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Searching for an absolute kinetic scale of antioxidant activity against lipid peroxidation

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ABSTRACT

The inhibition properties of a number of antioxidants against peroxidation, started by a 2,2′-azobis[2-(2-imidazolin-2-yl)propane] radical initiator, of linoleic acid in sodium dodecyl sulfate micelles, have been determined in terms of oxygen consumption by a Clark electrode in an oxygen-tight cell. For the 31 antioxidants investigated at variable concentrations, the experimental results well fit the kinetic equation for competitive reactions.

The ratio between the initial rates, monitored in the absence and in the presence of antioxidants, depends linearly on their concentration. From the slopes of these straight lines, an absolute scale of inhibition properties of the lipid peroxidation can be devised. Furthermore, the little difference of the time of complete oxygen consumption on concentration of different antioxidants has been found, indicating a restricted difference towards chemical structure and stoichiometric ratio.

Some considerations regarding the mechanisms of inhibition of the lipid peroxidation in micelles, in view of bibliographic data, have been made.

1. Introduction

The knowledge of the antioxidant properties of natural and synthetic molecules is an important requirement for investigations in the fields of biochemistry, biology, food chemistry, and other disciplines. It is of particular significance in i) the nutritional, pharmacological and health fields, for the prevention of diseases due to unbalanced diet and cell aging (Fang, Yang, & Wu, 2002; Huang, Ou, & Prior, 2005; Karadag, Ozcelik, & Saner, 2009; Perez-Lopez, Chedraui, Haya, & Cuadros, 2009; Prior, Wu, & Schaich, 2005); in ii) the food industry, for the preservation of the foodstuffs by natural products instead of synthetic additives (Huang et al., 2005; Pokorny, Yanishlieva, & Gordon, 2001); and, in iii) the chemical and manufacturing industries, in order to preserve finished products from oxidative degradation (Huang et al., 2005).

In order to determine the antioxidant properties of pure molecules or finished products, many assay procedures have been developed. These are principally spectrophotometric and electrochemical, where a number are based on kinetic measurements and others simply measure a parameter approximately correlated to the antioxidant properties. Some examples are the reducing capacity of the investigated solution (Gregoris et al., 2013; Huang et al., 2005), the total polyphenol content (Stevanato, Fabris, & Momo, 2004; Vianello et al., 2004) and the ability of a molecule to scavenge peroxyl radical species in homogeneous organic media (Burton & Ingold, 1981; Pryor et al., 1993). However, because of the high variability and complexity of the chemical background adopted and of the measurement procedures due to the complexity of *in vivo* or actual systems with respect to an *in vitro* more simplified investigation, no universal quantitative scale has been developed. Rather, many different chemical systems are proposed that have been studied principally in a homogeneous phase, and many different assay procedures and descriptive parameters are presented within a selected chemical system as well (Niki, 2010; Roginsky & Lissi, 2005). One such chemical system is based on the determination of the oxidation rate of linoleic acid (LH) in sodium dodecyl sulfate (SDS) micelles by oxygen dissolved in water, in a system which, although carried out *in vitro*, simulates the *in vivo* natural phenomena which involve chemical structures of different physico-chemical characteristics, such as, for example, aqueous plasma or cytoplasm and lipidic layers (Pryor, Kaufman, & Church, 1985; Pryor, Strickland, & Church, 1988).

A most comprehensive reaction system, frequently proposed for the *in vitro* assessment of the contrast capacity of the generic antioxidant, is illustrated in Table 1. Some of these reactions are considered in research papers (Bedard, Young, Hall, Paul, & Ingold, 2001; Bowry & Ingold, 1999; Burton & Ingold, 1981;

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Table 1
Chemical reactions for the radical oxidation of linoleic acid LH and the inhibition by the antioxidant AOH. Competitive reactions, colored grey, are reported.

(a) Initiation	
a1	$InNNIn \xrightarrow{k_1} 2\,In^{\bullet} + N_2$
a2	$In^{\bullet} + O_2 \xrightarrow{k_2} InOO^{\bullet}$
a3	$InOO^{\bullet} + LH \xrightarrow{k_3} InOOH + L^{\bullet}$
a4	$In^{\bullet} + LH \xrightarrow{k_4} InH + L^{\bullet}$
(b) Propagation	
b1	$L^{\bullet} + O_2 \xrightarrow{k_5} LOO^{\bullet}$
b2	$LOO^{\bullet} + LH \xrightarrow{k_6} LOOH + L^{\bullet}$
(c) Inhibition by AOH	
c1	$In^{\bullet} + AOH \xrightarrow{k_7} InH + AO^{\bullet}$
c2	$InOO^{\bullet} + AOH \xrightarrow{k_8} InOOH + AO^{\bullet}$
c3	$L^{\bullet} + AOH \xrightarrow{k_9} LH + AO^{\bullet}$
c4	$LOO^{\bullet} + AOH \xrightarrow{k_{10}} LOOH + AO^{\bullet}$
(d) Terminations (NRP: Non Radical Products)	
d1	$AO^{\bullet} + AO^{\bullet} \longrightarrow NRP$
	$LOO^{\bullet} + LOO^{\bullet} \longrightarrow NRP$
	$InOO^{\bullet} + InOO^{\bullet} \longrightarrow NRP$
	$InOO^{\bullet} + LOO^{\bullet} \longrightarrow NRP$
	$LOO^{\bullet} + AO^{\bullet} \longrightarrow NRP$
	$InOO^{\bullet} + AO^{\bullet} \longrightarrow NRP$

Denisov & Khudyakov, 1987; Huang et al., 2005; Pryor et al., 1993; Yu, Liu, & Liu, 1999), while other reactions are ignored, because of their presumed negligible contribution to the overall reaction rate.

2. Materials and methods

2.1. An oxygen-tight cell

The kinetics of oxygen depletion are measured in an unbiased manner if they are referred to the uncontaminated initial reserve of oxygen, with no or negligible intrusion from the atmosphere during monitoring. For this reason, we used a home-made thermostated cell, built as tightly as possible. The cell has a volume of 2.3 mL and it is provided with a magnetic stirrer. The Clark electrode (Clark, Wolf, Granger, & Taylor, 1953) (MI-730 oxygen electrode, from Microelectrodes, Inc., Bedford, NH, USA) is inserted at the correct depth and tightened with an o-ring. Notwithstanding all the care adopted, the cell is not perfectly airtight. The impermeability to air of the sealed cell was checked by monitoring oxygen recovery after its complete removing and recovery is found to be no greater than 1% h^{-1}, decisively smaller than the error relative to the determination of the initial rates and then negligible.

2.2. Experimental conditions and tested antioxidants

A solution of 50 mM SDS and 10 mM LH in sodium phosphate buffer, pH 7.4, obtained using Milli-Q water, thermostated at 37 °C, was left in equilibrium with atmospheric oxygen for 30 min. Subsequently, in order, the selected concentration of the antioxidant in the range from 0.5×10^{-3} to 5×10^{-3} mM and the radical initiator InNNIn (in our case ABIP, 2,2′-azobis[2-(2-imidazolin-2-yl)propane) at 4 mM, as last reagent, were added. In the initiation step *a*, the thermal decomposition of the radical initiator InNNIn generates a steady amount of the radical In·, reaction *a1*. Many different radical initiators have been proposed, and some discussion has been offered (Pryor et al., 1988; Yu et al., 1999) regarding the mechanism and the rate of radical generation. ABIP is largely used and the decomposition parameters are well known (Hanlon & Seybert, 1997; Laguerre, Lecomte, & Villeneuve, 2007; Li, Yeo, & Tan, 2000; Zennaro et al., 2007). We chose ABIP for its practicalness and reproducibility of the results, also.

2.3. Measurement of the O_2 consumption rate

Oxygen consumption was monitored by the Clark electrode reported above. The working electrode was set at −800 mV vs. Ag/AgCl and current was translated by a home built amperometric stand. The output was digitalized every 1 s by a data acquisition board and stored in a personal computer. The oxygen concentration was then given in arbitrary units, as $[O_2]_{au}$ (Rigo et al., 2000; Zennaro et al., 2007). Antioxidants dissolved in ethyl alcohol were added to the measurement solution and preliminary measurements demonstrated that the little aliquot of ethyl alcohol used to this purpose did not affect the experimental data. All measurements are in triplicate and standard deviations have been calculated. Pedex 0 and pedex A specify measurements carried out in the absence and in the presence of the investigated antioxidant, respectively.

3. Results and discussion

3.1. Choice of the antioxidants and analysis of the oxygraphic traces

In the present work 31 molecules, principally polyphenols, have been tested, for which different antioxidant characteristics in literature are reported (Table 2). Of these, nine have been utilized to investigate the mechanisms of the antioxidant activity: a-tocopherol, chromanol, trolox, BHT, lauryl gallate, resveratrol, pyrogallol, l-ascorbic acid and gallic acid. The choice of these particular compounds was suggested by the following different molecular characteristics, in order to verify the contribution of specific functional groups or chemical structure or physicochemical characteristics to the overall antioxidant properties:

- the different class of compounds: chromanols (tocopherol, chromanol and trolox); stilbenes (resveratrol); phenols (BHT, pyrogallol); gallic acid derivatives (gallic acid, lauryl gallate); ascorbic acid. Their different chemical structures can in different way delocalize the unpaired electron;
- the different hydrophilicity which affects the chemical compatibility with the SDS lipid layer (see, e.g. tocopherol, very liposoluble, and ascorbic acid, very hydrosoluble, but also tocopherol and chromanol, the second relatively soluble, or resveratrol and piceid, this last differing from resveratrol for a very soluble glycoside unit);
- the presence in the molecular structure of long aliphatic chains which can facilitate the insertion of the antioxidant molecule into the micellar lipid moiety (see, e.g. tocopherol and chromanol or lauryl gallate and pyrogallol);