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Review

In vitro measurements and interpretation of total antioxidant capacity ☆

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ABSTRACT

Background: One of the strategies most commonly used to assess a free radical-antioxidant balance in chemical and biological systems is the determination of the total antioxidant capacity (TAC). A large amount of research has been published using TAC. However, it remains unclear which is the significance of these investigations for understanding the biological importance of free radical reactions.
Scope of review: This review discusses the relevance and limitations of TAC for the assessment of the antioxidant activities present in food and food derivatives, and in body tissues and fluids.
Major conclusions: TAC determinations are simple, inexpensive, and able to evaluate the capacity of known and unknown antioxidants and their additive, synergistic and/or antagonistic actions, in chemical and biological systems. However, different TAC assays correlate poorly with each other, since each TAC assay is sensitive to a particular combination of compounds, but exclude many others. The TAC values for foods cannot be translated to the in vivo (human) antioxidant defenses, and furthermore, to health effects provided by that food.
General significance: Up to date, conclusions that can be drawn from the extensive amount of research done using TAC of foods or populations should not be considered when used for making decisions affecting population health. This article is part of a Special Issue entitled Current methods to study reactive oxygen species - pros and cons and biophysics of membrane proteins. Guest Editor: Christine Winterbourn.

1. Introduction

One of the strategies most frequently used to assess the free radical-antioxidant balance in biological systems is the determination of the total antioxidant capacity (TAC). Several assays are used to evaluate TAC, some widely accepted and others just tailored for a particular experimental design. When using bibliographical engines as a source it can be found approximately 20,000 citations for "antioxidant capacity," and around 5,000 for "total antioxidant capacity." The main question that arises from these impressive figures is, which are the contributions of these investigations for the advancement of the scientific knowledge in terms of free radical reactions and their biological significance? Two major facts should be considered: i) no univocal associations have been observed between TAC values of a food and TAC values in the body after consumption of that food, and ii) TAC determinations in tissues are of limited or no value in human studies. Thus, the answer to that question is that TAC has not contributed much to the field of redox biology and human health. More importantly, TAC-derived results have sometimes misled to wrong conclusions regarding the proper diet or conditions to improve health and/or prevent disease.

In this paper we will discuss the meaning of TAC determinations, their relationships with individual antioxidants, and the relevance of TAC measurements in foods or food components, and in tissues or body fluids.

2. Total antioxidant capacity

Methodological approaches to measure "antioxidant capacity" were first used to characterize the capacity of plasma to diminish the chemically induced oxidation of an easily detectable substrate [1]. Wayner et al. called their method "total peroxyl radical-trapping antioxidant capacity," and was meant to provide a holistic antioxidant evaluation, including additive, synergistic, and/or antagonistic relationships among the components of a biological system (plasma in their case). Years later, and driven by the increased use of antioxidant capacity strategies and assays, the term "TAC" was introduced as a generic denomination [2]. TAC determinations currently include several different assays (revised in Ref. [3]) used to test not only plasma or body fluids, but foods, beverages, and a number of other biological and chemical systems.

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Chemically, the assessment of TAC is mostly based on challenging an (oxidizable) substrate (e.g. fluorescent probes, lipids, plasma, semen, saliva) with an oxidant to obtain a maximum level of oxidation in the absence or presence of the "antioxidant sample" to be evaluated. The extent of inhibition of the oxidation defines the amount of "antioxidant sample" that provides certain degree of protection, e.g. 50%. Most of these assays are technically correct and simple to perform, which explains the massive use of TAC.

3. TAC versus individual antioxidants

There are several ways to define an antioxidant substance. An antioxidant molecule can be identified by its chemical structure that allows a free radical scavenging reaction and/or the chelation of redox-active metals. However, antioxidants are often defined as molecules that experimentally prevent the oxidation of a biological or chemical system. In simple chemical systems, both definitions frequently agree; but in more complex conditions (e.g. organisms) chemical and experimental definitions diverge [4]. Then, when it is not possible to measure individual antioxidant compounds (essentially in samples with unknown composition), TAC assays present the advantage that can integrate the individual antioxidant actions of different compounds and their additive, synergistic, or antagonistic interactions [5,6]. This integrative strategy could be of value, but TAC assays do not consider several aspects that intervene in the complex road between a chemical compound that prevents the oxidation of a chemical system, and the actual possibility of this compound to be an antioxidant in vivo and/or to benefit health. Furthermore, TAC is normally evaluated using a single assay which imposes the chemical limitations of that assay. Even when several assays are used to characterize a particular sample, a number of molecules that provide antioxidant protection may not be assessed. This is relevant in complex biological systems, where the participation of enzymes, signaling molecules, membrane components, etc., will define a network of antioxidant defenses that cannot be evaluated by any TAC assay [7].

Concerning the chemical specificity of TAC assays, in one of the most commonly used, the oxygen radical absorbance capacity (ORAC), the sample is challenged with peroxyl-radicals (produced by the thermal decomposition of an azo-compound) and the peroxyl-radicals not trapped by the sample are detected using different detection conditions (usually fluorescent probes) [8]. Thus, ORAC only evaluates substances able to trap peroxyl-radicals (Fig. 1). In other commonly used assay, the ferric ion reducing antioxidant power (FRAP) assay, the sample is incubated in the presence of complex ferric ions, which are reduced to the ferrous form and detected spectrophotometrically [9]. More specific TAC assays can also be designed to evaluate the capacity of a system to react with determined chemical species (e.g. singlet oxygen, superoxide anion, hydrogen peroxide) [10]. However it should be taken into account that not all the compounds defined as antioxidants, actually just a few, would be reactive with the same efficiency in all these assays. That is, depending on the assay/s performed, the conditions of the different assays, and the composition of the tested sample, the TAC of the sample under study can be markedly different. In addition, none of the current TAC assays are sensible to other quantitatively important antioxidant defenses, e.g. antioxidant enzymes, metal-binding proteins, and other molecules/strategies that contribute to the antioxidant network of a biological system (Fig. 1). To add complexity to this scenario, some of the assays can be carried out under conditions that provide information about processes mostly taking place in lipophilic or hydrophilic environments. In summary, information provided by TAC with respect to the antioxidant function of compounds being part of complex

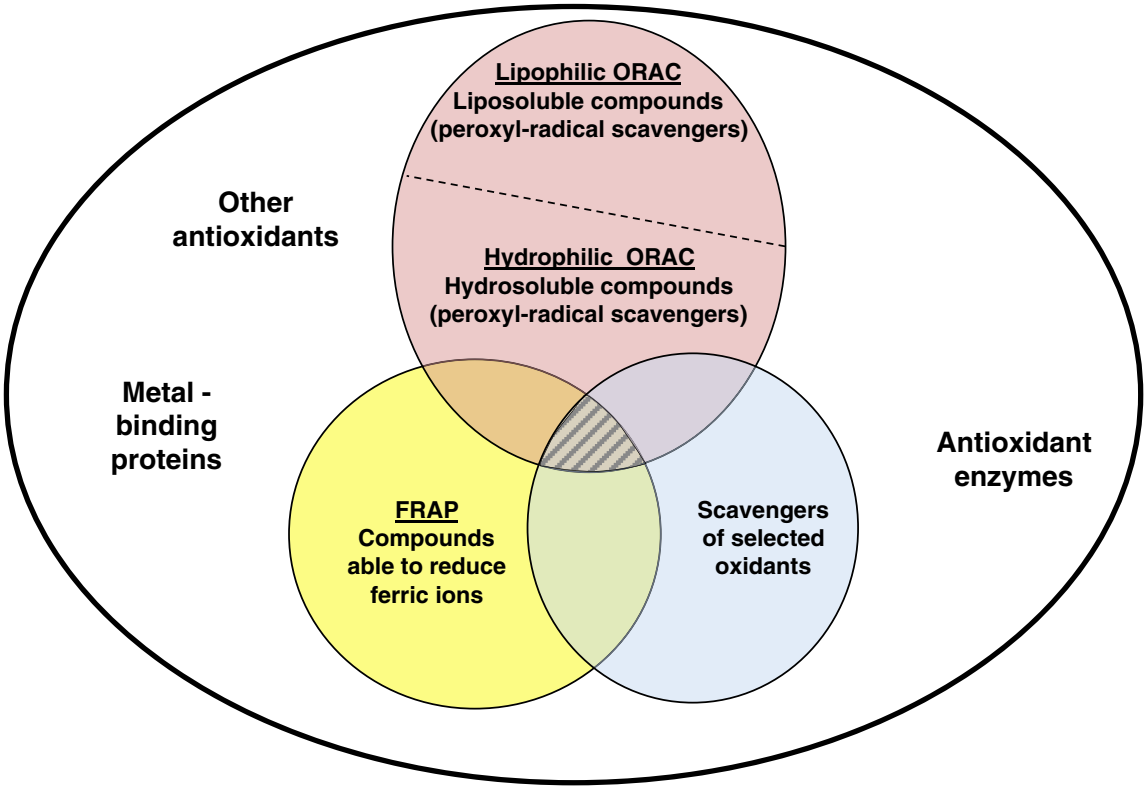

Fig. 1. Scheme of an antioxidant defense system. Total oval area corresponds to the complete antioxidant defense network of a system. The colored areas correspond to the molecules detected by each of three selected TAC assays. The non-colored area corresponds to the antioxidant defenses do not detected by any of the selected TAC assays. The stripped area represents the antioxidants that are detected by all the selected TAC assays. Areas do not quantitatively reflect the actual contributions of each set of antioxidants that react in the selected TAC assays.

systems is rather partial, in both qualitative and quantitative terms, and should be carefully interpreted.

4. TAC in food/beverages/supplements

TAC measurements have been carried out on a variety of food-derived samples: plants, plant extracts, preparations obtained after hydrolysis of plant components, fruits, vegetables, processed foods and beverages. TAC in foods can be measured using chemical or biological oxidizable substrates (e.g. plasma, cells in culture). ORAC has been the most frequently used method, but FRAP and others as Trolox equivalent antioxidant capacity (TEAC) [11] are also used to measure TAC in foods.

The usefulness of TAC in foods largely depends on the intended purpose of the assay, and on the experimental design. Similar values of TAC, even measured using the same method, can be due to the presence of different antioxidant molecules with different reactivity towards the oxidant source. TAC could be useful when comparing particular situations (e.g. assessing food oxidation during collection, storage and processing, comparing varieties and harvest conditions). In this context TAC is a simple and low cost method to assess multiple samples. The parallel measurement of samples for the content of the major antioxidant molecules, e.g. polyphenols, tocopherols, ascorbate, would further validate TAC values. However, it should be also considered that a direct measurement of these molecules can be more robust than extrapolating its presence from TAC values, e.g. to determine ascorbate concentrations vs. extrapolating ascorbate concentration from a TAC value.

Another situation is created by the extrapolation of the antioxidant capacity of a food to its antioxidant action at body level. For many compounds, the relevance of such extrapolation is strictly limited to the potential action of the antioxidant components of foods at the oral cavity and the gastrointestinal tract. This is mostly due to the fact that food antioxidant components can undergo extensive transformation and metabolism by tissues after absorption, including the gut microbiota. These events can lead to a decrease or disappearance of potential antioxidant activity, and/or to a limited capacity of the compound/metabolites to reach target tissues. Accumulating evidence have demonstrated that in most cases the beneficial actions of food components with antioxidant capacity in vitro occur through highly specific mechanisms rather than direct antioxidant effects [12]. A very important example of these indirect antioxidant effects are polyphenols, which constitute a big family of plant compounds present in large amounts in human diets, and which antioxidant action measured with TAC methods has driven numerous and misleading health claims.

With regard to the TAC of complex systems, for years the FDA recognized ORAC as a reference to compare the antioxidant value of foods, especially fruit and vegetables. The ORAC Database for Selected Foods from the USDA's Nutrient Data Laboratory included values for several hundreds of fruits, vegetables, and food preparations. This database was recently withdrawn because of technical and conceptual reasons [13]. The technical shortfalls, mostly related to the use of different ORAC conditions (different mechanisms to generate oxidants or different oxidizable substrates), make comparisons of results very difficult. Conceptually, it is argued that: i) the values indicating antioxidant capacity in vitro have not demonstrated to be relevant for the biological effects of specific bioactive compounds, and ii) the metabolic pathways associated to the prevention or amelioration of chronic diseases by bioactive compounds are only partially and non-antioxidant mechanisms may be involved.

Nevertheless, it should be noted that recently population studies found significant associations between the TAC of the food consumed (obtained from food-frequency questionnaires) and a lower risk of heart failure [14], and strokes [15,16]. Even when these studies could be of significance validating the use of TAC for foods, they do not allow ruling out that the observed associations are with the intake of fruit and vegetables, and TAC is just a reflection of that consumption.

In summary, the assessment of TAC in foods can be useful for select purposes and upon validation with other methods, including an initial characterization of the individual components with antioxidant capacity. In the case of using food TAC values to assess biological effects, its validity would be restricted to the oral cavity and the gastrointestinal tract. This assumption would be only applicable for those antioxidant compounds that remain unmetabolized at these levels.

5. TAC in tissues and body fluids

TAC has been measured in different tissues and biological fluids, as blood/plasma/serum, cerebrospinal fluid, seminal plasma, follicular fluid, amniotic fluid, saliva, tears, and urine [17].

TAC in tissues and body fluids has been measured with two major objectives: i) to associate the level of antioxidants and TAC provided by a food/food component with TAC in the body; and ii) to associate TAC in tissues, and body fluids with a particular condition (e.g. oxidative stress or altered redox status, disease states), or treatments (e.g. diet, supplementation, pharmacological treatment).

Given the simplicity of TAC assays and the easy access to human blood, TAC measurements in plasma flourished in the last years generating a large amount of data. However, the absence of concrete associations between measured values and functions makes that, as discussed below, the disadvantages outnumber the advantages of these determinations.

The limitations of the TAC measurements do not arise from technical (chemical) problems, but from the experimental design and the interpretation of the results. Each situation requires a careful design of the experimental protocol and, even after that, very cautious conclusions should be taken. For example, the administration of a diet (or food component) to a subject with the purpose of evaluating its impact on plasma TAC requires strictly controlled conditions for diet, life style, environment, etc. In humans, this can only be achieved in an acute or short-term protocol. Even under such controlled conditions, an increase in plasma TAC may have several explanations: i) an antioxidant action of a compound present in the diet that is absorbed and is present in the plasma; ii) an increase in plasma of molecules with antioxidant capacity resultant of the metabolism (or body processing) of the ingested compound; iii) a decrease in the degradation of molecules with antioxidant capacity present in plasma resultant of the metabolism (or body processing) of the ingested compound. Given that these three conditions are not mechanistically equivalent, drawing a definitive conclusion from the TAC results, could be premature and of limited validity.

Analysis of the contribution of the different compounds present in plasma/serum to TAC, as evaluated with different assays (e.g. ORAC, FRAP or TEAC), indicates that the highest contribution to TAC is provided by urate, and protein thiols [18]. This is exemplified in Table 1, in which are depicted the relative rates of reaction of peroxyl radicals with urate, ascorbate, and the polyphenol (−)-epicatechin. The three compounds have chemical structures that are thermodynamically appropriate to react with peroxyl radicals. However, the high urate and ascorbate plasma concentrations makes the potential contribution of (−)-epicatechin to the antioxidant capacity of plasma too low to be physiologically significant.

The most relevant question is if in humans (or animals) an increase in TAC values can be responsible for a health benefit. Based on the present evidence, the answer that can be drawn is that such association does not exist.

6. Conclusions

In summary, the advantages of TAC determinations are: i) its simplicity and low cost per sample; and ii) the possibility of evaluating known and unknown antioxidants and their additive, synergistic and/or antagonistic actions in a complex chemical or biological system. The

Table 1
Relative theoretical contribution of urate, ascorbate and (−)-epicatechin to plasma antioxidant capabilities.

Compound (AH)	$-E^{\circ\prime}$[a] (mV)	k[b] ($\mu M^{-1}\ s^{-1}$)	Plasma concentration[c] (μM)	Relative rate[d] (s^{-1})
Urate	590[e]	1.0[g]	200	200
Ascorbate	282[f]	1.0[f]	50	50
(−)-Epicatechin	570[f]	7.3[f]	0.3	2

Data correspond to the reaction ROO• + AH → ROOH + A•, where ROO• is a peroxyl radical and AH is a compound able to act as an antioxidant by scavenging ROO•. Modified from Fraga et al., 2010 [12].

[a] Reduction potentials defined for A• → AH where AH is any of the compounds of the table; the minus sign makes $E^{\circ\prime}$ consistent with the half-reaction AH → A• which is the process involved in the reaction.

[b] Second order rate constant for the reaction according to the rate (v) equation: $v_{AH} = k_{AH}$ [ROO•] [AH].

[c] Values taken from literature.

[d] Relative rate defined as reaction rate divided by [ROO•].

[e] Becker, 1993 [19].

[f] Fraga et al., 2010 [12].

[g] Buettner and Jurkiewicz, 1996 [20].

main disadvantages are: i) different TAC assays poorly correlate with each other; ii) different TAC assays detect a particular combination of compounds, but exclude the contribution of antioxidant enzymes, metal binding proteins, and other indirect antioxidants; and iii) the data for antioxidant capacity of foods generated by in vitro (test-tube) methods cannot be extrapolated to in vivo (human) health effects. Furthermore, molecules with antioxidant characteristics in foods have a wide range of functions, many of which are unrelated to the ability to react with free radicals. TAC methods can be used only under highly controlled conditions and provide results which interpretation should be backed by considering the characteristics of the studied model.

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