



## Antioxidant-spotting in micelles and emulsions

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### ABSTRACT

A simple protocol is described for locating the site of action of an antioxidant (AO) in a micro-heterogeneous mixture, based on the pattern of the reactivity curve towards the AO of a series of 4-alkanoyl TEMPO radicals. The resulting cut-off curves yield information regarding the hydrophobic microenvironment surrounding the reactive AO group, and its accessibility by the probe. Convex curves are an indication of an AO located in a more hydrophilic environment, while concave plots originate from AOs in a more hydrophobic location in the micro-heterogeneous system.

### 1. Introduction

Measuring the effectiveness of antioxidants (AOs) present in food stuffs has been the subject of a large variety of papers, promoted by the increasing understanding of the role played by these compounds in human health. The presence of these antioxidants also imparts an additional value to various foods and beverages, which can be ultimately recommended for exhibiting significant antioxidant activities.

Such a plethora of data on the antioxidant activities of a large variety of food preparations naturally raises the question of comparing their effectiveness with general, common standards. This is a difficult task, frequently eluded by many reports that simply ascribe particular values of antioxidant activity to complex mixtures, without any concern for the origin of such an activity or of their site of action. An example of such a simplification is the frequent use of 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Prior, Wu, & Schaich, 2005), the ferric-ion reducing antioxidant power (FRAP) (Ryan & Prescott, 2010), or Trolox Equivalent Antioxidant Capacity (TEAC) (Muñoz-Acevedo, Vargas Méndez, Stashenko, & Kouznetsov, 2011; Prior et al., 2005; Sproston & Akoh, 2016) methodologies, the latter being based on a barely water-soluble compound, and applied to heterogeneous mixtures, such as micelles or emulsions (González-Hidalgo, Bañón, & Ros, 2012; Sólyom et al., 2014). Frankel's conclusion that "there cannot be a simple and rapid method to evaluate antioxidants in complex foods and biological systems" (Frankel, 2001), or Niki's critical reflections on how correctly we are measuring antioxidant activities (Niki, 2002), have been ignored in the past fifteen years by a large portion of food chemists. The latter's observations that "the antioxidant efficacy depends markedly on the type

of oxidant" and that "the efficacy of radical scavenging depends on the localization of the antioxidant" have found support, with time, from other research groups. It is now increasingly accepted that the antioxidant activity of a particular compound in a micro-heterogeneous environment depends on the distribution of the antioxidant and of the measuring probe in such a medium (Aliaga, Rezende, & Arenas, 2009; Bridi, Aliaga, Aspée, Abuin, & Lissi, 2011; Galan, Losada-Barreiro, & Bravo-Díaz, 2016; Kiralan, Dog, Kittipongpittaya, McClements, & Decker, 2014).

This is the reason why different orders of effectiveness are obtained for the same series of compounds, even when the same methods of antioxidant evaluation are employed by a research group (Alemán et al., 2015; Aliaga, Juárez-Ruiz, Scaiano, & Aspée, 2008).

Thus, groups concerned with the antioxidant effectiveness of food preparations have been increasingly conscious of the need to determine the site of action of AOs in these heterogeneous mixtures (Costa, Losada-Barreiro, Paiva-Martins, Bravo-Díaz, & Romsted, 2015; Losada-Barreiro, Bravo-Díaz, & Romsted, 2015).

In doing so, subtle effects of the distribution, structure and orientation of these antioxidants and/or their evaluating probes on the measured effectiveness have been detected (Aliaga et al., 2016). One of them, the amphiphobic nature of radical probes, or of phenolic antioxidants, has been invoked to rationalize the long known paradoxical or "cut-off" effect of many of these compounds in food mixtures. By using the series of probes 1a–f, derived from the nitroxyl TEMPO radical, their cut-off effect *vis-à-vis* various antioxidants has been rationalized in micellar solutions (Aliaga, Lopez de Arbina, & Rezende, 2016) and food emulsions (Lopez de Arbina, Rezende, & Aliaga, 2017).

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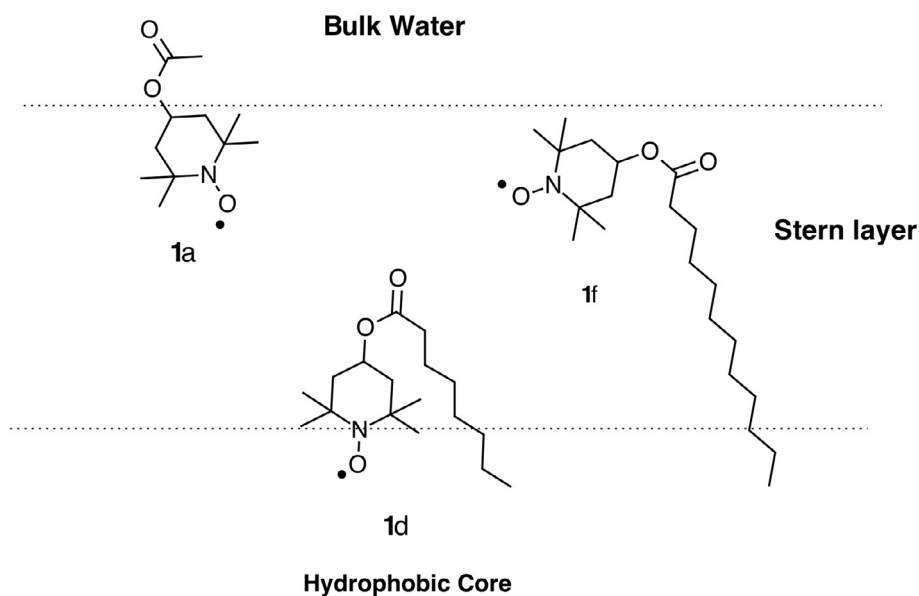


Fig. 1. Schematic representation of the different orientations and locations of probes **1a**, **1d** and **1f**, in the Stern layer of a neutral micellar system (Triton X-100) (Aliaga et al., 2016).

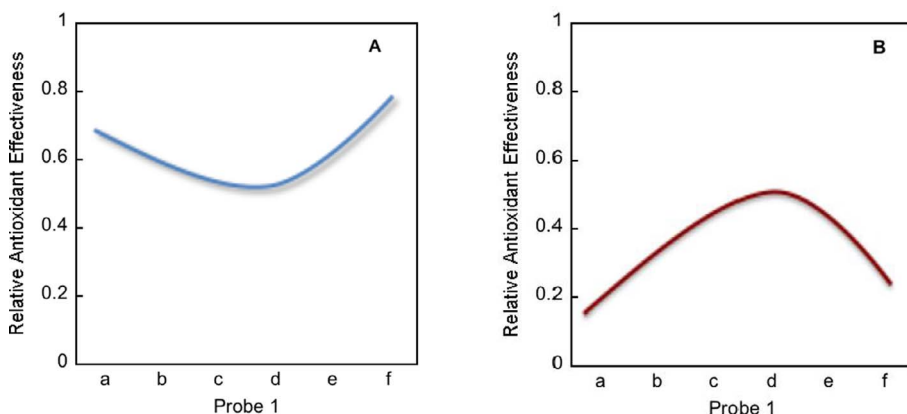


Fig. 2. Schematic characterization of the location of an antioxidant that reacts with the probe series **1a–f** in a micro-heterogeneous mixture. A convex plot (A) originates from a hydrophilic AO, while a concave curve (B) is characteristic of a hydrophobic antioxidant.

In spite of possessing the same nitroxyl fragment, these radicals are quenched differently by a given antioxidant through a hydrogen-abstrating process in a micro-heterogeneous medium, depending on the size of the 4-alkanoyl chain. Irrespective of the evaluated antioxidant, “cut-off” patterns are observed in plots of Relative Antioxidant Effectiveness vs. chain-size, with extreme values detected for probes with an intermediate chain-size, or hydrophobicity.

In the present communication we show that this paradoxical response can be used to determine the location and site of action of an antioxidant in a micellar system or an emulsion, if its reactivity towards radicals **1a–f** is compared for various members of the probe series.

## 2. Experimental

EPR spectra of probes **1a–f** were recorded on a Bruker EMX-1572 operating at X-band (9.0–9.9 GHz), at  $21 \pm 1$  °C. The EPR parameters were the same in all experiments: microwave power, 1 mW; modulation amplitude, 5 G; time constant, 10.24 ms; and conversion time, 40.96 ms.

Reduced glutathione, L-ascorbic acid, ( $\pm$ )-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), *p*-cresol,  $\alpha$ -tocopherol (vitamin E), 3,5-di-tert-4-butylhydroxytoluene (BHT) and reduced Triton X-100 were purchased from Sigma-Aldrich. Probes **1a–f** were obtained as described in (Aliaga et al., 2016).

The determination of the relative antioxidant effectiveness of

glutathione dicarboxylate (**2**) and of *p*-cresol (**5**) *vis-à-vis* the radical probes **1a–f** followed the same protocol described previously for the other antioxidants: L-ascorbate (**3**), trolox (**4**),  $\alpha$ -tocopherol (**6**) and BHT (**7**) (Aliaga et al., 2016).

Control solutions of probes **1a–f** were prepared by adding 10  $\mu$ l of a methanolic solution of the probe (1 mM) to a phosphate-buffered micellar solution (pH 7) of reduced Triton-X100 (11.0 wt%, 20 mM). The resulting solutions had a final volume of 200  $\mu$ l and a probe concentration of 50  $\mu$ M.

Test solutions of probes **1a–f** were prepared by adding 10  $\mu$ l of a methanolic solution of the probe (1 mM) to a phosphate-buffered micellar solution (pH 7) of reduced Triton-X100 (11.0 wt%, 20 mM), containing 10 mM of AO. The resulting solutions had a final volume of 200  $\mu$ l, a probe concentration of 50  $\mu$ M and an AO concentration of 10 mM.

Spectra of the radical probe in the control and in the test solutions were then recorded by transferring 80- $\mu$ l aliquots of these solutions to a capillary tube in the EPR cavity.

Negligible variations in the intensity of the TEMPO triplet after 20–30 min were an indication of the attained equilibrium in the test solutions. The relative antioxidant effectiveness was then calculated as the difference between the signal intensity of the control solution and the intensity recorded after equilibrium was attained in the test solution. The final relative antioxidant effectiveness was obtained as an average of three measurements.

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