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The ORAC (Oxygen Radical Absorbance Capacity) index does not reflect the capacity of antioxidants to trap peroxyl radicals

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Abstract. In the present work we demonstrate from kinetic studies that under the experimental conditions proposed for the ORAC protocol, ORAC values do not correlate with the capacity of antioxidants to trap peroxyl radicals ($ROO^{\bullet}$), suggesting a dominant role of alkoxyl radicals ($RO^{\bullet}$) in the assay.

A large number of studies has been devoted to determine the antioxidant capacity (AC) of polyphenol-rich fruits, vegetables and beverages.[1] For this purpose, different methodologies have been developed,[2] the ORAC (Oxygen Radical Absorbance Capacity) method being one of the most employed assays.[1-3] In fact, databases of the ORAC index of fruits have been recently built to emphasize the benefits of establishing the antioxidant capacity of polyphenol-rich foods.[4] The ORAC methodology was reported by first time in 1993 by Cao and collaborators.[5] The method was based on the ability of antioxidants to prevent the consumption of beta-phycoerythrin mediated by peroxyl radicals ($ROO^{\bullet}$) generated during the aerobic thermal decomposition of AAPH (2,2'-azo-bis(2-amidinopropane) dihydrochloride).[5] However, the currently employed assay is based on the work published in 2001 by Ou et. al.,[6] which proposed the use of fluorescein (FLH) as target molecule. Several aspects associated with the use of beta-phycoerythrin, including its direct reaction with procyanidins and reproducibility inconsistency between different batches, contributed to favor the use of FLH as probe in the ORAC assay. Nonetheless, more recently we have reported that the ORAC index is strongly influenced by the type of probe employed.[7] In particular, the use of FLH is not free of drawbacks. In the first place, the ORAC index is not necessarily related to the total free radical scavenging capacity of the tested compound(s) and, in complex mixtures, is determined not only by the concentration, but also by the chemical nature of, and possibly the interaction between, the antioxidants present in the sample.[8] Secondly, not less important, it has not been established yet whether the ORAC index of a specific sample is determined by the capacity of the antioxidants to trap AAPH-derived peroxyl and/or AAPH-derived alkoxyl radicals.[9] In the present work, we discuss this point and conclude that, under the conditions recommended by Ou and co-workers,[6] the ORAC index of different antioxidants is associated with the capacity of these compounds to remove different free radicals generated during the thermolysis of AAPH, precluding a meaningful interpretation of the results, particularly when complex mixtures such as foods or beverages are tested.

The minimal set of reactions involved in the ORAC assay of an antioxidant (XH) is given in **Scheme 1**.

Scheme 1:

$$AAPH \xrightarrow{O_2} 2ROO^{\bullet} + N_2 \quad [1]$$

$$FLH + ROO^{\bullet} \longrightarrow FL^{\bullet} + ROOH \quad [2]$$

$$XH + ROO^{\bullet} \longrightarrow X^{\bullet} + ROOH \quad [3]$$

The incubation of FLH in the presence of AAPH and simple phenolic compounds (as example, coumaric acid, sinapic acid, ferulic acid and Trolox) gave time profiles of FLH consumption as those presented in **Figure 1**.

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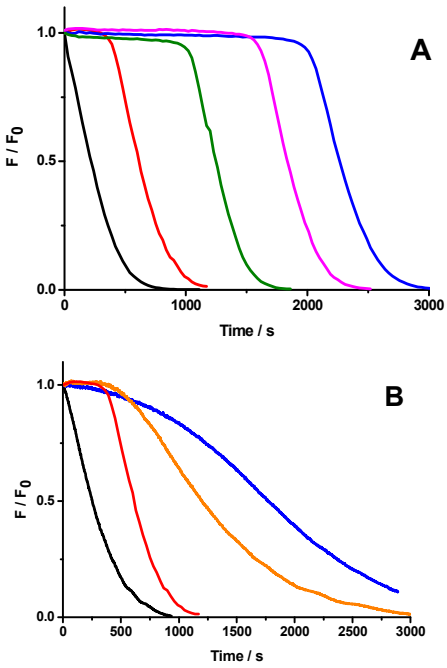

Figure 1: Graphic A: Protection of FLH (70 nM) elicited by Trolox at 1 (red); 5 (green); 7.5 (magenta); and 10 (blue) µM (data taken from ref.8). Graphic B: Protection of FLH (70 nM) elicited by Sinapic acid (orange), coumaric acid (blue), and Trolox at 1 µM concentration. Solutions were incubated in phosphate buffer (75 mM, pH 7.4) at 37ºC in the presence of AAPH (10 mM). Control experiments, in the absence of antioxidants; black line.

These data (**Figure 1**) show that lag times (T) at a given XH concentration, and hence ORAC values follows the order

Trolox < sinapic acid < coumaric acid

This trend is opposite to that expected considering their radical trapping capacity. In fact, the bond dissociation energies (BDE) of compounds with similar chemical structures such as phenol, 2,6-dimethoxyphenol, and 6-hydroxy-2,2,5,7,8-pentamethylchroman[10] suggest that the reactivity towards free radicals (defined as the initial rate of the free radicals-XH reaction), follows the order:[11]

Trolox > sinapic acid > coumaric acid

allowing to conclude that the most reactive phenol is the one that affords the minimum protection. This apparent anomaly cannot be explained in terms of the **Scheme 1** and requires to consider the role of alkoxyl radicals ($RO^{\bullet}$) (**Scheme 2**) to explain these results.[12,13]

Scheme 2:

$$2ROO^{\bullet} \longrightarrow ROO\text{-}OOR \quad [4]$$

$$ROO\text{-}OOR \longrightarrow 2RO^{\bullet} + O_2 \quad [5]$$

$$FLH + RO^{\bullet} \longrightarrow FL^{\bullet} + ROH \quad [6]$$

$$XH + RO^{\bullet} \longrightarrow X^{\bullet} + ROH \quad [7]$$

If it is considered that $RO^{\bullet}$ are more reactive than $ROO^{\bullet}$,[14] and also the very low FLH concentration employed in the assay (70 nM), we can assume that FLH is only removed by alkoxyl radicals. This is stressed by the data given in **Figure 2**. These data show that FLH consumption reaches a plateau when the dye consumption rate amounts to ca. 10 % of the total rate of radicals associated to the AAPH pyrolysis. Similar results are obtained employing phenols of low reactivity (such as coumaric acid). On the other hand, Trolox is more reactive and hence is able to trap both alkoxyl and peroxyl radicals and its maximal rate of removal is considerably higher than that of coumaric acid. The fast removal of Trolox reduces its induction time (see **Fig. 1B**), rendering it less efficient than less reactive compounds.

From induction time values (T) generated at a given antioxidant concentration ([XH]), the number (n) of free radicals removed by each reacted antioxidant molecule (between 1 and 2 for compounds bearing a single OH group), and the rate of production of azo-derived radicals (R), it is possible to estimate the fraction (f_T) of azo-derived free radicals that can be trapped by each XH molecule employing **Equation 1:**

$$f_T = n\,[XH] / T\,R \quad \textbf{[Eqn.1]}$$

As depicted in **Figure 1**, Trolox, coumaric, and sinapic acid generate T (defined as the time at which intercept the straight lines drawn to the data corresponding to the slow and fastest consumption rates) at low concentrations (1-10 µM). Taking into account these T data, the rate of azo-derived free radicals production (0.8 µM/min),[15] fractions (f_T) can be obtained from **Equation 1**. These values are presented in **Table 1** together with the values of several antioxidants usually present in foods and beverages. Additionally, **Table 1** shows f values of antioxidants obtained from the initial consumption rate (ri), evaluated from kinetics followed by high performance chromatography (HPLC), *versus* initial concentration plots (**Figure 3**) and determined by **Equation 2** (f_{ri}).FLH and pyrogallol red (PGR, a target molecule employed in an ORAC-like assay) data (**Figure 2**) are also included in **Table 1**.[8]

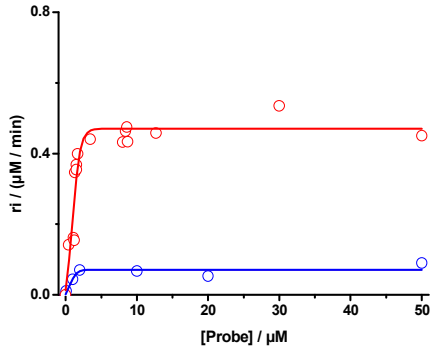

Figure 2: Dependence of the initial consumption rate (ri) of pyrogallol red (red) and fluorescein (blue) with their initial concentration. Consumption of Pyrogallol red and fluorescein was followed by visible spectroscopy (at 540 nm), and fluorescence (exc. = 493, and em. = 515 nm), respectively. Solutions were incubated in phosphate buffer (75 mM, pH 7.4) at 37ºC in the presence of AAPH at 10 mM concentration.

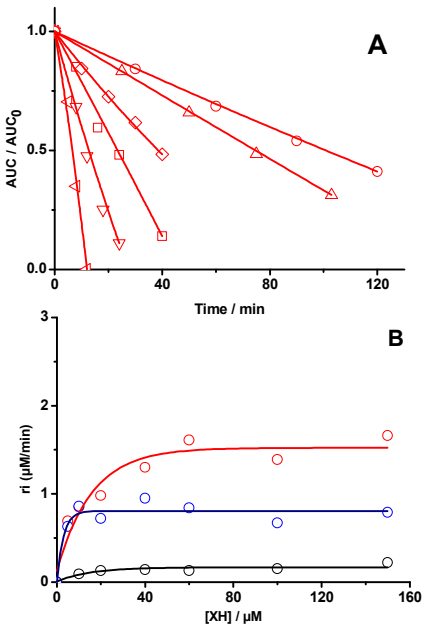

Figure 3: Consumption of polyphenols mediated by AAPH-derived free radicals. Graphic A: Kinetic profiles of kaempferol consumption. Kaempferol was incubated with AAPH (10 mM) in phosphate buffer (75 mM, pH 7.4) at 37ºC, and its consumption was followed by HPLC technique. Kaempferol concentrations: 10 (◁); 20 (▽); 40 (□); 60 (◇); 100 (△); 150 (○) μM. Graphic B: Dependence of the initial consumption rate (μM/min) with the initial concentration of quercetin (red); kaempferol (blue) and apigenin (black).

$$f_{ri} = n\,(r_i) / R \qquad \textbf{[Eqn.2]}$$

The data given in **Table 1** show that f_T values ranged from low (0.06) to values close to 1.0 (Trolox and gallic acid). The latter are the values expected when the phenol traps all azo-derived radicals. It is interesting to note that f_T values for Trolox and gallic acid were similar to those obtained from initial consumption rates (**Equation 2**, **Table 1**). This fact supports the validity of the reaction schemes. On the other hand, compounds such as cinnamic acids, some flavonoids and a phenolic acid (protocatechuic acid) showed f_T values between 0.06 and 0.1. Furthermore, the data depicted in **Table 1** show that compounds with low reactivity, such as cinnamic acids, render ORAC values larger than that of gallic acid, a compound that reacts with peroxyl radicals at rates almost that the obtained by diffusion-controlled reactions.[16] As a whole, these data can be explained only if it is considered that a small fraction (for example 0.1) of the AAPH-derived free radicals leads to RO$^\bullet$ radicals, and that those radicals are removing the antioxidants and FLH. This predominance of RO$^\bullet$ is compatible with the results published by Sueshi and coworkers,[9] which reported that almost all azo-derived radicals mostly generate RO$^\bullet$ radicals.[9]

In the case of f_{ri} values, which were obtained throughout the direct consumption of antioxidants elicited by AAPH-derived free radicals; values between 0.18 and 3.6 were obtained. Values near 1.0 (or higher) would indicate a total trap of ROO$^\bullet$radicals. By contrast, low values would imply reactions exclusively with RO$^\bullet$. In fact, the lowest value (0.18), obtained for FLH (**Figure 2** and **Table 1**), implies that, under the employed conditions, the consumption of this probe is exclusively associated with its reaction towards RO$^\bullet$. By contrast, at the concentrations employed in the ORAC-PGR index,[8] the f_{ri} value of 1.18 of PGR (**Figure 2** and **Table 1**) indicates that this probe is trapping both ROO$^\bullet$ and RO$^\bullet$ species. This leads to ORAC-PGR indexes very different from those provided by the ORAC-FLH approach.[8]

The ratio of f_T and f_{ri} reflects the influence of the reaction towards RO$^\bullet$ when FLH is employed as probe. A high ratio would indicate a high influence of RO$^\bullet$ radicals on the ORAC assay. Conversely, ratio values near 1.0 should indicate that ORAC assay is mainly influenced by ROO$^\bullet$. As can be seen in **Table 1**, almost all tested compounds showed high values of this ratio. Interestingly, Trolox and gallic acid presented values close to 1.0, showing that their reactions, in the presence or absence of FLH, are mainly related to ROO$^\bullet$. By contrast, quercetin, the antioxidant with the highest (f_{ri}/f_T) ratio would protect FLH from its reaction towards RO$^\bullet$. This result would explain that very low concentrations (0.1-1 μM) of quercetin are able to generate a very large protection on the kinetic profiles of FLH consumption. Interestingly, f_{ri} / f_T data depicted in **Table 1** showed a direct correlation (y= -8.7 + 5.1x; r = 0.5780) with ORAC values, supporting our hypothesis that ORAC assay, employing FLH as probe, gives values representing the reaction of antioxidants towards RO$^\bullet$and, in a low number of compounds

Compounds	*ORAC*	f_T	f_{ri}	f_{ri}/f_T
Caffeic acid	4.37[a]; 6.63[b]	0.13	1.26	9.7
Sinapic acid	2.8	0.32	2.22	6.9
Ferulic acid	3.5	0.24	1.54	6.4
Coumaric acid	4.1	0.20	0.62	3.1
Trolox	1	1.0	1.12[d]	1.1
Luteolin	7.9	---	0.62	---
Apigenin	8.2	---	0.62	---
Kaempferol	10.2[c]	0.08	2.22	27.8
Quercetin	10.7[c]; 7.28[a]	0.06	3.60	60.0
Ellagic acid	3.1	0.22	2.22	10.1
Protocatechuic acid	6.7[c]	0.09	1.44	16.0
Myricetin	1.8	0.13	---	--
Gallic acid	1.2[c]	1.17	1.40	1.2
PGR	---	---	1.18	---
FLH	---	---	0.18	---

towards ROO$^\bullet$.

Table 1. ORAC and *f* values of pure antioxidants and probes. *f* values were estimated by ORAC-FLH (f_T) and direct consumption (f_{ri}, of pure polyphenols or probes. f_T and f_{ri} values were estimated considering a n = 2. The consumption of FLH and PGR was assessed by UV-visible spectroscopy. Data taken from [a]Dávalos, et. al.[17]; [b]Pérez, et al.[18]; [c]López-Alarcón et al.[8]; [d]Atala, et. al.[19]

Conclusions

In conclusion, our data indicate that the FLH-based ORAC index is determined by the reactivity of XH towards both the RO$^\bullet$ and ROO$^\bullet$radicals, and that the relative contribution of these two species depends upon the reactivity of the probe and antioxidant,

rendering very difficult a rationalization and interpretation of the ORAC values, particularly in the case of complex mixtures.

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References

1 C. López-Alarcón and A. Denicola, *Anal. Chim. Acta,* 2013, **763**, 1-10.
2 D.Huang, B.Ou and R.L. Prior, *J. Agric. Food Chem.,* 2005, **53**, 1841-1856.
3 R.L. Prior, X. Wu, and K.Schaich, *J. Agric. Food Chem.,*2005, **53**, 4290-4302.
4 H. Speisky, C. López-Alarcón, M. Gomez, J. Fuentes, and C.Sandoval-Acuna, *J. Agric. Food Chem.,*2012, **60**, 8851-8859.
5 G. Cao, H.M. Alessio, and R.G. Cutler, *Free Radic. Biol. Med.,*1993, **14**, 303-311.
6 B. Ou, M. Hampsch-Woodill and R.L. Prior, *J. Agric. Food Chem.,*2001, **49**, 4619-4626.
7 C. López-Alarcón, A. Aspee and E. Lissi, In Emerging Trends in Dietary Components for Preventing and Combating Disease, ed B.S. Patil, G.K. Jayaprakasha, K.N.Ch. Murthy and N.P. Seerasm, American Chemical Society pres., 2012, vol. 1093, ch24, pp 417-429.
8 C. López-Alarcón and E. Lissi, E. *Free Radic. Res*., 2006, **40**, 979-985.
9 Y. Sueishi, D. Yoshioka, S. Oowada, N. Endoh, S. Kohri, H. Fujii, M. Shimmei and Y. Kotake, *Z. Phys. Chem*., 2010, **224**, 921-928.
10 M. Lucarini, P. Pedrielli and G. Pedulli, *J.Org. Chem.,* 1996, **61**, 9259-9263.
11 C. López-Alarcón, A. Aspée, and E. Lissi, *Free Radic. Res*. 2007, **41**, 1189-1194.
12 J. Werber, Y.J. Wang, M. Milligan, X. Li and J. Ji, *J. Pharm. Sci.*, 2011, **100**, 3307-3315.
13 S. Kohri and H. Fujii, *J.Clin. Biochem. Nutr.,* 2013, **53**, 134-138.
14 G. R. Buettner, *Arch. Biochem. Biophys.*, 1993, **300**, 535-543.
15 E. Niki, *Methods Enzymol*., 1990, **186**, 100-108.
16 T. Marino, A. Galano, and N. Russo, *J. Phys. Chem. B.*, 2014, **118**, 10380–10389.
17 A. Dávalos, C. Gómez-Cordovés, and B. Bartolomé. *J. Agric. Food Chem*., 2004, **52**, 48–54.
18 D. Pérez, F. Leighton, A. Aspée, C. Aliaga, and E. Lissi. *Biol. Res.*, 2000, **33**, 71–77.
19 E. Atala, A. Aspee, H. Speisky, E. Lissi and C. López-Alarcón, *J. Food Compos. Anal.*, 2013, **32**, 116-125.