DOI 10.1002/mnfr.200600018 Mol. Nutr. Food Res. 2006, *50*, 714 – 724

Lipid peroxidation inhibition capacity assay for antioxidants based on liposomal membranes

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An *in vitro* antioxidant assay has been developed to better reflect the *in vivo* conditions of antioxidants interacting with membrane and lipid surfaces. The lipid peroxidation inhibition capacity (LPIC) method measures the ability of both lipophilic and hydrophilic antioxidants to protect a lipophilic fluorescent probe 4, 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid, incorporated in the membrane, from 2,2′-azobis(2-amidinopropane)hydrochloride generated radicals in the surrounding aqueous solution. Antioxidant activities of test compounds were measured either after they were mixed with preformed liposomes ($LPIC_{Mixed}$) or after they were incorporated into liposomes ($LPIC_{Inco}$) as they were made. The results were analysed to determine how the method of mixing and the structures of the antioxidants influenced their protection of the membrane from free radical attack. The $LPIC_{Mixed}$ values were larger than the $LPIC_{Inco}$ values for a range of 12 structurally diverse antioxidant compounds. However, there was no linear correlation between the lipophilicities, as measured by their partition coefficient, log *P* and either $LPIC_{Inco}$ or $LPIC_{Mixed}$ values. A strong correlation was found between $LPIC_{Inco}$ and $LPIC_{Mixed}$ values.

Keywords: Antioxidant / Flavonoids / Lipid peroxidation / Lipophilicity / Phenolic acid

Received: February 11, 2006; revised: March 20, 2006; accepted: March 22, 2006

1 Introduction

Oxidative damage is thought to be a fundamental cause of, or contributor to, a wide variety of pathological conditions such as cancer, heart disease and complications associated with diabetes. Many diet-derived compounds such as flavonoids, β-carotene and α-tocopherol have been recognised to act as antioxidants and are thought to be contributors to the health promoting effects of fruit and vegetables [1]. A large number of assays have been developed to assess the *in vitro* activity of antioxidants. They are usually carried out in aqueous systems [2 – 4]. However, dietary antioxidants have structures that range from highly hydrophilic to highly lipophilic. They would therefore be expected to associate differently with membrane and lipid surfaces. Thus results of *in vitro* antioxidant assays should reflect and measure these differences in behaviour in order to better predict *in vivo* activities.

Lipophilic antioxidants, such as carotenoids and tocopherols, can prevent peroxidative damage to membranes. A correlation between lipophilicity and the degree of mobilisation and localisation of some lipophilic compounds, such as carvedilol, has been shown using both model [5] and brain membrane bilayers [6]. An antioxidant assay, which accounts for partitioning effects, should give a better indication of the potential *in vivo* benefit of the antioxidant. To this end, a new assay, the lipid peroxidation inhibition capacity (LPIC) assay, based on unilamellar liposomes, was developed. This assay simulates free radical attack on cell membranes and measures the ability of antioxidants to protect them without requiring the use of blood or tissue-derived material. The partitioning behaviour of antioxidants into the aqueous and membrane phases was studied by measuring the partition coefficient (log *P*) by HPLC [7] to determine the correlation between lipophilicity and LPIC antioxidant activity. The results allowed interpretation of the influence of structure and lipophilicity on antioxidant activity in a model membrane system.

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Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane)hydrochloride; **AUC,** area under the curve; **C_{11}-BODIPY,** 4, 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid; **DOPC,** 1,2-dioleoyl-*sn*-glycero-3–phosphocholine; **DSC,** differential scanning calorimetry; **LPIC,** lipid peroxidation inhibition capacity; **Trolox,** 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

2 Materials and methods

2.1 Chemicals

The fatty acid fluorescent probe 4, 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid (C_{11}-BODIPY) was obtained from Molecular Probes (Eugene, OR). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was purchased from Sigma (St. Louis, MO). Morin was obtained from May & Baker (England). Myricetin and caffeic acid were purchased from Acros Organics (Milwaukee, WI, USA). Ferulic acid and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Aldrich Chem. (Milwaukee, WI, USA). All other chemicals were obtained from Sigma. All solvents were of HPLC grade. Water used in experiments was deionised (MilliQ).

2.2 LPIC measurement

The unilamellar vesicles were prepared following the extrusion method described by MacDonald *et al.* [8]. The antioxidants were either incorporated into or mixed with unilamellar vesicles and the LPIC assay was performed with both procedures.

2.2.1 Antioxidants incorporated into unilamellar vesicles ($LPIC_{Inco}$)

DOPC (5.0 mM) and the probe C_{11}-BODIPY (4.8 μM) were dissolved in chloroform. Lipophilic antioxidants such as α-tocopherol or β-carotene were added to the mixture of DOPC and C_{11}-BODIPY in a round-bottomed flask. Chloroform was removed by gently blowing with nitrogen while rolling the flask to deposit a thin lipid film on the wall. Tris-HCl buffer (20 mM, pH 7.4) was added to the dried film to give the original volume. Water-soluble samples, such as caffeic acid and L-ascorbic acid, were added to the Tris-HCl buffer. The flask was sonicated for 60 s (Sonorex Super, Bandelin Electric, Berlin, Germany) to bring the dried film into suspension. The suspension was freeze-thawed three times by placing the flask in a freezer (−20°C) to ensure solute equilibration between trapped and bulk solutions. The final suspension consisted of 5.0 mM DOPC, 4.8 μM C_{11}-BODIPY and an appropriate amount of antioxidant. The suspension was introduced into a Liposofast apparatus (Avestin, Ottawa, Canada) and passed through two-stacked 100 nm polycarbonate filters 11 times. The resulting unilamellar vesicles were diluted with the same volume of Tris-HCl buffer. The test mixture (1840 μL) comprised unilamellar vesicles (1000 μL, 2.5 mM DOPC, 2.4 μM C_{11}-BODIPY) which contained an antioxidant and 840 μL of 20 mM Tris-HCl buffer. This test mixture was incubated at 37°C in a thermostatted cuvette with stirring for 15 min to allow equilibration between unilamellar vesicles and Tris-HCl buffer.

After equilibration, 160 μL of 500 mM 2,2′-azobis(2-amidinopropane)hydrochloride (AAPH) was added to start the reaction and the zero point was immediately recorded using a fluorescence spectrophotometer (Perkin Elmer LS50B, Wellesley, MA, USA) at 540 nm excitation and 600 nm emission. The fluorescence was recorded every minute until the fluorescence of the last reading declined to less than 2% of the first reading or reached the zero point. Each sample was assayed in triplicate and a blank run was carried out for each batch of unilamellar vesicles. For a blank, the same mixture without antioxidant was used.

2.2.2 Antioxidants mixed with preformed unilamellar vesicles ($LPIC_{Mixed}$)

The unilamellar vesicles were prepared by following the same procedures as above but no antioxidant was incorporated into the unilamellar vesicles during their preparation. The $LPIC_{Mixed}$ assay was carried out using the same procedure except that 100 μL test compound in Tris-HCl buffer was added to the reaction mixture with a corresponding reduced volume of Tris-HCl buffer (740 μL). The $LPIC_{Mixed}$ procedure was not suitable for lipophilic antioxidants, such as α-tocopherol and β-carotene as there was no emulsifier to disperse them.

2.2.3 Calculation of results

Activity was calculated using the difference between the area under the curve (AUC) of the C_{11}-BODIPY fluorescence decay in the presence of an antioxidant and the blank. The use of the 'AUC' technique to quantify activities combines both the inhibition time and inhibition percentage of the reactive species action by antioxidants into a single value [3]. The final results ($LPIC_{Inco}$ or $LPIC_{Mixed}$ value) were calculated by using a regression equation between the Trolox concentration (in μM) and the net area under the C_{11}-BODIPY decay curve. The AUC was evaluated by applying Simpson's 1/3 rule using parabolas to approximate the area under a curve as in Eq. (1)

$$\mathrm{AUC} = \frac{\Delta t}{3}[f(t_0) + 4f(t_1) + 2f(t_2) + 4f(t_3) + \cdots + 4f(t_{n-1}) + f(t_n)] \quad (1)$$

where $f(t_0)$ is the initial fluorescence reading at 0 min, $f(t_n)$ is the fluorescence reading at t_n minutes and Δt was 1 min. The calculation of the 'net protection area' (ΔAUC) of a sample is illustrated in Fig. 1. The relative LPIC value was obtained from the Trolox standard calibration curves and expressed in Trolox equivalents (μmol *per* μmol antioxidant).

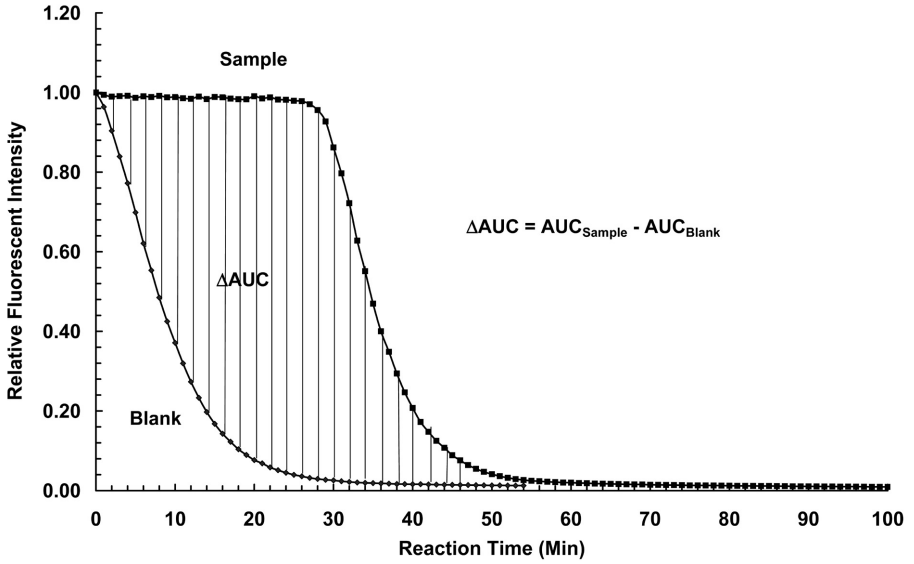

Figure 1. Illustration of ΔAUC calculation.

2.3 Differential scanning calorimetry (DSC)

DSC was used to determine whether the BODIPY probe and membrane components formed a stable system. The lipid vesicles were prepared by the same procedure as used in the LPIC assay. DSC was performed by using a Rheometrics Scientific DSC-SP (Piscataway, NJ, USA). The calibration with respect to temperature and caloric load was made by the use of an indium standard. The unilamellar vesicles (20 μL) were added to an aluminium sample pan, and the pan was sealed. An empty aluminium pan was used as a reference material. The scan rate was 2°C/min and the temperature range was −40 to 10°C. A cooling/heating cycle was repeated three times. The onset temperatures were obtained for both pure DOPC and DOPC-C_{11}-BODIPY vesicles.

2.4 Measurement of the partition coefficient (log *P*)

The log *P* value was calculated from HPLC retention times by comparison with 12 reference compounds whose log *P* values were known from octanol-water partitioning (hydroxyquinone, 0.55 [9]; phenol, 1.49; 4-nitrophenol, 1.91; 2-methylphenol, 1.95; 4-chlorophenol, 2.39; bromobenzene, 2.99; napthalene, 3.37; biphenyl, 4.04; anthracene, 4.45 [10]; benzoic acid, 1.83 [11]; tyrosine, 2.32 [12]; toluene, 2.76 [7]). The retention times (t_R) of antioxidants were determined by a Hewlett Packard 1100 series HPLC system equipped with vacuum degasser, quaternary pump, autosampler, and photodiode-array detector. An ALLTECH Spherisorb ODS-2 (5-μm particle size, 250 mm × 4.6 mm) column was used. The mobile phase was methanol/water (70/30 v/v), and the flow rate was 0.4 mL/min. Each compound was injected three times and the average log k' values (where k' is the capacity factor) were calculated using the equation:

$$\log k' = \log [(t_R - t_U)/t_U] \quad (2)$$

where t_R and t_U represent the retention times of the solute and an unretained compound (sodium nitrate), respectively. The range of log *P* values of reference compounds was from 0.550 to 4.450 at intervals less than 1.0. The dependence of log *P* on log k' was calculated by regression analysis on the 12 reference compounds as in Eq. (3).

$$\log P = 1.92 + 2.27 \log k' \ (n = 12, r^2 = 0.976) \quad (3)$$

This equation was used to calculate the log *P* values of the antioxidants tested.

3 Results and discussion

3.1 Effect of C_{11}-BODIPY on the phase transition of DOPC unilamellar vesicles

Changes in the phase behaviour of DOPC vesicles before and after the incorporation of C_{11}-BODIPY probes were investigated by measuring the peak onset temperatures for the two samples by DSC. A single endothermic transition peak was observed, which corresponded to the gel to liquid-crystalline phase transition arising from the melting of the lipid acyl chain [13]. The phase transition temperature for DOPC unilamellar vesicles was −14.4°C which was close to the reported phase transition temperature of fully hydrated DOPC bilayers of −16.5°C [13]. This observation indicated that the DOPC unilamellar vesicles, prepared by the extrusion technique, were nearly fully hydrated. The phase transition temperature of DOPC unilamellar vesicles

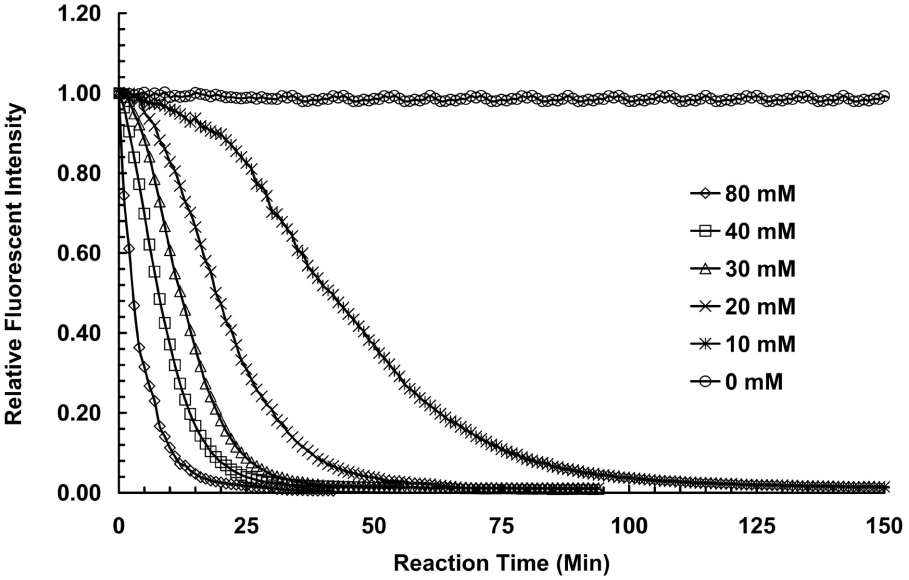

Figure 2. Time course of changes in C_{11}-BODIPY fluorescence in Tris-HCl buffer on incubation at 37°C alone or in the presence of varying amounts of AAPH.

was raised from −14.43 to −11.79°C by the incorporation of C_{11}-BODIPY indicating that C_{11}-BODIPY and DOPC were fully incorporated and their resulting unilamellar vesicles were more stable than that of pure DOPC unilamellar vesicles. Therefore, C_{11}-BODIPY is a suitable fluorescent probe for detection of lipid peroxidation in unilamellar vesicles.

3.2 Effects of free radical concentration

The unilamellar vesicles were both thermally and photochemically stable after incubation at 37°C for 2 h in the light path of the fluorimeter and in the absence of both antioxidants and peroxyl radicals from AAPH (Fig. 2). It has been reported that the red fluorescence of C_{11}-BODIPY shifts to green upon oxidation [14]. Oxidation of C_{11}-BODIPY resulted in a shift in the maximum wavelengths for both excitation and emission. The excitation and emission wavelengths selected were 540 and 600 nm, respectively. In the presence of peroxyl radicals derived from thermolysis of AAPH, the probe C_{11}-BODIPY lost its fluorescence, and the fluorescent decay rate increased as the concentration of added AAPH increased. Under the experimental conditions, peroxyl radicals generated by thermolysis of AAPH oxidised C_{11}-BODIPY to completion in less than 40 min at 40 mM AAPH (Fig. 2). The rate of oxidation of C_{11}-BODIPY was directly dependent on the AAPH concentration.

AAPH can be used to provide a constant rate of free radical production [15]. However, when the AAPH concentration was 10 mM, a lag period of approximately 20 min was observed before the fluorescence of the BODIPY probe began to fall. It is generally assumed that, because AAPH is water soluble, AAPH-derived peroxyl radicals will form in the aqueous phase of a reaction mixture. As a result, liposomes and incorporated fluorescent probe (BODIPY) would be expected to be attacked by AAPH peroxyl radicals after diffusion from the aqueous phase. However, in SDS micelles AAPH partitions approximately 91% into the micellar phase and has a distribution that is similar to the 95% partitioning observed for linoleic acid [16]. These facts suggest that the majority of AAPH peroxyl radicals in our experiments would form either within or at the surface of the lipid bilayers, not in the aqueous phase. Therefore, hydrophilic antioxidants could react either with the small fraction of AAPH radicals produced in the water phase, or at the surface of the lipid bilayers, with either primary radicals or resulting lipid peroxyl radicals. In contrast, lipophilic antioxidants that bind to the surface of, or are incorporated into the membrane, could react either with the larger fraction of radicals formed in the lipid phase from AAPH or at the surface of the lipid bilayers. Thus, lipophilic antioxidants occurring at high concentration in the membrane react with the highly localised concentrations of free radical and thus delay the oxidation of the BODIPY probe. On the other hand, hydrophilic antioxidants, which are not at high concentration at the surface of the membrane, are not able to rapidly consume the high concentrations of free radical at the surface and thus are not able to efficiently protect the probe from oxidation.

3.3 Protective effects of antioxidants on unilamellar vesicles

3.3.1 Effect of Trolox on the peroxidation of unilamellar vesicles

The structure of Trolox (Fig. 3) is essentially a hindered phenol in a ring system. Compounds with this structure,

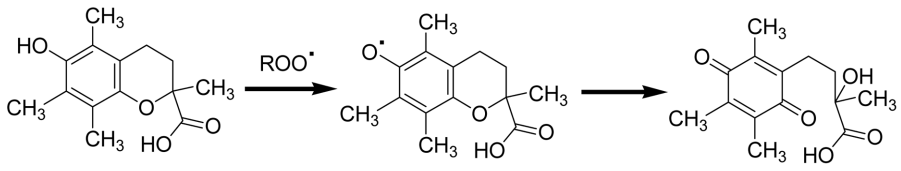

Figure 3. Chemical structure of Trolox and its radical and radical product.

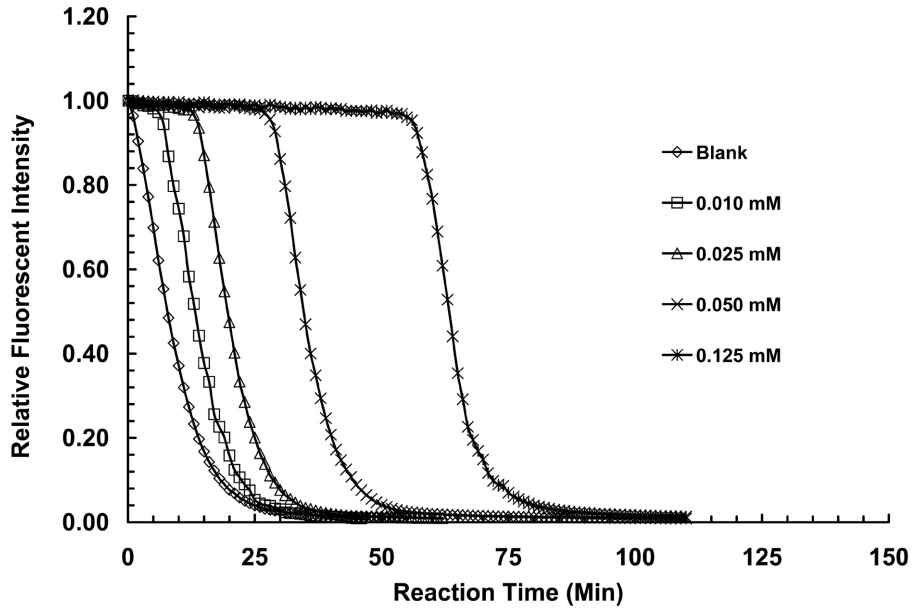

Figure 4. Time course of changes in C_{11}-BODIPY fluorescence in Tris-HCl buffer on incubation at 37°C in the presence of varying amounts of incorporated Trolox and 40 mM AAPH as the free radical generator.

such as α-tocopherol and Trolox, can react rapidly with peroxyl radicals, and therefore give high antioxidant activity [17]. This is because this structure possesses all the requirements for the stabilisation of the resulting phenoxyl radical (Fig. 3) [18].

The chromanol structure of Trolox provides the antioxidant activity [18], and the carboxylic group provides Trolox with a moderate water solubility. This makes Trolox the compound of choice for calibrating the activity of both water-soluble and lipid-soluble antioxidants. Upon incorporation of Trolox into unilamellar vesicles, the fluorescence decay of C_{11}-BODIPY showed a plateau region followed by a decrease in the fluorescence signal in a manner similar to that of the blank C_{11}-BODIPY and AAPH system (Fig. 4). The length of the initial plateau lag phase increased as the concentration of Trolox increased and a linear correlation was found between ΔAUC and Trolox concentration. The regression equation was used to calculate the $LPIC_{Inco}$ value (Eq. (4)).

$$LPIC_{Inco} = \frac{(\Delta AUC - 0.4562)}{0.4622} \times \frac{1000}{\text{molarity of sample}} \quad (r^2 = 0.996) \tag{4}$$

When Trolox was mixed with unilamellar vesicles rather than incorporated at formation, the fluorescence decays of C_{11}-BODIPY showed very similar patterns (Fig. 5) compared with Trolox incorporated into unilamellar vesicles (Fig. 4). There was, again, a linear correlation between the amounts of Trolox added and ΔAUC. The regression equation (Eq. (5)) was used to calculate the $LPIC_{Mixed}$ value.

$$LPIC_{Inco} = \frac{(\Delta AUC - 0.0201)}{0.4617} \times \frac{1000}{\text{molarity of sample}} \quad (r^2 = 0.998) \tag{5}$$

The slopes of the equations used to calculate $LPIC_{Inco}$ and $LPIC_{Mixed}$ values were almost the same (0.4622 and 0.4617, respectively). These results demonstrated that the antioxi-

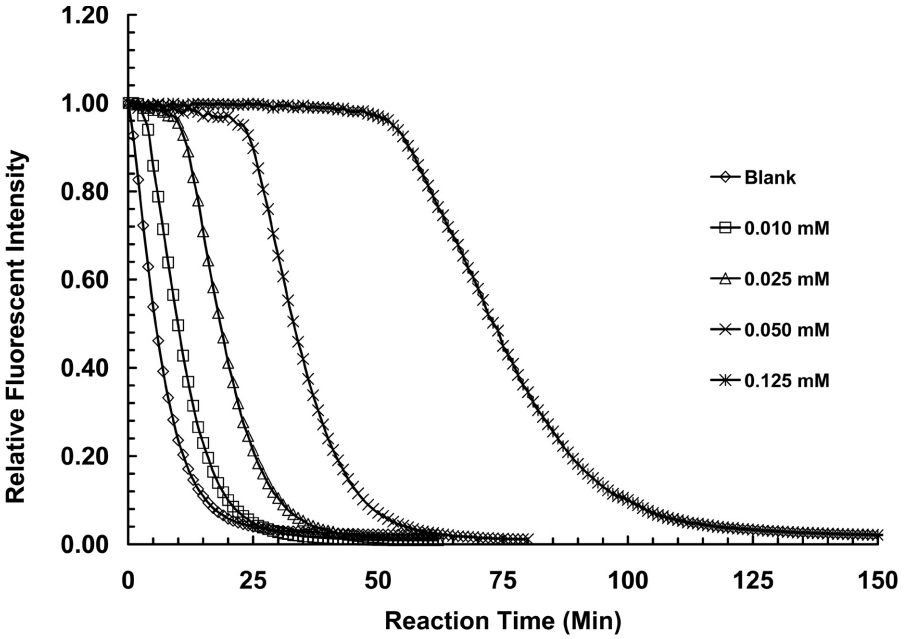

Figure 5. Time course of changes in C_{11}-BODIPY fluorescence in Tris-HCl buffer on incubation at 37°C in the presence of varying amounts of mixed Trolox and 40 mM AAPH as the free radical generator.

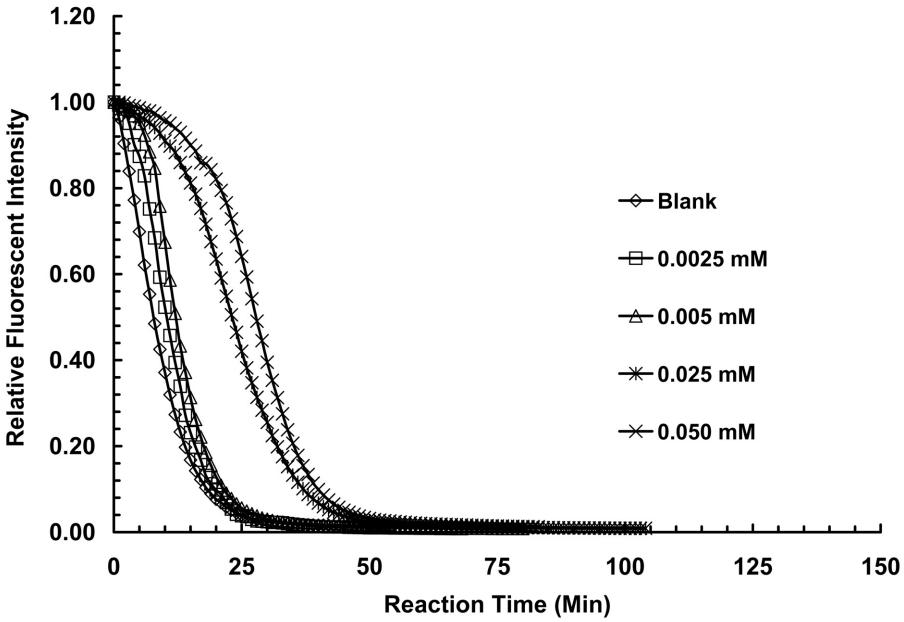

Figure 6. Time course of changes in C_{11}-BODIPY fluorescence in Tris-HCl buffer on incubation at 37°C in the presence of varying amounts of incorporated α-tocopherol and 40 mM AAPH as the free radical generator.

dant behaviour of Trolox was similar in both the $LPIC_{Inco}$ and the $LPIC_{Mixed}$ assays and indicated that the phase partitioning and membrane association of Trolox is similar no matter which way the liposome was formed. This is not surprising considering that the bulk of the Trolox resides in the aqueous phase even when it was coevaporated from an organic solvent with a lipid [19].

3.3.2 Effect of α-tocopherol on the peroxidation of unilamellar vesicles

The correlation between ΔAUC and antioxidant concentration was also evaluated using α-tocopherol (Fig. 6). When α-tocopherol was incorporated into unilamellar vesicles, a linear correlation was found between ΔAUC and α-tocopherol concentration up to 25 μM ($r = 0.999$) but reached a plateau at higher concentrations. The protective effect of α-tocopherol was 1.7 times stronger than that of Trolox. α-Tocopherol is an amphiphilic molecule consisting of two functional domains, a chromanol nucleus with a hydroxyl group and a hydrophobic phytyl side chain (Fig. 7).

It is known that the phytyl side chain is required for the incorporation and retention of α-tocopherol in a lipid membrane [20] and the interaction between polyunsaturated phospholipid acyl chains and the phytyl side chain of α-

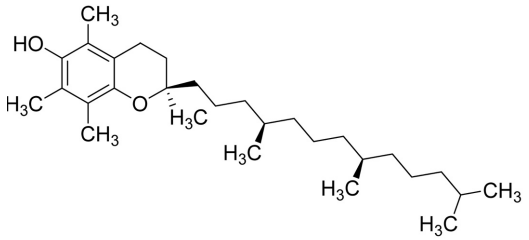

Figure 7. Chemical structure of α-tocopherol.

tocopherol stabilises membranes [21]. α-Tocopherol (log *P* of 9.9) is much more lipophilic than Trolox (log *P* of 3.2). α-Tocopherol can therefore be more easily incorporated into a lipid bilayer, and hence, can be more evenly distributed in the entire lipid bilayer. When α-tocopherol is incorporated into lipid bilayers, the hydrophobic phytyl side chain of α-tocopherol associates with the hydrocarbon chain of the phospholipids and positions the reducing hydroxyl group in the water phase [22]. This hydroxyl proton is in dynamic equilibrium between the phosphate oxygens and the interfacial water. Peroxyl radicals approaching the lipid interface come in contact with a reservoir of reducing protons that reduce the radicals before they can diffuse into the hydrophobic core of the membrane and cause any damage to the lipid molecules. It has been reported that α-tocopherol is located within membranes in which its chromanol ring is stabilised by the hydrogen bonding with ester carbonyl group of phospholipids [23]. Therefore α-tocopherol is capable of reacting with both AAPH-generated radicals and lipid peroxyl radicals at the surface and inside lipid bilayers.

3.3.3 Effect of β-carotene on the peroxidation of unilamellar vesicles

A strong linear relationship was also found between the ΔAUC and β-carotene concentrations with a correlation coefficient of 0.997 (Fig. 8). β-Carotene was a much less effective antioxidant than either α-tocopherol or Trolox. Upon incorporation of β-carotene in the unilamellar vesicles, the chromophores of β-carotene are probably localised in the hydrophobic region of the lipid bilayers [24]. β-Carotene can mainly scavenge peroxyl radicals within the lipid bilayers, *i. e.* quench radicals in the hydrophobic part of the membrane [25]. It is unable to interact with the interfacial water and phospholipid polar groups effectively. The miscibility of β-carotene with the phospholipid bilayer is very small and β-carotene has the tendency to form bulk-phase aggregates [26]. Because of these factors, it appears that β-carotene is not fully available to react with peroxyl radicals, and therefore is reduced in its antioxidant activity.

3.3.4 Effect of phytochemicals on the peroxidation of unilamellar vesicles

The protective effects of a range of structurally diverse phenolic acids and flavonoids were tested by either direct addition or incorporation into unilamellar vesicles and the results evaluated in terms of lipophilicity and their chemical structures.

3.3.5 Gallic acid and their derivatives

Gallic acid and three of its esters were separately incorporated into unilamellar vesicles. The log *P* and $LPIC_{Inco}$ values (Table 1) increased as the acyl chain length of the gallic acid esters increased. However, log *P* increased significantly, but $LPIC_{Inco}$ values increased only slightly between octyl gallate and lauryl gallate. The longer side-chain facilitated better incorporation and retention in membranes. However, long side-chains decrease the mobility of antioxidants within and between the membranes [27]. It has been proposed that the autoxidation of lipid membranes is more sensitive to the fluidity in the hydrophobic domain of

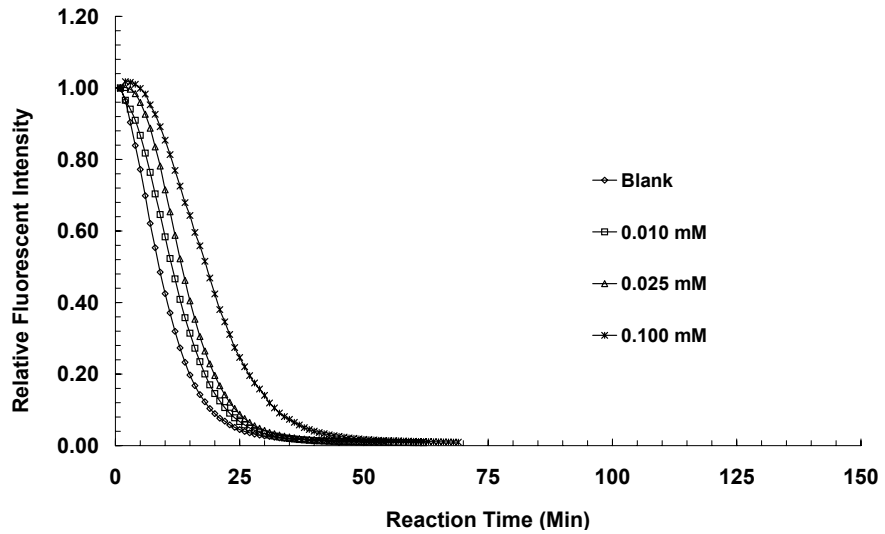

Figure 8. Time course of changes in C_{11}-BODIPY fluorescence in Tris-HCl buffer on incubation at 37°C in the presence of varying amounts of incorporated β-carotene and 40 mM AAPH as the free radical generator.

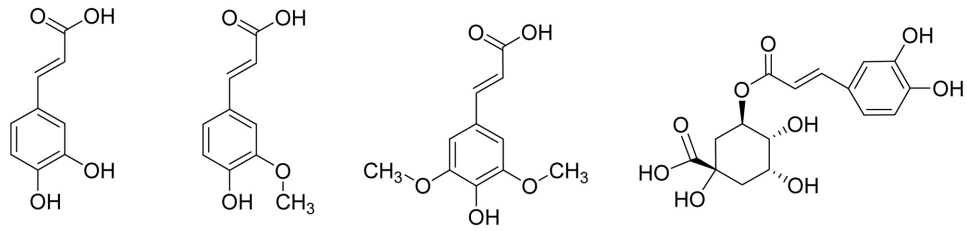

Figure 9. Chemical structures of dihydroxycinnamic acids.

membranes and compounds with longer lipophilic side-chains decrease the membrane fluidity in the hydrophobic domain, and thus decrease the rate of autoxidation [28].

3.3.6 Hydroxycinnamic acids and their derivatives

Four dihydroxycinnamic acids were tested (Fig. 9).

The $LPIC_{Inco}$ value of caffeic acid was 1.5 times higher than that of chlorogenic acid (Table 1). Rancimat tests have also indicated that chlorogenic acid is a less effective antioxidant than caffeic acid [29]. Caffeic acid is more lipophilic than chlorogenic acid. Substitution of the 3-hydroxyl group of caffeic acid by a methoxy group (ferulic acid) considerably reduced the antioxidant effectiveness but had little influence on the log *P* value. The $LPIC_{Inco}$ activity of sinapic acid, with an additional 5-methoxy group, was almost the same as that of ferulic acid although the 5-methoxy group had little effect on the log *P* value. Sinapic acid, with two methoxy substitutions and only a similar log *P*, had a much higher peroxyl radical scavenging ability than that of gallic acid, with three free hydroxyl groups. This indicated that the presence of the –CH=CH–COOH groups in sinapic acid ensured greater hydrogen-donating ability and subsequent radical stabilisation than the carboxylate group in gallic acid. In contrast, the $LPIC_{Mixed}$ value of cholorogenic acid was 1.5 times higher than that of caffeic acid. All four dihydroxycinnamic acids had much higher $LPIC_{Mixed}$ values than their $LPIC_{Inco}$ values. The anomalously high $LPIC_{Mixed}$ value of chlorogenic acid may be due to its amphiphilic nature, which could allow it to 'anchor' at the lipid bilayer/water interface. Both ferulic and sinapic acids were less effective than caffeic acid because of their single hydroxyl groups. However, ferulic acid was more active than sinapic acid. This was the opposite order compared with their $LPIC_{Inco}$ values.

3.3.7 Flavonoids

Dietary flavonoids are diverse and vary according to hydroxylation pattern, conjugation between aromatic rings, glycosidic moieties, and methoxy groups. In this study, the protective activities of a range of structurally diverse flavonoids (Fig. 10) were measured (Table 1).

Table 1. Trolox equivalent antioxidant activities and log *P* values of phenolic acids, flavonoids and their derivatives

Compound	$LPIC_{Inco}$	$LPIC_{Mixed}$	log *P*
Caffeic acid	3.92 ± 0.04	5.59 ± 0.09	0.96
Ferulic acid	2.23 ± 0.01	4.28 ± 0.01	1.09
Sinapic acid	2.31 ± 0.08	3.66 ± 0.10	1.08
Chlorogenic acid	2.57 ± 0.02	8.26 ± 0.10	0.49
Gallic acid	0.58 ± 0.09		0.43
n-Propyl gallate	1.41 ± 0.09		1.99
Octyl gallate	1.90 ± 0.03		4.63
Lauryl gallate	1.97 ± 0.06		6.75
Quercetin	4.34 ± 0.07	8.64 ± 0.05	2.29
Isoquercetin	3.82 ± 0.05	5.02 ± 0.07	1.56
Rutin	3.62 ± 0.03	4.68 ± 0.04	1.53
Kaempferol	1.58 ± 0.08	3.18 ± 0.13	2.77
Morin	2.16 ± 0.09	3.55 ± 0.14	1.22
Myricetin	1.78 ± 0.08	3.40 ± 0.04	1.93
Apigenin	0.36 ± 0.02	2.38 ± 0.04	2.85
Catechin	3.11 ± 0.02	6.48 ± 0.07	1.04

Data are expressed as mean ± SD, all samples were analysed in triplicates. Log *P* is the partition coefficient measured by HPLC.

The flavonoids can be classified into several families according to their structures. The spatial arrangement of substitutions is probably a greater determinant of antioxidant activity than the nuclear backbone alone. Among the flavonols studied, which possessed the 3-hydroxypyran-4-one C ring, quercetin gave the highest $LPIC_{Inco}$ and $LPIC_{Mixed}$ values. The order of LPIC activities was the same for these flavonols regardless of whether the compound was incorporated into ($LPIC_{Inco}$) or mixed later with ($LPIC_{Mixed}$) the unilamellar vesicles. Kaempferol, quercetin, and myricetin have the same chemical structure on the A and C-rings but the log *P* value decreased as the number of hydroxyl groups increased on the B-ring. Both morin and quercetin have two hydroxyl groups on the B-ring, but morin lacks the conjugated hydroxyl substitution, which resulted in the weaker LPIC activity of morin. Kaempferol, with only one hydroxyl group on the B-ring had even weaker activity than that of morin. However, myricetin which possesses three

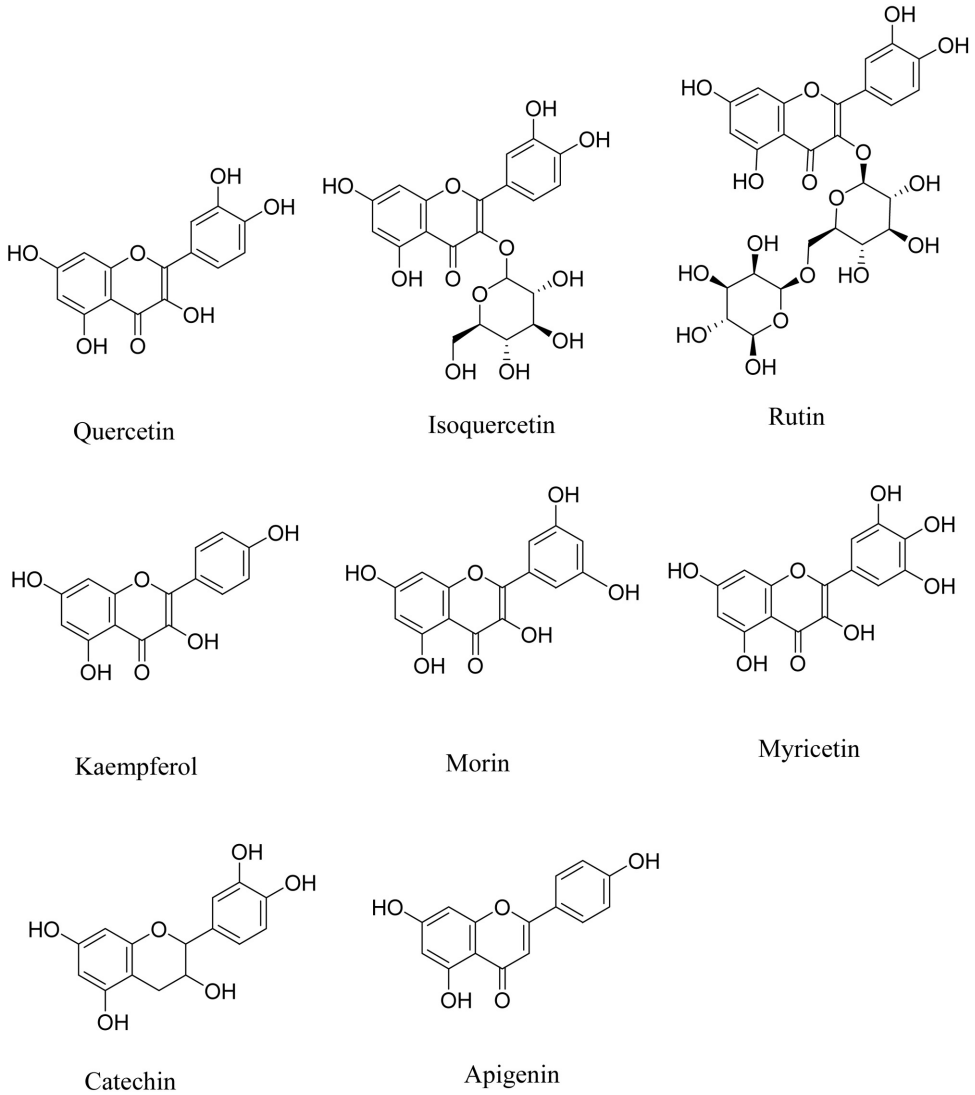

Figure 10. Chemical structures of flavonoids.

hydroxyl groups on its B-ring, was only half as less effective as quercetin in the LPIC assay. It has been reported that myricetin is less effective than quercetin in inhibiting oxidation of LDL [30] and the mechanism of lipid peroxidation in both LDL and microsomal membranes is similar [31]. Our results suggest that the mechanism of lipid peroxidation in unilamellar vesicles has some degree of similarity with that of LDL.

Catechin has the same number and location of hydroxyl groups as quercetin, but catechin lacks the 2,3-double bond and the 4-keto group, so there is no electron delocalisation between the A-ring and B-ring because of the saturation of the heterocyclic C-ring. This caused a reduction of both LPIC activity and log *P* compared with quercetin. Interestingly, catechin was still more effective in both $LPIC_{Inco}$ and $LPIC_{Mixed}$ systems than myricetin. This result suggests that the additional hydroxyl groups in the B-ring of myricetin lower the lipophilicity and consequently the affinity for lipid bilayers. The hydroxyl group at position 3 of the C-ring, which is shared by all flavonols, may account for the major inhibiting activity of lipid peroxidation. This observation is best illustrated by the comparison of apigenin and kaempferol, which had similar log *P* values.

Replacement of the hydroxyl group at the C-3 position of quercetin by glucose in isoquercetin led to a slight decrease in both $LPIC_{Inco}$ and $LPIC_{Mixed}$ values. The disaccharide rutinose in rutin caused a further decrease in LPIC activity. The lower LPIC activity of these glycosylated derivatives may have been due to the decrease of lipophilicity, in addition to the loss of the free hydroxyl group at the 3-position of C-ring. However, isoquercetin and rutin retain significant inhibiting activities presumably because of the catechol

structure of their B-ring and their affinity for phospholipid membranes. Quercetin possesses high affinity for phospholipid membranes [32] and could penetrate the lipid bilayers of a liposomal suspension [33]. Rutin can also effectively partition into the hydrophobic core of phospholipid membranes [34]. Morin, in which the dihydroxyl groups in the B-ring are arranged *meta* to each other, was much less effective than quercetin, which possesses two adjacent hydroxyl groups on the B-ring. Morin was also less effective than myricetin. This indicates the importance of the adjacency of the hydroxyl groups in the B-ring to the antioxidant activity of flavonoids [35].

It is known that the fluorescent group of BODIPY is located at the surface of lipid bilayers [36]. Consequently, the oxidation reaction between BODIPY and AAPH free radicals probably takes place at the surface of lipid bilayers. The relationship between the structure and the $LPIC_{Inco}$ or $LPIC_{Mixed}$ activity suggested that, in addition to lipophilicity, the interaction of the polyphenol moieties with the surface of the lipid bilayer affected their antioxidant activities.

3.3.8 Comparison of $LPIC_{Mixed}$ and $LPIC_{Inco}$ values

Unlike Trolox the $LPIC_{Mixed}$ values of other antioxidants are on average larger than those of the $LPIC_{Inco}$ values (Table 1). The mobility and location of antioxidants in the lipid bilayer could affect their ability to protect the fluorescent probe, which is located at the interfacial surface [36]. Amphiphilic compounds such as chlorogenic acid with a very high $LPIC_{Mixed}$ activity could, interact by forming high concentrations at the surface of the lipid bilayers. Incorporated lipophilic antioxidants would be located predominantly inside the lipid bilayers, away from the interface, whereas, when mixed with preformed vesicles, they have more interaction at the interface. Flavonoid aglycones are rather lipophilic antioxidants among the plant phytochemicals but they are more hydrophilic than α-tocopherol. Ratty *et al.* [37] have demonstrated that flavonoids interact in the polar surface region of phospholipid bilayers. It appears likely that quercetin is mostly localised near the surface of membranes where aqueous peroxyl radicals are trapped and are accessible to chain-initiating peroxyl radicals more readily than α-tocopherol. The planar structure of quercetin may explain its high affinity for the phospholipids bilayers [38]. Catechin was also more hydrophilic than α-tocopherol and assumed to be localised near the membrane surface. Chlorogenic acid is relatively hydrophilic but it was very active in the $LPIC_{Mixed}$ assay. Higher lipophilicity could cause some flavonoids to penetrate into lipid bilayers to some degree and then reduce their concentration at the surface. This capability would, in turn, decrease their $LPIC_{Mixed}$ and $LPIC_{Inco}$ activities. Laranjinha *et al.* [39] reported that chlorogenic and caffeic acids undergo chemical structural modifications upon interaction with lipid peroxyl radicals to form alkyl quinone radicals. This structural change increases electron stabilisation and hence antioxidant activity. When chlorogenic acid is incorporated into lipid bilayers, it may be trapped inside the aqueous region of lipid bilayers, and they are not available to react with free radicals. Our results demonstrate that antioxidant efficacy in one set of experimental conditions may not necessarily translate into a similar degree of protection in another set of conditions.

3.3.9 Correlation between log *P* and LPIC activity

While log *P* values can explain differences in LPIC values for compounds that are structurally related, the effect of lipophilicity on the behaviour of antioxidants might not be linear. This idea is supported by the findings of Beyeler *et al.* [40], who reported that the effects of cianidanols on rat hepatic monooxygenase increased with lipophilicity, reached a plateau, decreased and levelled off for the most lipophilic compounds. These findings indicated that the antioxidant activity of phenolic compounds is associated with their affinity and distribution in lipid membranes. This is presumably because (a) at high values of log *P*, the antioxidant is dispersed in a lipid phase and not located at the lipid-water interface; (b) at low value of log *P*, the antioxidant is located in an aqueous phase and has insufficient solubility in the lipid phase. This characteristic could be important in terms of paracellular transport of phenolic compounds and the ability to enter the cell to participate in *in vivo* protection from oxidative damage. It has long been recognised that for a chemical to be biologically active, it must first be transported from its site of administration to its site of action and then it must bind to or react with its receptor or target, *i.e.* biological activity is a function of both partition and reactivity [41].

4 Concluding remarks

In conclusion, a new *in vitro* antioxidant assay based on LPIC was validated. The assay employed the lipid-like fluorescent probe C_{11}-BODIPY, which is incorporated into 100 nm DOPC liposomes. Various antioxidant compounds were either introduced as the liposomes were fabricated ($LPIC_{Inco}$) or mixed with preformed liposomes ($LPIC_{Mixed}$). The azo initiator AAPH (a peroxyl radical generator) was used to trigger lipid peroxidation and effects on integrated C_{11}-BODIPY red fluorescence intensity (600 nm) were tracked. Inhibition of fluorescence decay (reflecting oxidation of probe) indicated antioxidant potency. The apparent advantage of this approach is that it tested antioxidants in lipid membrane settings instead of homogeneous solution used for many existing assays. In addition, the LPIC can be used to measure the activities of both lipophilic and hydrophilic antioxidants by using membrane lipids as the carrier.

This work was funded by the Foundation for Research Science and Technology Wellness Food Programme Contract C06X0405. We thank Dr. Margot Skinner and Dr. David Stevenson for critically reviewing the manuscript.

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