



Cut-off effect of radical TEMPO derivatives in olive oil-in-water emulsions



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ABSTRACT

Three oil-in-water emulsions were prepared from mixtures of olive oil and Tween 20 in water. The effectiveness of a series of radical 2,2,6,6-tetramethylpiperidinoxyl (TEMPO) derivatives of variable lipophilicity in reactions with antioxidant Trolox, and as pyrene-fluorescence quenchers, was compared in the three emulsions. A “cut-off” effect was observed for the pyrene quenching by the probes, but not for their reaction with Trolox. The results were rationalized in terms of the amphiphobic nature of the probes, and the different locations of probe, pyrene and Trolox in the three-phase microheterogeneous systems.

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1. Introduction

Characterization of new antioxidants in foodstuff (Almeida et al., 2016; Bakota, Winkler-Moser, Berhow, Palmquist, & Liu, 2015; Din, Sarfraz, & Shahid, 2015; Farvin & Jacobsen, 2015; Pazos, Alonso, Sánchez, & Medina, 2008; Vhangani & Van Wyk, 2016) for the evaluation of known AO's in food emulsions (Chen, Zou, Liu, & McClements, 2016; Espinosa, Inchingolo, Alencar, Rodríguez-Estrada, & Castro, 2015; Gonzalez, Medina, Maldonado, Lucas, & Morales, 2015; Jia, Shin, & Lee, 2015; Yang, Wang, & Lee, 2015) are subjects of permanent interest. Some of the studied systems are rather complex, allowing only measurements of bulk activities, which are poorly rationalized in chemical terms. Thus, for example, it is not uncommon to observe for the same group of antioxidants, distinct order of activities, depending on the employed emulsion (Pazos et al., 2008). On the other hand, detailed physicochemical analysis of emulsions are found in the literature, that nevertheless fail to shed light on the chemical factors that are responsible for distinct activities of the same compound in different media (Pande & Akoh, 2016).

Thus, when trying to understand the factors that govern the antioxidant activity of chemicals in food emulsions, some balance must be reached between two extremes: on the one hand,

renouncing to describe the system in detail, by simply measuring and comparing bulk properties of a complex mixture; on the other hand, attempting to arrive at an in-depth view of the chemical system, by resorting to simplified models.

Following the last approach, we have resorted in the past to micellar solutions of known antioxidant and radical probes, and have related their structures with their partitioning and reactivity in these microheterogeneous media (Aliaga, Lopez de Arbina, et al., 2016; Aliaga, Rezende, & Arenas, 2009; Aliaga, Bravo-Moraga, et al., 2016; Gomes, Costa, de Assis Perrechil, & da Cunha, 2016). Taking advantage of a protocol for the determination of partition constants in micellar solutions, based on the EPR spectra of probes from TEMPO derivatives (Aliaga, Torres, & Silva, 2012), we have shown that the partitioning of antioxidants and/or probes in microheterogeneous media determines to a significant extent the measured bulk antioxidant activities of these systems (Aliaga et al., 2009; Gomes et al., 2016). Further investigation of these model systems, with a systematic variation of the lipophilicity of the TEMPO probes, led us to an interpretation of the well-known “cut-off”, or paradoxical polar effect (Almeida et al., 2016; Balgavý & Devínsky, 1996; Costa, Losada-Barreiro, Paiva-Martins, Bravo-Díaz, & Romsted, 2015; Losada Barreiro, Bravo-Díaz, Paiva-Martins, & Romsted, 2013), based not only on the probe insertion, but also on its orientation within a hydrophobic microenvironment (Aliaga et al., 2009; Aliaga, Bravo-Moraga, et al., 2016). For a “cut-off effect” to be observed in a micellar solution, a particular structural requirement should be fulfilled by the probes and/or antiox-

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idants: they should comprise two hydrophobic moieties, competing for the hydrophobic core of a micelle (Aliaga, Bravo-Moraga, et al., 2016). This “amphiphobic” nature should give rise to such an effect not only in aqueous micellar solutions, but also in more complex systems, consisting of emulsified foodstuffs.

This has in fact been observed in studies of the antioxidant activities of alkyl gallates in three different emulsions (Gonzalez et al., 2015), and, more recently, of the activities of lipophilic alkyl esters of hydroxytyrosol in intact olive oil-in-water emulsions (Almeida et al., 2016). The authors' interpretation of the observed cut-off effect in these systems relied on kinetic measurements, and emphasized partitioning, more than the antioxidant orientation, in the microheterogeneous system.

As a natural extension of our previous work, we have therefore employed the series of amphiphobic radical probes 4-alkanoyl-2,2,6,6-tetramethylpiperidinoxyl (4-alkanoyl TEMPO) **1** and the well-known hydrophilic antioxidant Trolox **2** to confirm the existence of a cut-off effect in olive oil-in-water emulsions (see Scheme 1).

Besides varying the radical probe in these studies, instead of the phenolic antioxidant, we have also carried out a more detailed characterization of the obtained emulsions, relating the measured activities with the oil droplet sizes. Finally, by profiting from the quenching ability of radicals, we have additionally measured their interaction with a hydrophobic fluorescent compound (pyrene), a process for which the close proximity between reagents is not a requirement as obvious as for the probe reactions with Trolox.

The present study on the reactivity of TEMPO and TEMPO derivatives **1** in olive oil-in-water emulsions thus aims at applying a systematic approach in basic chemical terms to a more realistic description of antioxidants and probes in food matrices.

2. Experimental

EPR spectra were recorded with a Bruker EMX-1572 equipments, operating at X-band (9.0–9.9 GGz) at 21 ± 1 °C. Fluorescence measurements employed a Perkin Elmer LS 55 fluorometer. Emulsions were prepared with an Omni-Ruptor 4000 sonicator. Dynamic light-scattering measurements were carried out with a Malvern Nano-S90 Zetasizer equipment.

Tween 20 was purchased from Acros. Trolox (98% purity), pyrene and 2,2,6,6-tetramethylpiperidinoxyl (TEMPO) were purchased from Sigma Aldrich. The TEMPO derivatives **1a–f** were prepared as previously described (Aliaga, Bravo-Moraga, et al., 2016).

2.1. Emulsion preparation

Three different olive oil-in-water emulsions with distinct droplet sizes, were prepared by mixing appropriate proportions of olive oil and Tween 20. An appropriate weight of an aqueous buffer solution (pH 3.6) of citric acid/citrate was then added, up to a final weight of 18 g. The resulting mixtures were transferred to stoppered 25-ml vials and cooled at 5 °C for 2–3 min. Then they were subjected to 3 repeated cycles, each of them comprising a 3-min

sonication, followed by a 3-min cooling in an ice-bath. This protocol yielded emulsions which were stable at room temperature for 1000 h, and which exhibited reproducible characteristics, as shown by light-scattering measurements of their average drop sizes.

2.2. Pyrene quenching

Blank pyrene solutions were prepared by diluting 30 µl of the appropriate emulsion in water (3 ml), and adding to this solution 10 µl of a 100 µM methanolic solution of pyrene. To these blank solutions were added increasing concentrations of the TEMPO quencher **1a–f**. The resulting solutions, excited at 337 nm, had their emission spectra recorded at 360–400 nm.

2.3. Reaction with Trolox

Blank probe solutions were prepared by adding 3 µl of a 10-mM methanolic solution of the TEMPO probe to 300 µl of the appropriate emulsion. The reaction with Trolox was followed by adding to these blank probe solutions 3 µl of a 500-mM methanolic solution of Trolox, to a final volume of 300 µl.

Probe consumption was followed by the decay of its EPR signal, with the following constant parameters: microwave power 1 mW, modulation amplitude 5 G, time constant 5.12 ms, conversion time 20.48 ms.

3. Results and discussion

3.1. Emulsion preparation and characterization

Three different olive oil-in-water emulsions A, B and C were prepared in aqueous buffer solutions (pH 3.6). They were stable at room temperature for 1000 h, and exhibited reproducible characteristics, as shown by light-scattering measurements of their average droplet sizes.

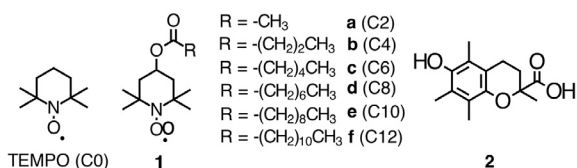
Table 1 lists the characteristics of the three emulsions, with the proportions (% w/w) of their components, and their droplet sizes, obtained as z-average values from DLS measurements.

3.2. Pyrene quenching by TEMPO derivatives **1a–f**

Quenching of the pyrene emission at 360–380 nm by increased concentrations of added TEMPO derivatives **1a–f** allowed a comparison to be made of the relative quenching abilities of these radical probes in the employed emulsions. Stern-Volmer plots of I_0/I vs. quencher concentration were linear (graphs not shown), and the corresponding slopes allowed the determination of quenching constants for all TEMPO derivatives. These observed constants (k_{SV}^{obs}) had to be corrected, to take into account the effective pyrene and probe concentrations in each emulsion. In fact, the amount of added pyrene was the same in all systems, but its effective concentration depended on its distribution among the three emulsion microphases and their corresponding volumes.

Analysis of its emission spectra in all emulsions, by measuring the ratio between the intensities of their first and third bands (Dong & Winnik, 1984; Thomas, 1977) led to the conclusion that in all cases the hydrophobic pyrene was totally dissolved in the oil droplet. Thus, the resulting pyrene concentration in emulsions A, B and C increased in the order $A < B < C$, according to the percentage of olive oil in the emulsion, listed in Table 1. Conversion of oil masses to volumes, with a density of 0.9 g cm^{-3} for olive oil, led to correction factors of 11.1, 3.3, and 1.1 for the pyrene concentrations in emulsions A, B and C.

The location of the probes in the emulsions also had to be known. We resorted to the g-factor values of their EPR spectra to



Scheme 1. Structures of the TEMPO radical, TEMPO derivatives **1** and antioxidant Trolox **2** employed in the present work.

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