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Analytical criteria to quantify and compare the antioxidant and pro-oxidant capacity in competition assays: The bell protection function

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ABSTRACT

The development of a convenient mathematical application for testing the antioxidant and pro-oxidant potential of standard and novel therapeutic agents is essential for the research community and food industry in order to perform more precise evaluations of products and processes. In this work, a simple non-linear dose–time tool to test the effectiveness of compounds for competitive assays is presented. The model helps to describe accurately the antioxidant and pro-oxidant response as a function of time and dose by two criteria values and allows one to perform easily comparisons of both capacities from different compounds. The quantification procedure developed was applied to two well known in vitro competition assays, the β-carotene and crocin bleaching asymptotic reactions. The dose–time dependency of the response of commercial antioxidants and some expected pro-oxidant compounds was evaluated in this study and the results showed low experimental error. In addition, as an illustrative example of the capabilities of the criteria proposed, the quantification of the combined effect of an antioxidant and a pro-oxidant was analyzed. Afterwards, the model was verified for other relevant competitive methods, using available experimental data from the bibliography. Its application is simple, it provides parametric estimates which characterize the response, and it facilitates rigorous comparisons among the effects of different compounds and experimental approaches. In all experimental data tested, the calculated parameters were always statistically significant (Student's *t*-test, $\alpha = 0.05$), the equations were consistent (Fisher's *F*-test) and the goodness of fit coefficient of determination was higher than 0.98.

1. Introduction

Antioxidants and pro-oxidants are compounds that can delay or accelerate oxidation processes. Living organisms have developed a complex network (Kalyanaraman, 2004) of antioxidants (enzymes such as superoxide dismutase, catalase, glutathione peroxidase or non-enzymatic compounds such as uric acid, bilirubin, albumin, metallothioneins); they are essential for a healthy life in order to counteract various harmful (Hussain, Hofseth, & Harris, 2003) pro-oxidants or reactive species (i.e. O_2, H_2O_2, $ROO^{\bullet}$, $OH^{\bullet}$). Apart from these endogenous antioxidants, there are exogenous ones that can derive from natural sources (vitamins, flavonoids, anthocyanins, some mineral compounds), or from synthetic compounds (such as butylhydroxyanisole, butylhydroxytoluene, etc.). There are also exogenous compounds such as metal ions that can promote or accelerate the oxidation processes (Carocho & Ferreira, 2013). Clinical trials and epidemiological studies have established an inverse correlation between the intake of natural exogenous antioxidants and the occurrence of oxidative stress diseases such as inflammation, cardiovascular problems, cancer, and aging-related disorders (Gutteridge & Halliwell, 2010). Thus, the analysis of natural antioxidants for disease prevention (Chatterjee, Poduval, Tilak, & Devasagayam, 2005; Notas et al., 2005) and the identification of possible pro-oxidant substances have become topics of increasing interest.

Several in vivo and in vitro methods have been developed for determining the total antioxidant and pro-oxidant (oxidation modifiers, OM) capacity of compounds. The capacity of OM is frequently determined in competition assays, in which the OM and indicators of the reaction (in general another OM) compete for the reactive species. Competition assays are performed to describe OM capacity and to rank the affinity of OM to counteract or increase the action of reactive species against an indicator. In general, these assays differ in the mechanism of generation of different radical species and/or target molecules and in the way end-products are measured. At present, there is no convenient assay that enables the evaluation of the OM capacity (Halliwell, 2013; Naguib, 2000; Tsuchihashi, Kigoshi, Iwatsuki, & Niki, 1995) for different compounds. The current methods used to test the OM capacity still have left many open questions (Frankel & Meyer, 2000; Halliwell, 2012). The in vitro assays can only rank OM capacity for their particular reaction system and their relevance to in vivo activities is uncertain. Thus, it is logical that in the last decade, researchers have claimed

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unity of the approaches (Frankel & Finley, 2008; Murado & Vázquez, 2010) and have tended to standardize the protocols to increase the effectiveness of methods for in vitro and in vivo responses (Dawidowicz & Olszowy, 2010; Frankel, 1993, 1994; Ordoudi & Tsimidou, 2006; Prior & Cao, 1999; Prior, Wu, & Schaich, 2005).

Additionally, the arbitrary use of simple analytical procedures to calculate molecular properties, occasionally without a validation study, as well as a lack of statistical significance, has caused much controversy (Frankel, 1993, 1994; Huang, Boxin, & Prior, 2005; Koleva, Van Beek, Linssen, De Groot, & Evstatieva, 2002; Laguerre, Lecomte, & Villeneuve, 2007; Naguib, 2000; Roginsky & Lissi, 2005). Commonly, the mathematical determinations of the OM capacity are based on a fixed endpoint without proper considerations of the kinetic behavior. The most typical and incorrect practice is to use the single-time dose–response of one commercial OM as a calibration curve (normally focusing on the linear range), and afterwards to compute the equivalent OM capacity of any type of sample by testing it only at one single-time–dose, assuming too many false aspects as true.

In the current study, a simple non-linear mathematical application for competitive OM assays, in which the responses have one common asymptote (majority of ones) is presented. It helps to describe accurately the response as a function of time and dose by two criteria values and facilitates convenient comparisons of the capacity of different compounds. The model was validated in well known in vitro competition assays, evaluating the dose–time-dependency of the response of OM compounds.

2. Material and methods

2.1. β-Carotene bleaching method

The protocol has been recently revised and improved (Prieto, Rodríguez-Amado, Vázquez, & Murado, 2012). The reagent is prepared by dissolving 4 mg of β-carotene (βC), 0.5 mL of linoleic acid and 4 g of Tween-40 in 20 mL of chloroform. In aliquots of 1 mL, the solution was distributed into 30 mL tubes, and the chloroform was evaporated simultaneously in all of them in a rotary evaporator (40 °C/~15 min), adapted to work with multiple tubes. The resulting oily residue was washed with N_2 and stored at −18 °C. At the time of use, a tube provides sufficient reagents for 120 samples by adding 30 mL of buffer Briton 100 mM, pH = 6.5 in Milli-Q water at the reaction temperature (45 °C). The absorbance at 470 nm of the reagent thus prepared is ~1.4, stable for a week and the specific value should not be corrected for dilution. The concentration of βC in the final solution of the reaction is 1 μM.

The procedure is performed by adding 50 μL of sample and 250 μL of reagent into the wells (330 mL) of a microplate of 96 units (it is advisable to use a multichannel pipette). The device is programmed to 45 °C with agitation for reading only interrupting at intervals of 3, 5 and 10 min (initiation, propagation and asymptotic phase), during a period of 200 min. The *OM* standards and samples are analyzed kinetically for different doses. Under these conditions the method can be applied to analyze antioxidants and pro-oxidants separately or even simultaneously.

2.2. Crocin bleaching assay

Recently, the protocol has been revised and its quantification procedure improved and transferred to microplate readers (Prieto, Murado, & Vázquez, 2014; Prieto, Murado, Vázquez, Anders, & Curran, 2013) The reagent is prepared by dissolving Cr (5 mg; 125 μM in the final reaction) and AAPH (75 mg; 7.68 mM in the final reaction) in 25 and 5 mL, respectively, of 100 mM Briton buffer, pH = 5.5, in Milli-Q water at 40 °C. To avoid any initial degradation, both solutions must be prepared and mixed just before use. The absorbance at 450 nm of the mixture (~1.4) is very dependent on the origin and conservation state of Cr. The concentration of Cr in the final solution of the reaction is 100 μM. When applying the method to analyze pro-oxidants the AAPH compound must not be included in the reagent preparation, all other conditions are maintained.

Each well of a preheated (37 °C) microplate (96 wells, 350 μL) contains 250 μL of reagent, and 50 μL of sample solution in water:ethanol (9:1). The apparatus was programmed for 200 min at 37 °C, with agitation at 660 cycles/min (1 mm amplitude), only interrupted for readings at intervals of 3, 5 and 10 min (initiation, propagation and asymptotic phase).

2.3. Standard OM compounds for an illustrative analysis

2.3.1. Antioxidants

(a) Butyl-hydroxyanisole (BHA): a synthetic food additive (E320) mainly used as an antioxidant and preservative. Its known capacity is suitable in lipophilic and hydrophilic environments.
(b) Butyl-hydroxytoluene (BHT): a synthetic lipophilic (fat-soluble) organic compound, chemically a derivative of phenol, that is useful for its antioxidant properties. It is primarily used as a food additive (E321).
(c) Propyl 3,4,5-trihydroxybenzoate or propyl gallate (PG): an antioxidant that has been added to foods containing oils and fats to prevent oxidation (E310).
(d) (2R)-2,5,7,8-tetramethyl-2-[(4R,8R)-(4,8,12-trimethyltridecyl)]-6-chromanol or α-tocopherol (TOC): a natural fat-soluble organic compound (E306) consisting of various methylated phenols (a type of tocopherol or vitamin E), that is useful for its antioxidant properties.
(e) 6-Ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline or ethoxyquin (ETX): commonly used as a food preservative (E324) in pet foods to prevent the rancidification of fats, in spices to prevent color loss due to oxidation of the natural carotenoid pigments and as a pesticide.
(f) L-Hexuronic acid (vitamin C) or Ascorbic Acid (AA): a naturally occurring hydrosoluble organic compound with antioxidant properties. Ascorbic acid and its sodium, potassium, and calcium salts are commonly used as antioxidant food additives (E300–304)
(g) Tert-Butylhydroquinone (TBHQ): It is a derivative of hydroquinone, substituted with tert-butyl group. TBHQ is a highly effective antioxidant in foods (E319). It is added to a wide range of foods, with the highest limit (1000 mg/kg) permitted for frozen fish and fish products.
(h) Manganese sulfate (Mn^{+2}): a required trace mineral for all known living organisms, also extensively present as possible interference in salts may be able to act as a metal chelator (e.g., iron-sequestrants) and inhibit Fenton-type reactions that produce hydroxyl radicals through complexation/chelation reactions.
(i) 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Tr): A water-soluble analog of vitamin E used in biological or biochemical applications to reduce oxidative stress or damage.

The concentration ranges in μM of the antioxidants used for the βC reaction are: BHA: 0(0.5)–5, BHT: 0–(3)–30, ETX: 0–(0.0004)–0.004, TOC: 0–(0.004)–0.04, and PG: 0–(8)–80. The concentration ranges in μM of the antioxidants used for the Cr reaction are: AA: 0–(30)–300, ETX: 0–(3)–30, Tr: 0–(15)–150, TBHQ: 0–(80)–800, and Mn^{+2}: 0–(12.5)–125. All compounds were purchased from Sigma S.A. (St. Louis, MO, USA).

2.3.2. Potential pro-oxidant agents

(a) Iron (II) sulfide (Fe^{+2}): much attention has been paid to its oxygen complexes (ferryl and perferryl radicals) in the food industry as they are considered as primary catalysts (initiators) of lipid peroxidation in meat products and others that contain lipids.
(b) Porcine Hemoglobin (Hb) in reduced form (Fe^{+2}): the iron-containing oxygen-transport metalloprotein in the red blood