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A selective Assay To Measure Antioxidant Capacity in Both The Aqueous and Lipid Compartments of Plasma

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The measurement of the total antioxidant capacity (TAC) of human plasma has been widely applied in nutritional science, for example to evaluate the antioxidant contribution of dietary components and to study, although indirectly, the bioavailability of dietary antioxidants. Several methods have been proposed for the measurement of TAC, most of them based on the ability of plasma to withstand the oxidative damage induced by aqueous radicals. Although plasma contains both hydrophilic and lipophilic antioxidants that interact through extensive cross-talk, in most of the methods employed for the TAC measurement, the hydrophilic antioxidants such as ascorbic acid, uric acid, and protein thiols mainly contribute to the total antioxidant plasma capacity (almost 70%) while lipophilic antioxidants embedded in the lipoproteins (carotenoids, α-tocopherol, ubiquinol-10) participate only in a negligible amount (less than 5%).
The present paper reviews the analytical methods used to assess the TAC and in particular focuses on new approaches that are capable of distinguishing the antioxidant capacity of both the aqueous and lipid compartments of plasma. The general principle of the method as well as some *in vitro* and *ex vivo* applications will be discussed within the text.

Key word : total antioxidant capacity, aqueous and lipid plasma compartments

PLASMA ANTIOXIDANTS

Human plasma is a heterogeneous system, made up of the aqueous and the lipid (lipoproteins) compartments. Based on the hydrophilic / lipophilic character, plasma antioxidants are located in three main domains: (I) water-soluble antioxidants such as ascorbic acid, uric acid and bilirubin in the aqueous phase, (II) water-insoluble antioxidants such as the carotenoids located in the core of lipoproteins and (III) α-tocopherol and ubiquinol-10 at the interface between the aqueous and lipid compartment at the surface of lipoproteins.

These antioxidant nutrients located in different sites extensively interact, as summarized in Fig 1. At the core of lipoproteins, carotenoids scavenge lipid radicals by addition to the double bond, yielding radical derivatives[1)] which are scavenged by α-tocopherol through a H-transfer mechanism. α-Tocopherol can then be recycled from the tocopheroxyl radical by ascorbic acid[2)].

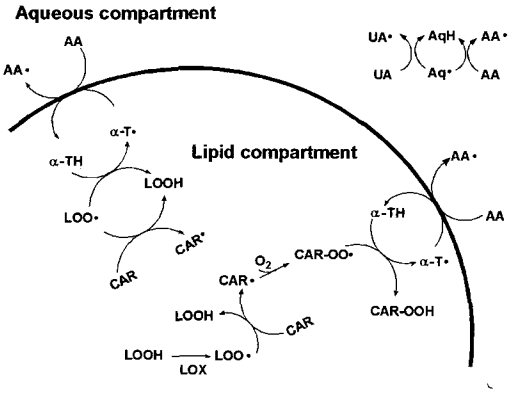

Fig 1. Proposed mechanism of lipid and aqueous-soluble antioxidants in human plasma.
AA· : ascorbyl radical, AA; ascorbate, Aq· : aqueous radical, AqH : aqueous hydrogen donor, α-T· : α-tocopheroxyl radical, α-TH : α-tocopherol, CAR : carotenoids, CAR· : carotenoid alkyl radical, CAR-OO· : carotenoid peroxyl radical, CAR-OOH : carotenoid hydroperoxide, LOO· : lipid peroxyl radical, LOOH : lipid hydroperoxide, UA· : uric acid radical, UA· : uric acid radical.

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HOW TO MEASURE THE PLASMA ANTIOXIDANT STATUS / EFFICIENCY

For the measurement of the plasma antioxidant status/ efficiency, two main approaches can be used:(I) measure

each aqueous and lipid soluble antioxidant in plasma;(II) measure the total antioxidant capacity of plasma. The measurement of each individual antioxidant requires several analytical instruments and good analytical skills. Moreover, measurement of plasma antioxidants yields limited data since it does not give any information about the possible cooperative / synergistic mechanisms among hydrophilic/lipophilic antioxidants. In addition, polyphenol micronutrients such as procyanidins, anthocyanidins and catechins which are widely present in fruits and vegetables are not easily measured in plasma because of a lack of suitable analytical methods. However, these compounds could significantly contribute to the total antioxidant capacity in plasma[3]. To overcome these limits the measurement of the total antioxidant capacity (TAC) of plasma has been considered.

THE MEASUREMENT OF THE TOTAL ANTIOXIDANT PLASMA CAPACITY

For the measurement of the total antioxidant capacity in plasma, two main approaches have been reported as extensively reviewed by Cao[4] and Schlesier[5]. The first group of approaches is represented by those assays involving oxidants that act as pro-oxidants, the second group, assays involving oxidants that do not act as pro-oxidants.

The methods belonging to the first group measure the ability of plasma to counteract the oxidation of an exogenous oxidizable substrate induced by an added pro-oxidant. A typical output of a TAC assay is shown in Fig 2. Plasma, which is challenged with a radical inducer such as 2,2'-azobis(2,4-amidinopropane) dihydrochloride (AAPH) generating radicals at a constant rate, is monitored by an oxidizable substrate 2',7'-dichlorodihydrofluorescein (DCFH). Curves A and B represent the oxidation of DCFH in the absence and presence of plasma respectively. The lag phase observed in curve B is due to endogenous plasma antioxidants, which delay the oxidation of DCFH by scavenging the peroxyl radicals induced by AAPH. Once the endogenous antioxidants are consumed, the substrate, DCFH, starts to be oxidized. Therefore, the length of lag phase depends on the total antioxidant capacity of plasma: the longer the lag-phase, the greater the antioxidant efficiency of plasma. Trolox, a water-soluble vitamin-E analogue, is generally used as the internal standard, whose addition to the incubation mixture induces a second lag phase. The TAC value is then calculated by comparing the lag phase of plasma to that of Trolox using the following proportion: $C_{trolox} : T_{trolox} = X : T_{plasma}$ where C_{trolox} is the concentration of the Trolox added, T_{trolox} is the lag phase induced by Trolox,

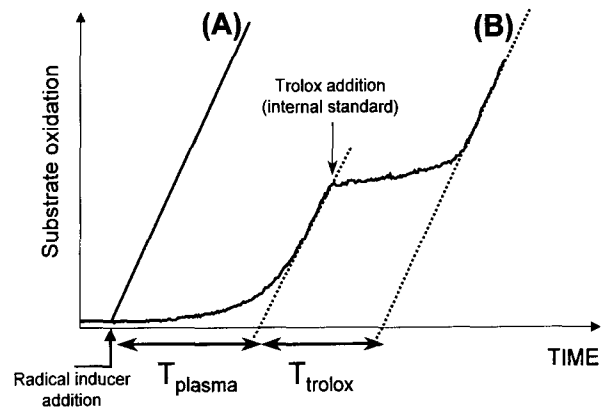

Fig 2. Time-dependent oxidation of DCFH induced by the aqueous radical inducer AAPH (3 mM) in the absence (A) and presence (B) of human plasma (final dilution in PBS 1 : 60). Trolox (2.2 μM) is added as the internal standard.

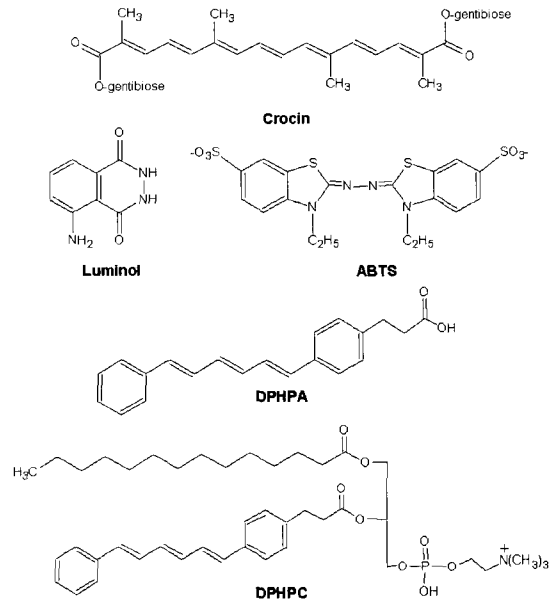

Fig 3. Chemical structures of some oxidizable substrates used for the total antioxidant capacity measurement. DPHPA = 1,6-diphenylhexatriene propionic acid); DPHPC = 1-palmitoyl-2-((2-(4-(6-phenyl-*trans*-1,3,5-hexatrienyl)-phenyl)ethyl)-carbonyl)-sn-glycero-3-phosphocholine.

X the TAC of plasma and T_{plasma} the lag phase induced by plasma. The resulting X value is then multiplied by 2 (the stoichiometric factor of Trolox) and by the plasma dilution factor. Values are expressed as µM (micromoles of peroxyl radical trapped by 1 liter of plasma)[6].

Several methods based on this general principle have been reported in the last decade using various hydrophilic oxidizable substrates, as shown in Fig 3, and using different calculation methods (e.g. lag phase, area under the curve,

competition kinetics). AAPH is a hydrophilic azo-compound that spontaneously decomposes at 37℃ with a known rate constant (R_i=1.36×10^{-6} [AAPH] mol / liter / sec), giving carbon centered radicals, which then react with oxygen yielding the corresponding peroxyl radicals. Crocin, isolated from saffron and characterized by a polyene chain with a high extinction coefficient, has been used as an oxidizable substrate in the assay developed by Tubaro[7] and then automated by Kampa[8]. It is a hydrophilic carotenoid due to the presence of two gentiobiose moieties. The reaction of crocin with peroxyl radical leads to a loss of the double bond conjugation and hence to bleaching that can be readily monitored at 445 nm. R-phycoerythrin (R-PE) is a protein isolated from *Corallina officinalis*, and is used as the oxidizable substrate in the TRAP (Total Radical-Trapping Antioxidant Parameter)[9] and ORAC (Oxygen Radical Absorbance Capacity)[10] assays. R-PE is a fluorescent protein that emits in the visible region (λexc 495 nm, λem 595 nm) and is characterized by fluorescence quenching upon reaction with peroxyl radicals.

The methods belonging to the second group of approaches measures the capacity of plasma to quench a stable and pre-formed radical that does not act as pro-oxidant. In the TEAC assay (Trolox Equivalent Antioxidant Capacity) first reported by Miller[11], the antioxidant plasma capacity expressed as Trolox equivalents is determined by measuring the ability of plasma to quench the radical cation of 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS). The quenching reaction is monitored by measuring the decay of the radical cation at 734 nm. The FRAP (Ferric Reducing Ability of Plasma) assay, which does not involve a radical species (neither pro-oxidant or stable), has recieved a great deal of attention since it is quick and simple to perform[12]. The FRAP assay measures the reducing (antioxidant) ability of plasma by monitoring the ability of plasma to reduce ferric to ferrous ions monitored at 593 nm as a tripyridyltriazine (TPTZ) complex.

Table 1. Total Antioxidant Capacity Using Aqueous Initiators

Assay	Food/beverage	Plasma Antioxidant Capactiy (TAC)
TRAP(R-PE/AAPH)	Green and black tea[19]	↑
	Red wine[22]	↑
	Tomato puree[25]	-
	Tomato juice[26]	-
TRAP(oxygen electrode/AAPH)	Tomato juice[27]	-
Luminol/AAPH	Chocolate[21]	↑
Crocin/AAPH	Red wine[7]	↑
	Coffee[23]	↑
ORAC(R-PE/AAPH)	Strawberries, spinach, red wine[24]	↑
FRAP	Carotenoid-rich vegetables[28]	-
	Green tea[20]	↑

APPLICATIONS AND LIMITS

The measurement of TAC in plasma has recently found several applications: (I) to study the involvement of oxidative stress in pathological conditions such as atherosclerosis[13], ischemia[14], Parkinsons disease[15] and diabetes[16]; (II) to evaluate the antioxidant contribution of dietary components (Tab. 1) ; (III) to indirectly investigate the bioavailability of dietary antioxidants for which no analytical methods for their detection in biological matrices are available (e.g. procyanidins)[17],[18]. The effects of some foods and beverages on TAC are summarized in Tab 1. It is evident that foods such as green tea[19],[20], cocoa[21], red wine[22],[7], coffee[23], and strawberries[24] that contain significant amounts of polyphenol constituents increase the TAC significantly. By contrast, diets enriched with the lipophilic antioxidants such as lycopene or β-carotene were not found to affect TAC as measured by the TRAP, FRAP or ORAC assays[25-28]. These results are in contrast to the well known antioxidant activity of carotenoids as reported both in *in vitro* and in *ex vivo* conditions[29].

An explanation for this apparent contradiction resides in the fact that most of the TAC methods described up to now, measure primarily the antioxidant capacity of the aqueous compartment of plasma. For example in the R-PE / AAPH based assay, the percentage contribution of water-soluble antioxidants (uric acid, protein thiols, ascorbic acid) is almost 70% while that of lipophilic antioxidants is less than 5%: (α-tocopherol 4.8% while carotenoids do not contribute at all)[9]. A similar percentage contribution has been reported for other assays such as the luminol[30] and crocin-based methods[7] and the FRAP assay[12]. The lack of contribution from the lipophilic antioxidants could be ascribed to the relative low amount of plasma lipophilic antioxidants as compared to the hydrophilic antioxidants(Tab 2). How-

Table 2. Hydrophilic and lipophilic non-enzymatic plasma components contributing to the antioxidant plasma capacity (concentration in brackets expressed as μM).

Hydrophilic		Lipophilic	
SH groups	[450 700]	α-tocopherol	[15-40]
Uric acid	[120-450]	Ubiquinol-10	[0.4-1.0]
Ascorbic acid	[30-150]	Carotenoids	[1-3]
Bilirubin	[2-20]		

ever, it is conceivable that the antioxidant activity of lipophilic antioxidants in the plasma lipid compartment can be greatly increased by a synergistic action through "cross-talk" with water-soluble antioxidants. A methodological explanation should also be taken into account: a hydrophilic radical generator, which produces hydrophilic peroxyl radicals, coupled with a hydrophilic oxidizable substrate such as R-PE, crocin or DCFH are used in most of the assays used for the TAC measurement. It is quite clear that under these experimental conditions, the hydrophilic antioxidants mainly contribute to the total antioxidant plasma capacity while the lipophilic antioxidants, deeply embedded in the lipid core, cannot participate in the antioxidant effect.

HOW TO SELECTIVELY MEASURE THE AQUEOUS AND LIPID COMPARTMENT OXIDIZABILITY

A general approach to selectively measure the lipid compartment oxidizability of plasma is to measure LDL oxidizability induced by aqueous (AAPH, transition metal ions) or lipid radical inducer 2,2'-azobis (2,4-dimethylvaleronitrile) (AMVN). By using AMVN as a radical initiator and luminol as an oxidizable substrate, the percentage contribution of the lipophilic antioxidants to the antioxidant activity of isolated LDL was greater than 70% (tocopherol 73%; ubiquinol-10 2.5%)[30]. However this approach is limited since, by using isolated LDL, it does not take into account the potential cross-talk between aqueous- and lipid-antioxidants, a mechanism that could greatly increase the antioxidant activity of the lipid compartment through a recycling mechanism. For example, it has been reported that carotenoids cross-talk with α-tocopherol, which can be recycled from the tocopheroxyl radical by ascorbic acid[2]. In addition several polyphenol micronutrients, such as procyanidins, have been found to spare/recycle α-tocopherol and thus to increase the efficacy of lipid-soluble antioxidants[31].

Considering the methodological limits described above, it is crucial to have a selective method to measure the oxidizability of both the hydrophilic and lipophilic components in biological systems. This could be relevant not only to study diets supplemented with lipophilic antioxidants, but also to investigate lipoprotein oxidation in whole plasma and the activity and mechanism of lipid- or water-soluble antioxidants.

Recently Mayer et al[32] proposed a continuous spectroscopic method based on selective fluorescence markers able to monitor the aqueous and lipid phases in human serum. In particular DPHPA (Fig 3) was used as an appropriate probe for the aqueous phase since it preferentially binds to albumin while DPHPC (Fig 3), which incorporates into lipoproteins such as LDL, VLDL and HDL, monitors the lipid compartment oxidizability. AAPH was selected as the radical inducer for both compartments. By using this method, the authors reported that intake of apples (1 Kg) induces a more pronounced antioxidant protection in the aqueous phase than in the lipid phase of serum. An improved TEAC assay has been reported by Re et al.[33]. By using a pre-formed radical mono-cation of 2,2'-azinobia(3-ethylbenzothiazoline-6-sulphonic acid diammonium salt, ABTS, (generated by oxidation of ABTS with potassium persulfate) and an appropriate solvent system, the assay is applicable to both aqueous and lipophilic systems. Also the ORAC assay has been expanded for reflecting lipophilic antioxidants by using randomly methylated β-cyclodextrin as a solubility enhancer and fluorescein as the oxidizable substrate[34].

Our group recently developed a fluorometric method able to selectively measure the oxidizability of both the aqueous and lipid compartments in human plasma[35]. To

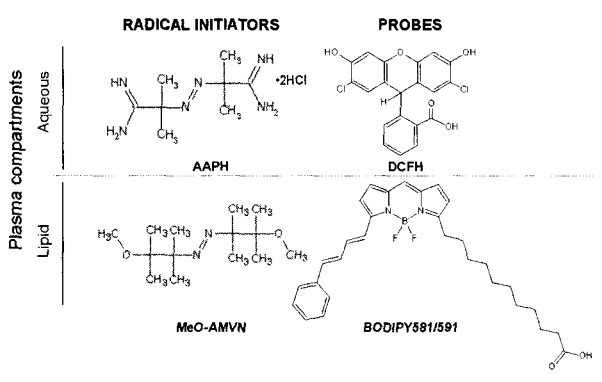

Fig 4. Chemical structures of hydrophilic and lipophilic azo-compounds and probes for measuring aqueous and lipid compartments lipid oxidizability.

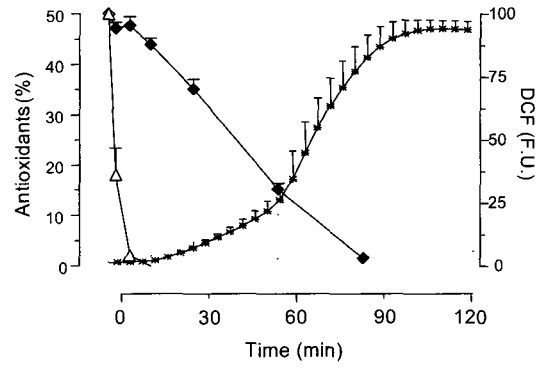

Fig 5. Hydrophilic plasma compartment oxidation induced by AAPH: DCF (*), ascorbic acid (△) and uric acid (◆). Values are means ±SD of three replications. For the experimental conditions see [35]. F.U.= fluorescence units

selectively measure the antioxidant activity in the aqueous compartment of plasma, AAPH was used as a hydrophilic radical inducer and DCFH as a hydrophilic oxidizable substrate, as reported earlier[35),36)](Fig 4). DCFH is not a fluorescent compound but when it reacts with peroxyl radicals, it becomes fluorescent as a consequence of a H-abstraction mechanism that results in the conjugation of the two aromatic rings. In Fig 5, the kinetics of DCFH oxidation in human plasma is plotted with respect to ascorbic and uric acid depletion. As soon as AA is consumed, DCFH starts to be oxidized, and the oxidation rate significantly increases after the depletion of uric acid, indicating the contribution of hydrophilic antioxidants.

For the measurement of the lipid compartment, a lipophilic radical generator coupled with a selective probe capable of detecting the lipid peroxidation was used. 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN) was selected as a lipid-soluble radical inducer[37)]. It decomposes at 37℃ with a known rate, yielding peroxyl radicals able to induce the lipid peroxidation process in the lipophilic compartment. BODIPY581/591[4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid], a fluorescent fatty acid derivative, was used as a selective lipophilic oxidizable substrate[38),39)]. BODIPY581/591 has a high fluorescence quantum yield, is limited to the lipid phase, is stable for several hours in biological fluids at 37°C, absorbs/emits in the visible region, and is a sensitive and selective indicator of the oxidation in the lipid phase of plasma.

The selective distribution of the two radical initiators was initially confirmed by measuring the rate of consumption of hydrophilic (uric acid, ascorbic acid) and lipophilic (β-carotene, α-tocopherol) endogenous antioxidants in plasma. The amounts of free radicals generated from the two radical inducers were kept constant by adjusting the concentrations of the two radical inducers on the basis of their known rate constants.

In the presence of AAPH, we observed that uric acid was consumed earlier than β-carotene indicating the gradient of hydrophilic peroxyl radicals from the aqueous to the lipid compartment of plasma. When MeO-AMVN was used as radical inducer, the order was reversed since uric acid was consumed after β-carotene confirming the diffusion and activation of MeO-AMVN into the core of lipoproteins. To investigate the selective distribution of DCFH and BODIPY581/591, in the aqueous and lipid compartments of plasma, the oxidation rates of the substrates were measured in the presence of AAPH and MeO-AMVN (Fig 6). DCFH oxidation was delayed by 120 min when MeO-AMVN was used with respect to the water-soluble radical inducer, AAPH, indicating the primary localization of DCFH in the aqueous domain (Fig 6, A). By contrast, when BODIPY581/591 was used as the oxidizable substrate, MeO-AMVN immediately induced a significant oxidation of the substrate whose rate increased further after the consumption of -tocopherol and carotenoids (Fig 6, B). By contrast, in the presence of AAPH, BODIPY581/591 did not undergo a significant oxidation for up to 180 min of incubation, suggesting the localization of BODIPY 581/591 in the lipid phase of plasma.

APPLICATIONS

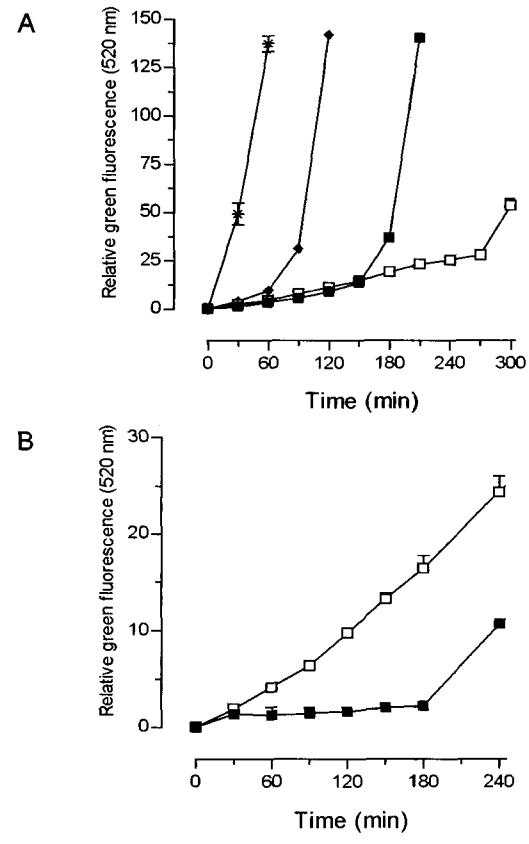

Fig 6. **A** : Oxidation of DCFH to DCF induced by AAPH or MeO-AMVN. The reaction mixture consisted of DCFH (1 μM final concentration), the azo-compound and human plasma (final dilution 1 : 5). Samples were incubated at 37℃ in the dark and at fixed times the DCF content measured by fluorescence (λex=502 nm, λem=520 nm). Legends to symbols: *(AAPH 20 mM; no plasma addition), ◆(AAPH, 20 mM), ■ (AAPH 10 mM), □ (MeO -AMVN, 2mM).
Values are means ±SD of five independent experiments.
B : Time-course of BODIPY green fluorescence in human plasma in the presence of 2 mM MeO-AMVN(□) or 20 mM AAPH(■).

The antioxidant mechanism of (-)-epigallocatechin-(3)-gallate (EGCG), the main polyphenolic component of green tea, was studied in human plasma[40]. Although several methods indicated the efficacy of EGCG in protecting plasma and lipoproteins from oxidative damage, they did not provide any information about the mechanism and the site of the antioxidant action. The aim of this study was to understand, by using the methods described above, in which plasma compartment (hydrophilic or lipophilic) EGCG acts as antioxidant, and the possible interaction between EGCG and other antioxidants (e.g. ascorbic acid, α-tocopherol, uric acid).

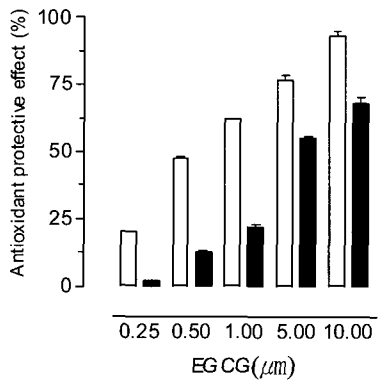

Fig 7. Dose-dependent protective effect of EGCG on aqueous (blank bar) and lipid (filled bar) compartment oxidation after 180 min of incubation.
Values are means ± SEM of five independent experiments.[40]

When human plasma was exposed to AAPH, the aqueous compartment oxidation (monitored by DCFH oxidation) was dose-dependently inhibited by EGCG (IC_{50}= 0.72 μM)(Fig 7), without sparing the hydrophilic antioxidants, ascorbic acid and uric acid (Tab. 3). In contrast, the lipophilic antioxidants (α-tocopherol and carotenoids) were significantly spared by 1-5 μM EGCG. When radical damage was selectively induced in the lipid compartment of plasma using MeO-AMVN as the lipophilic radical inducer, EGCG was found to spare α -tocopherol, but not the carotenoids (Tab. 3). In addition, EGCG inhibited lipid compartment oxidation, monitored by BODIPY581/591 as a selective lipid probe, but with a potency lower than that found in the aqueous compartment (IC_{50}= 4.37 μM)(Fig 7). Therefore, the results indicate that EGCG at concentrations found in human plasma after acute or chronic green tea ingestion is mainly localized in the aqueous compartment, where it is effective in quenching aqueous radical species, thus limiting their diffusion into the lipid compartment and preventing lipid-soluble antioxidant depletion. EGCG is also able to interact with α-tocopherol at the aqueous/lipid interface where recycling of α-tocopherol occurs through a H-transfer mechanism, as confirmed by ESR experiments, affording a protective mechanism to the lipid compartment of plasma.

Table 3. Antioxidant effect of EGCG in human plasma[40]

	Radical initiator	
	AAPH	**MeO-AMVN**
Sparing on hydrophilic antioxidants (AA, UA)	-	N.D.
Sparing on lipophilic antioxidants		
α-Tocopherol	+	+
carotenoids	+	-
Inhibition of aqueous compartment oxidation (IC50)	0.72 μM	N.D.
Inhibition of lipid compartment oxidation (IC50)	N.D.	4.37 μM

N.D ; not determined; (-) ; no significant sparing effect, (+) ; significant sparing effect

CONCLUSIONS

We report a selective fluorescence method to distinguish the oxidizability of the aqueous and lipid compartments of plasma, that is characterized by sensitivity, specificity and ease of evaluation. The method is useful in the evaluation of potential antioxidants and in particular for studying the lipophilic component of the total antioxidant capacity of plasma.

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