningful interpretation of antioxidant action requires: (a) specifying the oxidising substrate protected by the putative antioxidant; (b) measuring the correct extent of oxidation and inhibition by the antioxidant and choosing an appropriate end-point of oxidation; (c) assuring that the substrate and the mode of inducing oxidation are relevant as sources of oxidative damage; and (d) determining any possible adverse prooxidant effects from the antioxidants.

COMPOSITION OF THE TEST SYSTEM

Interfacial phenomena and antioxidant partitioning

The activity of different types of antioxidants can vary significantly depending on whether the lipids are triacylglycerols, methyl esters, free fatty acids or incorporated into various biological particles such as lipoproteins or liver microsomes. Whether the antioxidants function in aqueous, bulk lipid or in heterophasic systems is critically important. The oxidative

stability of most colloidal, lipid-bearing foods is greatly affected by a multitude of surface-active substances and their interfacial interactions with both oxidants and antioxidants. Antioxidant activity is thus strongly affected by the physical composition of the test system, and the relative activity of antioxidants of different polarity varies significantly in different multiphase systems.[6] The phenomenological observation that polar antioxidants are more active in bulk oil systems whereas non-polar antioxidants are more active in lipid suspended in aqueous systems was referred to as the 'polar paradox' by Porter.[55,75] This paradoxical behaviour of antioxidants was confirmed with the observations that the hydrophilic antioxidants Trolox (the carboxylic acid derivative of α-tocopherol) and ascorbic acid and the rosemary compounds carnosic acid and rosmarinic acid were better antioxidants in bulk oils than the corresponding lipophilic antioxidants α-tocopherol, ascorbyl palmitate and carnosol (Table 3).[11,48,50,56,76–80] In contrast, the order of activity was reversed in emulsified corn oil systems. This interfacial phenomenon was explained by differences in the affinity of hydrophilic and lipophilic antioxidants towards the air, oil and water phases as well as the interface.[56] Accordingly, in bulk oils the hydrophilic antioxidants may be more effective by being oriented in the oil–air interface where they can efficiently protect against lipid oxidation (Fig 1). In contrast, in oil-in-water emulsion systems, hydrophobic antioxidants are located in the oil and the oil–water interface where they are more protective than hydrophilic antioxidants, which are partitioned into the aqueous phase and are not able to adequately protect lipids in the water–oil interface. When dissolved in the water phase, the efficiency of hydrophilic antioxidants may be offset by their prooxidant activity resulting from reduction of metals into the more active lower valence state.

Measurement of partitioning properties of a number

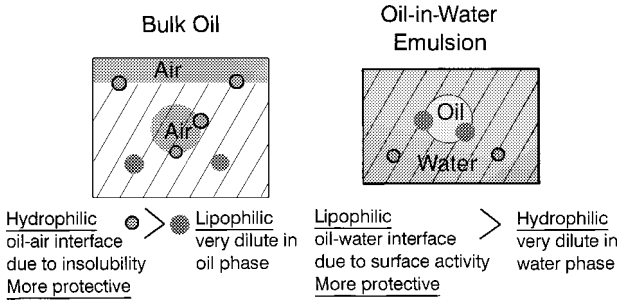

Figure 1. Interfacial distribution of hydrophilic and lipophilic antioxidants in bulk compared to oil-in-water emulsions (from Ref 56).

of antioxidants in model emulsions[58,81] and in mayonnaise[82] confirmed that antioxidants of different polarity partition into different phases in heterophasic systems. The partitioning of antioxidants between the oil and water phases occurs to different extent according to their chemical structures and polarity.[81–83] Thus, in a food emulsion like mayonnaise,[82] various relatively polar antioxidants were distributed between the lipid and aqueous phases according to their oil–water partition coefficients $P_{o/w}$ determined in unbuffered oil–water mixtures (20:80 w/w).[58] The antioxidants tested could be categorised according to their $P_{o/w}$ into three groups of increasing polarity.

Group 1. Trolox: $P_{o/w} \approx 3.5$.
Group 2. Propyl gallate, ferulic acid: $P_{o/w} \approx 0.7–0.9$.
Group 3. Gallic acid, caffeic acid, catechin: $P_{o/w} \approx 0.1$.

There is only limited knowledge on how partitioning and antioxidant effectiveness of natural phenolic compounds in heterophasic systems are governed by their chemical structures. Antioxidants may also be distributed into surfactant/emulsifier-rich interfacial layers in heterophasic food emulsions.[82,83] The partitioning properties of a particular antioxidant not only depend on the chemical structure and relative

Table 3. Effect of lipid systems on relative activities of antioxidants[a]

Lipid system	*Antioxidant trends*	*References*
Bulk corn oil	Trolox > α-tocopherol, ascorbic acid > ascorbyl palmitate	56
Oil/water emulsion	α-Tocopherol > Trolox ≈ ascorbyl palmitate > ascorbic acid	56
Bulk corn oil	Carnosic acid ≈ rosmarinic acid > carnosol	76
Oil/water emulsion	Carnosol > carnosic acid > rosmarinic acid	76
Bulk Me linoleate, corn oil	Trolox > α-tocopherol; carnosic acid > carnosol > α-tocopherol	77–79
Bulk linoleic acid	Trolox > α-tocopherol > carnosol > carnosic acid	77–79
Me linoleate emulsion, corn oil emulsion	α-Tocopherol > Trolox; α-tocopherol > carnosic acid > carnosol	77–79
Linoleic acid emulsion	Trolox > α-tocopherol ≈ carnosol > carnosic acid	77–79
Lecithin liposome	BHT > BHA > propyl gallate > TBHQ > gallic acid	80
DLPC liposome	Trolox = α-tocopherol	48
PC liposome	EG > EC > quercetin > α-tocopherol	50
Me linoleate Solution[b]	α-Tocopherol ≫ quercetin > EC = EG	50
SDS micelles of linoleic acid	α-Tocopherol = ascorbyl palmitate > Trolox > ascorbic acid	11
$HDTBr^+$ micelles of linoleic acid	Ascorbic acid > Trolox > ascorbyl palmitate > α-tocopherol	11

[a] Abbreviations: BHT, *tert*-butylhydroxytoluene; BHA, *tert*-butylhydroxyanisole; TBHQ, *tert*-butylhydroquinone; DLPC, dilinoleyl phosphatidylcholine; PC, phosphatidylcholine (egg); EG, epicatechin gallate; EC, epicatechin; SDS, sodium dodecyl sulphate; $HDTBr^+$, hexadecyltrimethyl ammonium bromide.
[b] Hexane–isopropanol (1:1 v/v).

polarity of the antioxidant, but also vary according to the lipid substrates, surfactants, pH, temperature and the composition of the phases.[12,49,58,81]

The following example illustrates the importance of partitioning behaviour for testing and predicting effectiveness of antioxidants in complex food systems. A patented formulation consisting of a mixture of ascorbic acid, soy lecithin and tocopherols (the A/L/T system)[84] performed as an effective synergistic antioxidant in bulk oil on the basis of Rancimat tests. The A/L/T mixture was superior to a large array of natural and synthetic antioxidants in retarding oxidation of bulk, refined fish oil based on both chemical and sensory analyses (Jacobsen and Meyer, unpublished data). In another study with bulk fish oil stored at room temperature, the A/L/T mixture was also very effective in preventing oxidation based on peroxide value, but not based on sensory measurements.[85] The antioxidant synergism of the A/L/T mixture in oils was explained based on interactions between all three components. The lecithin not only functions as an emulsifier to improve the contact between ascorbic acid and tocopherol, but also participates in redox cycling with the tocopheroxyl radical and ascorbic acid by radical exchange reactions of a primary amine group in phosphatidylserine or phosphatidylethanolamine.[86] However, the A/L/T ternary antioxidant mixture was ineffective in inhibiting the sensory deterioration of fish oil-enriched mayonnaises.[87] In a study of the fate of the A/L/T system in real mayonnaise, ascorbic acid was found to be located in the aqueous phase.[87] This result indicates that separation of the components of the A/L/T mixture could partly explain the loss of its antioxidant potency in mayonnaise. Recently, ascorbic acid was found to be inactive as an antioxidant in mayonnaises containing fish oil.[88] By interacting in the aqueous phase with iron released from the egg-yolk emulsifier, ascorbic acid promoted oxidative flavour deterioration. These studies illustrate how different results can be obtained depending on the composition of the system, the oxidation conditions and the methodology employed to evaluate antioxidant activity.

In summary, antioxidant effectiveness in multiphase food and biological systems is affected by important factors determined by interfacial phenomena governing the localisation and orientation of antioxidants by partitioning between the aqueous phase and the lipophilic phase and by interacting with the emulsifier at the interface. This concept was supported by studies carried out with different model emulsions[56,58,76–78] and with real food emulsions like mayonnaise.[82,87,88] Studies of partitioning properties of antioxidants also explained the efficiency of ferulic acid as an inhibitor of human LDL oxidation *in vitro*.[89] The phase distribution behaviour of antioxidants in multiphase systems is thus important to design test protocols for antioxidants and to interpret experimental results obtained with heterophasic systems. More knowledge is required on the partitioning behaviour and efficiency of antioxidants in different phases to improve our understanding of antioxidant properties in different colloidal food and biological systems.

The oxidising substrate to be protected

The relative activity of various antioxidants depends on the type of substrate (eg phospholipids vs triacylglycerols and free fatty acids), the degree of lipid unsaturation and the physicochemical state of the oxidisable substrate. Some of the observed differences are attributable to the degree of heterogeneity of the system as discussed above, and complicated by the colloidal properties of the lipid substrate. Such influence occurs for example when linoleic acid is used as an oxidising substrate suspended in aqueous emulsion systems.

The disadvantages of using emulsified linoleic acid and methyl linoleate as substrates to evaluate antioxidants have been reported previously.[6,78] Emulsified linoleic acid[8–10,90–97] and emulsified methyl linoleate[6,78,95,98–100] are widely used as lipid systems to test antioxidants. However, these lipids are not appropriate model substrates for foods, which consist mainly of triacylglycerols. In contrast to triacylglycerols, linoleic acid forms micelles in aqueous systems, which have different colloidal properties strongly affecting the behaviour of both oxidation initiators and antioxidants.[6] The greater polarity of methyl linoleate compared to triacylglycerols would also be expected to affect the relative activity of hydrophilic compared to lipophilic antioxidants.

The effectiveness of α-tocopherol and its water-soluble analogue Trolox was greatly affected by the lipid systems used for antioxidant testing.[78] Thus the effectiveness of Trolox was significantly greater in oil-in-water emulsions of linoleic acid compared to the corresponding triacylglycerol emulsion (Table 3). In triacylglycerol emulsified with Tween 20 the antioxidant activity of Trolox decreased because it partitioned into the water phase, whereas in the corresponding linoleic acid emulsion its antioxidant activity increased owing to formation of mixed micelles of linoleic acid and Tween 20. Because micelle-forming lipid substrates in aqueous systems greatly enhance the activity of hydrophilic and polar antioxidants, linoleic acid is not considered to be a valid substrate to test antioxidants in foods consisting of triacylglycerols.

Another artefact of using linoleic acid test systems was observed in studies evaluating superoxide dismutases (SOD) as antioxidant enzymes for lipid foods. Because SOD catalyse the disproportionation of superoxide to oxygen and hydrogen peroxide, these enzymes were tested for the protection of foods by determining if the removal of superoxide can retard the overall rate of oxidation. Although several studies proved that SOD exhibited antioxidant effects in dilute emulsions of linoleic acid,[101,102] these enzymes did not inhibit oxidation of either emulsified herring oil or emulsions of tocopherol-stripped vegetable

oils.[103] The data suggest that, in dilute linoleic acid aqueous systems, SOD is an effective antioxidant because it inhibits the action of superoxide, which initiates lipid peroxidation by the Fenton reaction.[104] The Fenton reaction is apparently not significant in triglyceride systems and in rapidly oxidising, dilute fish oil emulsions. In emulsions of fish oil or vegetable oils without endogenous tocopherols the amounts of new initiations are apparently surpassed by the propagation reactions that are initiated by metal-catalysed breakdown of lipid hydroperoxides rather than by superoxide.[103]

Many examples in the literature show that phenolic compounds can have either antioxidant activity or prooxidant activity depending on the oxidising target and conditions used in the test system. A number of examples illustrate the variation in activity of antioxidants tested in different lipid systems (Table 3). Hydrophilic polyphenolic compounds showed significantly different trends in antioxidant activity when tested in three different systems. Pure catechin and green tea catechin gallates were effective in inhibiting oxidation of LDL and lecithin liposomes but showed prooxidant activity in oil-in-water emulsions.[72,105,106] The marked variation in antioxidant activity of rosemary constituents (Table 3) and green teas is attributed to differences in their relative partition between phases in various lipid systems. The improved antioxidant activity observed for green teas in lecithin liposomes compared to corn oil emulsions was explained by the greater affinity of the polar catechin gallates for the polar surface environment of the lecithin bilayers, thus affording better protection against oxidation.[105] Phenolic extracts from fresh grapes inhibited both *in vitro* oxidation of human low-density lipoprotein (LDL) and of soybean lecithin liposomes. However, the ranking of activities of extracts of different grape types showed opposite trends in the LDL compared to the liposome systems.[107,108]

Propyl gallate stimulated hydroxyl radical-dependent deoxyribose degradation, measured as thiobarbituric acid-reactive substances (TBARS), but strongly inhibited the oxidation of rat liver microsomes mediated by iron/ascorbate.[109] When catechins were compared with oligomeric procyanidins, their antioxidant activity in a phosphatidylcholine liposome system decreased with polymerisation, in contrast with their reactivity with the ABTS cation radical in an aqueous system (see discussion of the TEAC assay below), increasing from monomer to trimer and decreasing from trimer to tetramer.[110] However, in these comparisons the authors also used different modes of inducing oxidation (ascorbate/iron vs metmyoglobin/H_2O_2) and different methods of measuring oxidation (TBARS vs quenching of radical cation at 734 nm).

The antioxidant effectiveness of rosemary extracts, carnosol and carnosic acid, was significantly influenced by the type of system tested, the oil substrates, the methods used to follow oxidation, and the concentrations of test compounds.[57] Although the rosemary extracts and compounds effectively inhibited oxidation in corn oil, soybean oil, peanut oil and fish oil, when tested in bulk, these compounds were either inactive or promoted oxidation in the corresponding vegetable oil-in-water emulsions. In fish oil emulsions, however, the rosemary compounds inhibited conjugated diene and pentenal formation, but not propanal. The greater affinity towards the more polar fish oil–water interface may explain the higher antioxidant activities of rosemary antioxidants observed in fish oil emulsions than in vegetable oil emulsions.

Emulsifier type and interacting components

The use of charged emulsifiers is another factor markedly affecting antioxidant activity. Sodium dodecyl sulphate (SDS) is often employed to test antioxidants with linoleic acid in aqueous systems (Table 1). Antioxidants such as ascorbic, benzoic and hydroxycinnamic acids, which are present largely as anions at test conditions around pH 7.0–7.4, are ineffective in aqueous systems emulsified with SDS owing to electrostatic repulsion by the negatively charged micelles at neutral pH values (Table 1). These antioxidants may not contact the oxidising lipid inside the SDS micelles because of repulsion exerted by the negative charge of the SDS emulsifier. Methyl cinnamates were more active as antioxidants than their corresponding free cinnamic acids in an SDS–linoleic acid micelle system at neutral pH. This difference is due to the electronic repulsion of the micelles.[13] This charge effect may thus shield true differences in antioxidant properties of the compounds tested.

Various fruit, vegetable and herb extracts were better antioxidants in inhibiting oxidation induced by iron/ascorbate of phosphatidylcholine compared to human liver microsomes.[53] This difference in antioxidant activity may indicate a possible diminishing effect on antioxidant activity in the presence of proteins or enzymes in human liver microsomes. In another study the addition of bovine serum albumin (BSA) to oxidising phosphatidylcholine liposomes decreased the antioxidant potency of phenolic compounds in de-alcoholised wine.[111] In the presence of 20% BSA, however, the order of activity of various phenolic compounds changed, and the phenolic compounds in red wines were more active in inhibiting lipid oxidation than those in grape extracts.[111] These results illustrate how the addition of other food components such as proteins to the test system may provide useful information about interacting substances.

In conclusion, the structure–activity relationship of natural phenolic antioxidants is not only significantly affected by the test system used and the biological targets to be protected, but also by the modes of inducing oxidation and by the method used to determine oxidation. Antioxidant activity may be further modulated by other components present in

the test system. For these reasons it is highly desirable to (a) compare antioxidant activities in several systems relevant to either foods or biological targets, (b) calibrate substrates for food and biological antioxidant testing according to the relevant lipid type and (c) avoid the use of free fatty acids, notably linoleic acid, as substrates because of their different colloidal behaviour from either triacylglycerols or phospholipids in aqueous systems.

THE MODE OF INDUCING OXIDATION

In standard tests for *oxidative stability*, several parameters are manipulated to accelerate oxidation, including temperature, metal catalysts, oxygen pressure, and variable shaking to increase reactant contact. Accelerated testing by increased temperature is beset by many pitfalls, which may occur from physical and chemical changes of the test system[5,6] and from invalid extrapolations of reaction kinetics between different temperatures.[4,5] Additional methods to accelerate oxidation include the use of iron/ascorbate, lipoxygenase and exposure to light to promote photosensitised oxidation by singlet oxygen. When lipoxygenases are employed, the enzyme inhibitory activity may be confounded as an antioxidant effect. Photosensitised acceleration invariably favours the effects of antioxidants that have multiple effects as singlet oxygen quenchers, and underestimates the effects of chain-breaking antioxidants. The two most commonly used parameters to accelerate lipid oxidation are discussed below in more detail.

Transition metals

Metal ion catalysts in the presence of small amounts of hydroperoxides are the most important initiators of lipid oxidation in foods and biological systems. Transition metals such as iron and copper catalyse both the initiation and decomposition of hydroperoxides, which are important in polyunsaturated lipids containing three or more double bonds.[6] The decomposition of polyunsaturated hydroperoxides is particularly significant in lowering the yield of hydroperoxides. The resulting high levels of volatile decomposition products have a high impact on flavour deterioration in food lipids and on biological damage.

The level of copper ions used as catalyst in an *in vitro* LDL oxidation test and in a lecithin liposome had a large impact on antioxidant effectiveness of different anthocyanins. When the oxidation of LDL was catalysed by 10 μM copper, the order of antioxidant effectiveness of the anthocyanins was malvidin > delphinidin > cyanidin > pelargonin. However, at 80 μM copper the antioxidant activity changed and decreased in the order delphinidin > cyanidin > malvidin > pelargonin.[54] In the liposome system, malvidin was the best antioxidant at both levels of copper. In these test systems, anthocyanins were assumed to operate by several mechanisms, including metal chelation, radical scavenging, and protein binding in LDL. In another study of green tea antioxidants tested in a liposome system oxidised at 50 °C, epigallocatechin gallate (EGCG) and propyl gallate (PG) were the best antioxidants, followed by epicatechin, epigallocatechin (EGC), epicatechin gallate (ECG), catechin and gallic acid (GA).[106] However, in liposomes oxidised at 37 °C with cupric acetate, catechin and epicatechin were better antioxidants than ECG, but EGCG, EGC, PG and GA promoted oxidation. In oil-in-water emulsions, all tea catechins, GA and PG were prooxidants. Tea catechins were considered better antioxidants in liposomes compared to emulsions because of the greater affinity and better protection afforded by the polar catechins toward the polar surface of the lecithin bilayers.[81,105]

The significance of the type of inducer on the activity of natural antioxidants was illustrated in a study showing that a methanolic extract of rosemary inhibited oxidation of human liver microsomal lipids 50 times better when oxidation was induced by NADPH compared to ascorbate/iron induction.[53] More recently, the efficacies of flavonoids (myricetin, quercetin, apigenin and chrysin, kaempferol, morin, taxifolin) showed either antioxidant or prooxidant activities depending on the concentration of iron and other metal catalysts used in a linolenic acid-containing hepatocyte test system.[112] Flavones, luteolin, apigenin and chrysin behaved as antioxidants at low iron concentration but as prooxidants at high iron concentration. These studies further illustrate how flavonoid and polyphenol antioxidants can operate by several different mechanisms and how the dominant activity in a test system depends on the protocol, notably the nature of the lipid substrate and level of metals used in the oxidation test. There is an urgent need therefore to better understand the mechanism of action of phenolic antioxidants in complex environments.

Azo initiators

Azo compounds (R—N=N—R) are commonly used as initiators in various *in vitro* assays for antioxidant testing (Tables 1 and 2). These compounds are useful to study quantitatively the kinetics of lipid oxidation and antioxidation because they generate radicals at a reproducible and constant rate. In the presence of initiators such as α,α-azobisisobutyronitrile (AIBN) the initiation can be directly related to the rate of production of the azo radical. By using either water-soluble or lipid-soluble azo dyes, these compounds can initiate radicals in known specific microenvironments.[113–115] For example, a mixture of α-tocopherol and ascorbic acid showed an additive protective effect in a liposome oxidised in the presence of the water-soluble 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), which generates free radicals in the aqueous phase. In contrast, the mixture of α-tocopherol and ascorbic acid showed a synergistic protective effect in the presence of the oil-soluble 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), which generates free

radicals within the lipid phase. This synergistic effect was interpreted by the initial trapping of radicals by α-tocopherol in the lipid phase, with the resulting α-tocopherol radicals becoming oriented at the surface bilayers and reduced by ascorbic acid in the water layer to regenerate α-tocopherol.[113] Better antioxidant activity was also observed with *tert*-butyl hydroxytoluene when tested in a liposome oxidised with Cu^{2+} in the presence of *tert*-butyl hydroperoxide than in the same liposome oxidised with AAPH or AMVN.[116] In another study using a liposome system oxidised with AAPH, the green tea catechin ECG had the highest activity, followed by epicatechin, quercetin and α-tocopherol, while the reverse trend was observed when methyl linoleate was oxidised in hexane–isopropanol solution in the presence of AMVN (Table 3).[50] These results were explained by a greater protection of the polar phopholipids by the flavonoids by being associated with the surface, while α-tocopherol was less protective by remaining in the core of the membrane.

The hydrophilic AAPH initiator is widely used in quantitative studies of oxidation kinetics. Although this kinetic approach may be useful to study free radical oxidation in simple model systems, the azo initiators are artificial systems that are not found in either foods or biological systems. The efficiency of these initiators is greatly affected by the solvent system used for oxidation, by its viscosity and by solvent cage effects that are not relevant to food lipids.[6] Diazo initiators produce a high flux of peroxyl radicals that do not have time to branch and proceed to other reactions observed in real lipid systems. In contrast to metal catalysts, the azo compounds have no effect in promoting decomposition of hydroperoxides to produce aldehydes. Azo initiators thus emphasise the propagation phase of lipid oxidation and minimise the decomposition of hydroperoxides, which is significant with polyunsaturated fatty acids containing three or more double bonds.[6] The quantitative kinetic data obtained with diazo initiators are thus oversimplified and not relevant to either foods or biological systems.

Careful design of *in vitro* protocols can aid in obtaining mechanistic information if relevant substrates and oxidation inducers are employed. Although the azo initiators have the advantage of generating localised radicals at a constant rate, their artificial nature and exaggeration of the hydroperoxide generation stage make them inappropriate to mimic reactions occurring in food and biological systems.

EFFECT OF NATURAL ANTIOXIDANTS ON OXIDATION OF HUMAN LOW-DENSITY LIPOPROTEIN (LDL) *IN VITRO*

A prevailing hypothesis postulates that the oxidation of unsaturated lipids in LDL particles initiates a complex sequence of reactions leading to the development of atherosclerosis. This hypothesis is supported by numerous studies *in vitro*, in animals and in humans.[117–120] The LDL particles contain cholesterol, cholesterol esters and triacylglycerols of n-6 and n-3 polyunsaturated fatty acids that are readily oxidised in the presence of various free radical initiators, metals such as copper ions, and radicals released by endothelial cells from the arterial wall. The hydroperoxides formed by lipid oxidation decompose into aldehydes that interact with the free lysine group of apolipoprotein B. The oxidatively modified LDL particles are no longer recognised by the normal LDL receptor, a well-regulated system in plasma, and are taken up by the 'scavenger' receptor system, which is unregulated and leads to the accumulation of oxidised LDL in subendothelial cells to form 'foam cells'. The oxidation of LDL cholesterol forms a mixture of products, including α- and β-epoxide-, triol-, 7-α- and 7-β-hydroxy- and 7-keto-cholesterol.[121,122] Cholesterol oxidation products account for most of the cytotoxicity of oxidatively modified LDL.[123] These products have been suggested as useful markers of atherosclerosis.

Epidemiological evidence and *in vitro* antioxidant studies have supported the hypothesis that natural phenolic antioxidants present in fruits and vegetables may be active *in vivo* to inhibit the oxidation of LDL and thus provide protection against coronary heart disease and thrombosis.[117,124] A wide variety of methods have been employed to assess antioxidant activity on human LDL *ex vivo*. Notably, the mode of induction and method used for oxidation measurement, including the type of oxidation inducer and assay reaction time, appear to influence significantly the ranking of antioxidant activity, but other factors may also have a large impact. A number of agents have been used to oxidise LDL, including Cu^{2+}, macrophages, cultured vascular cells, metmyoglobin and AAPH. As discussed in the previous section, azo initiators such as AAPH are not suitable for LDL oxidation *in vivo*. For example, although *in vivo* supplementation with vitamin E significantly inhibited LDL oxidation by endothelial cells, macrophages and copper, it promoted oxidation in the presence of AAPH.[124] Therefore the use of AAPH and other artificial azo compounds as oxidants to test antioxidants for their biological effects may be questionable. Although it is debated whether copper is a suitable initiator for LDL oxidation *in vivo*, copper-accelerated oxidation of human LDL is the most extensively studied *in vitro* mechanism. This oxidation requires both binding of Cu^{2+} ions by apolipoprotein B (apo B) and reduction of copper by LDL.[125]

Phenolic antioxidants may act as inhibitors of LDL oxidation by several different mechanisms, including scavenging of free radicals and reactive oxygen species (superoxide, oxidised lipids, oxysterols), metal chelation, protecting α-tocopherol present in LDL, and binding with proteins. Two other antioxidant mechanisms have been postulated with copper-mediated oxidation of LDL *in vitro*, namely the binding of phenols to tryptophans and/or histidines in apo B to block access to copper, and the oxidation of histidine to 2-oxo-histidine caused by secondary radicals of

Table 4. Relative antioxidant activity of selected phenolic compounds by different methods[a]

	Inhibition (%) LDL oxidation[b]	*TEAC (mM Trolox)*	*TAS*[c] *(Randox) (mM)*	*ORAC (μM Trolox)*
Phenolic compounds (5 μM GA equivalent)	Ref 72	Ref 131	Ref 132	Ref 41
Catechin	74.9	2.40	3.50	2.49
Myricetin	68.1	3.12	4.04	–
Epicatechin	67.6	2.50	4.96	2.36
Rutin	67.6	2.42	–	0.56
Gallic acid	63.3	3.01	3.00	1.74
Quercetin	61.4	4.72	4.24	3.29
Ellagic acid	36.6	–	–	–
Sinapic acid	35.1	–	–	–
α-Tocopherol	32.6	0.97	0.90	–
Anthocyanins	Ref 54			Ref 43
Cyanidin	79.4	4.42	–	2.2
Delphinidin	71.8	4.44	–	1.8
Malvidin	59.3	2.06	–	2.0
Pelargonin	39.0	1.30	–	1.1
Hydroxycinnamates	Ref 133			Ref 41
Caffeic acid	96.7	1.26	3.64	2.23
Chlorogenic	90.7	1.20	–	
Ferulic acid	24.3	1.90	1.84	1.33
p-Coumaric acid	24.5	2.22	1.56	1.09
Tea catechins	Liposomes[d] Ref 106			
Epigallocatechin gallate	82.0	4.75	–	–
Epicatechin	80.2	2.50	–	–
Epicatechin gallate	59.6	4.93	–	–
Epigallocatechin	22.2	3.82	–	–

[a] See Table 2 for abbreviations and conditions for TEAC and ORAC assays. GA, gallic acid.
[b] Copper-catalysed oxidation of human LDL monitored by hexanal determination by headspace gas chromatography[19].
[c] TAS, total antioxidant status. The Randox method is based on the TEAC assay (see Table 2).
[d] Contained 8 mg ml^{-1} soya lecithin in water, oxidised at 37 °C with 10 μM cupric acetate.

phenols.[126,127] The latter mechanism is supported by the observations that both ascorbic acid and dehydroascorbic acid retard copper-induced LDL oxidation *in vitro*.[127]

In our antioxidant evaluations on human LDL a number of *in vitro* systems were used including copper or biological catalysts (myoglobin, cytochromec, iron/ascorbate, endothelial cells) to compare the effects of wines and extracts of grapes, berries, grape juices, green tea catechins and various pure phenolic compounds. Diluted red wine containing 10 μM total phenols had the same antioxidant activity as 10 μM quercetin.[9,128] Evaluations of 20 Californian wines showed relative inhibition of LDL oxidation varying from 46 to 100% with red wines and from 3 to 6% with white wines.[20] Similarly, the antioxidant activities of phenolic extracts from 14 different types of fresh grapes inhibited LDL oxidation by 22–91%.[107] Samples of commercial grape juices inhibited LDL oxidation by 62–75%, and vitamin C had no significant effect on their antioxidant activity.[129] The relative activities of extracts of berries based on their protection of human LDL against oxidation decreased in the order blackberries >red raspberries >sweet cherries >blueberries >strawberries.[130] Pure phenolic compounds showed relative antioxidant activities decreasing in the order catechin > myricetin = epicatechin = rutin > gallic acid > quercetin (Table 4).[41,43,54,72,106,131–133] When pure anthocyanins were tested against LDL oxidation that was induced with 80 μM copper, cyanidin and delphinidin were better antioxidants than malvidin and pelargonin[54] (Table 4), when LDL oxidation was accelerated with 10 μM copper, malvidin was the best antioxidant, followed by cyanidin, delphinidin and pelargonin.[54] However, when tested in a liposome system oxidised with 10 μM copper, pelargonin was followed by malvidin in antioxidant activity, but cyanidin and delphinidin were prooxidants.

Chlorogenic acid (5′-caffeoyl quinic acid) was found to be a better antioxidant than caffeic acid on human LDL oxidation *in vitro* when oxidation was induced with AAPH.[24] In another study using an LDL fraction containing ~20 wt% VLDL oxidised with cupric acetate as catalyst, caffeic acid was a better antioxidant than chlorogenic acid.[134] However, these authors used the notoriously unspecific and unreliable TBA test to measure LDL oxidation. They also derived a 'phenol

antioxidant index' by testing at a constant phenol concentration and dividing the total phenol concentration by the concentration of phenols required for 50% inhibition (IC_{50}) of lipoprotein oxidation.[135] Since IC_{50} is already based on the total phenol content of the sample, this index actually expresses antioxidant potency at a specific phenol concentration, and not total potency as reported. Thus antioxidant data obtained by different test procedures for assessing antioxidant activity on human LDL *in vitro* are difficult to compare. The properties of various phenolic compounds are very system-dependent, and several experimental factors influence the results obtained. Much attention has been given in the literature to the structure–activity relationships of phytochemicals and other phenolic antioxidants.[13,16,69,131] Although these relationships are of theoretical interest, this information is limited from the practical viewpoint without considering in the evaluation the significant effects of the system, the substrate, the oxidation inducer and the methods used for end-point of oxidation.

FREE RADICAL-TRAPPING METHODS

The potential health benefits of phytochemicals in fruits and vegetables has led to an explosion of research into the antioxidant properties of polyphenolic compounds, especially the flavonoids, which constitute an estimated 4000 different compounds. A wide variety of one-dimensional methods have been developed using a broad range of conditions, oxidants, methods to measure oxidation and end-points of oxidation. This diversity of methodology used to evaluate natural antioxidants from plant extracts and pure phenolic compounds has led to widely conflicting results that are extremely difficult to interpret (Table 4).

Several protocols have been developed for measuring the free radical-trapping ability of antioxidants using a wide variety of radical-generating systems and methods for oxidation end-point observations (Table 2). These radical-trapping methods have been developed to test 'antioxidant capacity' of biological samples and various plant extracts. Many papers have reviewed the multiplicity of radical-trapping methods,[3,136–138] but there is much confusion in understanding the significance of results and possible biological implications. These non-specific and one-dimensional methods do not allow investigations of the mechanism of antioxidant protection. They do not take into account the complex multistep mechanism of phenolic antioxidants (reactions (1)–(6)), their multiple actions in complex biological systems, partitioning effects and the significant effect of substrates on antioxidant effectiveness, as previously discussed. The characteristics of several commonly used free radical assays (Table 2) are briefly described below.

DPPH radical assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was one of the earliest synthetic radicals used to study structural effects on the activity of phenolic antioxidants.[26] This commercially available radical serves as the oxidising radical to be reduced by the antioxidant (AH) and as the indicator for the reaction $DPPH^{\bullet} + AH \rightarrow DPPH\text{-}H + A^{\bullet}$. The disappearance of the DPPH radical absorption at 515 nm by the action of antioxidants is measured spectrophotometrically in a methanolic solution until the absorbance remains constant. Assay time may vary from 10–20 min up to ~6 h.[27] The assay was employed to determine the 'antiradical efficiency' of polyphenolic compounds,[27,28] of caffeic acid and related hydroxycinnamates[93,139] and of different wines, grape juices and grape pomace extracts.[29,30,140,141] The decay slope and the absorbance level reached by the remaining DPPH radicals vary significantly with different types and concentrations of antioxidants. An antioxidant index parameter was proposed to determine 'antiradical efficiency' based on the amount of antioxidant required for a 50% decrease in initial DPPH radical concentration and the time required to reach the steady state DPPH radical concentration.[28] This assay is limited because DPPH radicals interact with other radicals (alkyl), and the time response curve to reach the steady state is not linear with different ratios of antioxidant/DPPH.[27,28]

TRAP assay

The total radical-trapping parameter (TRAP) assay was developed to measure 'total antioxidant capacity' of plasma or serum.[31] This assay uses peroxyl radicals generated by 2,2′-azobis(2-amidinopropane) hydrochloride (ABAP) to oxidise plasma antioxidants, and the oxidation is monitored by oxygen absorption. The induction period is compared to that of Trolox used as a reference water-soluble antioxidant. This method was later modified by adding linoleic acid as an oxidisable lipid substrate, before oxidation with ABAP.[142] The decomposition of ABAP showed the following decreasing order of consumption of plasma antioxidants: ascorbate > thiols > bilirubin > urate > vitamin E. The use of an artificial water-soluble azo compound as a radical generator such as ABAP does not provide a useful estimate of the important protective activities of metal chelators such as urate and of lipophilic antioxidants such as vitamin E. To overcome the problem of an unsteady electrode end-point, several modifications were developed by using chemiluminescence methods. These methods were reviewed by Alho and Leinonen[143] who also described protocols for plasma and LDL. These authors concluded that the measurement of plasma TRAP might not be valid, since free radical production would have to be sufficiently extensive to disturb the steady state level of antioxidants and disrupt the compartmentalisation protection afforded by cell membranes.

Superoxide anion scavenging

Although the superoxide radical anion ($O_2^{\bullet -}$) cannot directly initiate lipid oxidation, these assays use $O_2^{\bullet -}$ scavenging, because in the presence of metal ions the highly reactive hydroxyl radical, $^{\bullet}OH$, can be generated by the Fenton reaction.[104] However, the scavenging of $O_2^{\bullet -}$ is not the only mechanism for inhibition of lipid oxidation in either biological or lipid food systems.[103,144] Therefore phenolic compounds in plant extracts having $O_2^{\bullet -}$-scavenging properties are not necessarily effective in preventing lipid oxidation. The same argument may apply in the measurement of inhibitory effects on xanthine oxidase, the enzyme that generates superoxide radicals. Inhibition of xanthine oxidase activity as well as $O_2^{\bullet -}$-scavenging properties was employed to assess the activity of polyphenolic berry extracts.[33] In addition to these shortcomings, measurements of $O_2^{\bullet -}$-scavenging should be interpreted with caution, because no equilibrium can be achieved when superoxide radicals are generated continuously during the test.

TEAC assay

In this assay the radical cation 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate) ($ABTS^{\bullet +}$) is produced continuously by reaction between the ferrylmyoglobin radical generated from metmyoglobin, H_2O_2 and peroxidase, and the activity of antioxidants to scavenge the $ABTS^{\bullet +}$ radical cation is measured by the decrease in its absorbance at 734 nm.[35,36,136] The Trolox equivalent antioxidant activity (TEAC) reflects the amount of Trolox (mM) required to produce the same activity as 1 mM of the test compound. The reaction of ferrylmyoglobin in this assay may be confounded by the reduction of the ferrylmyoglobin radical by the antioxidants to be tested. Since $ABTS^{\bullet +}$ is a relatively long-lived radical, the TEAC assay was modified by generating the $ABTS^{\bullet +}$ radical by direct oxidation of ABTS with potassium persulphate prior to reaction with test antioxidants.[38] Thus the high TEAC values obtained for quercetin and cyanidin in the original myoglobin/ABTS assay were significantly lower with the modified persulphate-activated $ABTS^{\bullet +}$ assay. This result is consistent with some interaction occurring between these flavonoid antioxidants and ferrylmyoglobin in the original assay.[38] Despite recent improvements and increased use, the TEAC assay has several limitations. The ability of an antioxidant to scavenge the artificial $ABTS^{\bullet +}$ radical may not reflect the antioxidant activity due to other mechanisms effective in complex food lipids or physiologically relevant substrates, including metal chelation, and effects of antioxidant partitioning among phases of different polarities.

ORAC assay

The oxygen radical absorbance capacity (ORAC) method of Cao *et al*[39] is a modification of the procedure measuring the fluorescence during AAPH-induced oxidation of porphyridium cruentum β-phycoerythrin (β-PE), a phycobiliprotein containing a red photoreceptor pigment.[145–147] In the ORAC assay the 'antioxidant capacity' is quantified by calculating the net protection area under the time-recorded fluorescence decay curve of β-PE in the presence of an antioxidant or serum. The decrease in fluorescence is not linear with time, and the shapes of fluorescence decay curves differ in the presence of different antioxidants and with different concentrations of the same antioxidant.[39,148] The calculation of the area under the curve apparently circumvents erroneous assumptions of linearity and response similarities. However, in a recent application of this method to the evaluation of antioxidants in oat extracts, the inhibition of AAPH-induced oxidation of β-PE was evaluated from the midpoint (30 min) of the assay.[149] A principal drawback of the ORAC method is that it is assumed that the oxidative deterioration and, in turn, the antioxidative mechanism and protection of the fluorescent protein β-PE can mimic critical biological substrates.[39,145,147] In the ORAC assay the effect of oxidation of the photoreceptor portion of phycoerythrin (34 covalently attached linear tetrapyrrole prosthetic groups) on fluorescence measurements does not necessarily reflect the extent of antioxidant protection afforded against oxidative damage of the protein itself.

FRAP assay

The ferric-reducing antioxidant power (FRAP) assay measures directly the ability of antioxidants to reduce a ferric tripyridyltriazine complex (Fe^{3+}-TPTZ) to the ferrous complex (Fe^{2+}-TPTZ) at low pH.[46,150] The resulting blue colour measured spectrophotometrically at 593 nm is taken as linearly related to the total reducing capacity of electron-donating antioxidants. The main disadvantage of this approach is that the measured reducing capacity does not necessarily reflect antioxidant activity. Since the method does not include an oxidisable substrate, no information is provided on the protective properties of antioxidants.

Comparison of radical trap methods with *in vitro* LDL oxidation

The relative antioxidant activity of many phenolic compounds varies widely according to different testing methods. Thus there is no agreement between the relative activity of various flavonoids, anthocyanins, hydroxycinnamic acids and tea catechins on the inhibition of copper-induced human LDL oxidation *in vitro* compared to the total antioxidant capacity measured by the TEAC and ORAC assays (Table 4). Contrasting results were also reported for the antioxidant potentials of green tea polyphenols to inhibit human LDL oxidation *in vitro* and their efficiency to scavenge the $ABTS^{\bullet +}$ radical by the TEAC assay.[51] In contrast to our evaluations of grape juices with LDL, which showed that vitamin C had no activity,[129] a significant effect was reported for vitamin C when evaluated by the antioxidant capacity TEAC method

of Miller *et al.*[36] These data emphasise that the ranking of antioxidant activity is strongly dependent on the test system and on the substrate to be protected by the antioxidants. As discussed previously, polyphenolic flavanols may inhibit LDL oxidation by several mechanisms in addition to free radical scavenging.[72] On the other hand, the total antioxidant capacity assays listed in Table 2 only measure radical-scavenging activity in aqueous systems. The discrepancy in ranking of antioxidants shown in Table 4 can be explained not only by the multiplicity of mechanisms effective for these polyphenolic compounds, but also by the influence of the interfacial properties of multiphase LDL particles compared to the aqueous test systems used for the TEAC and ORAC assays.

RECOMMENDED PROTOCOLS

We have seen in this survey that the effectiveness of antioxidants is strongly dependent on the test system, the physical states of the lipid substrates, the conditions of oxidation, the oxidising substrate, the localisation of antioxidants and the method employed to evaluate oxidation and the stages of oxidation. The activity of antioxidants is greatly affected by complex interfacial phenomena in emulsions and multicomponent foods according to their hydrophilic or lipophilic character. The methodology to evaluate natural antioxidants must be carefully interpreted according to the system and to the analytical method used to determine the extent and end-point of oxidation.

Each antioxidant evaluation should be carried out under various conditions of oxidation, using several methods to measure different products of oxidation related to real food quality or critical biological reactions. *There cannot be a short-cut approach to determining the activity of antioxidants*. Various testing protocols should consider the following parameters.

(1) *Substrates*. Use substrates relevant to foods and biological systems, including triacylglycerols and phospholipids, in bulk, emulsion or liposome systems. Free fatty acids should be avoided because they form micelles in which antioxidants behave differently than in triacylglycerols.
(2) *Conditions*. Test under various oxidation conditions, including different temperatures (below 60 °C), metal catalysts and surface exposures. Select conditions to simulate real food or biological systems as closely as possible, depending on the application.
(3) *Analyses*. Measure relatively low levels of oxidation (below 1%) and include both initial products (hydroperoxides, peroxide value, conjugated dienes) and secondary decomposition products (carbonyls, volatile compounds).
(4) *Concentrations*. Compare antioxidants at the same molar concentration of active components using structurally related reference compounds. Consider carefully the concentration ratios of catalytic inducers/antioxidants and antioxidants/substrates. With crude plant extracts the total phenol contents and compositional data are required to compare samples.
(5) *Calculations*. Quantify on the basis of induction period, % inhibition or rates of hydroperoxide formation or decomposition, or IC_{50} (antioxidant concentration to achieve 50% inhibition).

Because of the complexity of real foods, accelerated test systems are difficult to standardise, and each antioxidant test should be calibrated for each lipid or food. Accelerated oxidation conditions should be close to the storage conditions under which the food is to be protected. Ultimately, antioxidants should be evaluated on the food itself.

In biological systems, phenolic compounds can participate in several antioxidant defences, including preventing oxidant formation, scavenging activated oxidants, reducing active intermediates and inducing repair systems. To improve our understanding of these complex interactions in different systems, the use of non-specific and one-dimensional assays for antioxidant capacity would be risky because they do not provide information on the biological target(s) protected. The better approach is to measure specific products of oxidation in both relevant *in vitro* and *in vivo* biological systems.

CONCLUSIONS

The large amount of effort expended in testing new natural antioxidants emphasises the need for improved test methods. Several currently used methods and model systems may not evaluate the true protective effects of antioxidants, and the data obtained can be confounded by many factors, including the composition of the test system, the substrate to be protected and the mode of inducing oxidation. In simplified model systems, interfacial phenomena may be overridden when interpreting antioxidant mechanisms and activity that appear strongly influenced by complex interfacial and phase distribution properties. When testing antioxidant activity of potential food antioxidants or bioactive compounds, the first aim may be to develop a model system where basic chemical principles can be deduced. On the other hand, the true effectiveness of antioxidants cannot be properly assessed unless the conditions, ie the complexity of the system, are as close as practically possible to the conditions under which protection against autoxidation is required. Targeting of antioxidants to prevent particular free radical formation steps and oxidative deterioration processes requires detailed understanding of the mechanisms of oxidation. Specific lipid model systems should mimic the food or physiological target systems to be protected as close as practically possible. There are various sources and types of oxidation and we should first define the targets of

oxidation—lipids, protein, DNA—before selecting methods to assess the protective properties of antioxidants under the conditions of their potential action and use. The total antioxidant capacity of phytochemicals based solely on one property, such as their scavenging ability towards artificial radicals, provides no information on what lipid or other substrate is protected. There cannot be a short-cut approach to evaluate antioxidants. In view of the wide divergence of results of natural antioxidants in foods and biological systems, more valid guidelines and assay protocols are urgently needed to bring some order to the present chaos in this important field. Our understanding of the effects of antioxidant compounds can only be improved if more specific methodology is used capable of defining what products are formed and inhibited by antioxidants depending on conditions, systems and targets of protection.

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