

A new microplate procedure for simultaneous assessment of lipophilic and hydrophilic antioxidants and pro-oxidants, using crocin and β -carotene bleaching methods in a single combined assay: Tea extracts as a case study

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ABSTRACT

β -carotene and crocin bleaching reactions are the basis of two methods extensively used to quantify antioxidant and pro-oxidant activities. They are appropriate for lipophilic and hydrophilic matrices, respectively, and can provide useful complementary information in the study of complex natural extracts containing components with variable degrees of polarity. In this regard, a microplate procedure (Carotene Combined Bleaching) is proposed that enables the combination of both methods in a single, informative and less expensive method which is also faster to carry out. As an illustrative model, the method was applied to test a set of commercial lipophilic and hydrophilic antioxidants and some predictable pro-oxidant agents. Afterwards, as a food compound case study, the antioxidant activity of five types of tea

extracts (Green, Blue, White, Black and Red) were characterized and their equivalent potential activity was calculated using commercial antioxidants on the basis of the new procedure developed in this research. The activity of the tea extracts decreased in the following order: (a) In a predominantly lipophilic environment: White > Black > Red > Blue > Green tea extracts; and (b) In a predominantly hydrophilic environment: Green > Red > White > Black > Blue tea extracts.

Keywords: antioxidant activity; β -carotene method; crocin bleaching method; microplate analysis; antioxidant activity of tea extracts; non-linear responses; mathematical modeling.

1. INTRODUCTION

Antioxidants and pro-oxidants (hereafter oxidation modifiers: *OM*) are chemical entities that interfere with the oxidation of a given substrate through a certain mechanism in a specific chemical environment. The nature of the *OM*, the substrate and the environment, as well as the involved mechanism, are factors which can perturb significantly the profile of the process (Karen Schaich, 2004; Laguerre, Lecomte, & Villeneuve, 2007). Since all the combinations of these factors can be provided in diverse quantitative modalities by the actual conditions, it is not expected to find a universal method capable of assessing *OM* activities independent from the system under study. This is one of the reasons for the proliferation of procedures that has taken place in recent years, which has led to repeated calls by the research community for: 1) methodological standardization (Dawidowicz & Olszowy, 2010; Frankel, 1993; Frankel, 1994; Ordoudi & Tsimidou, 2006; R. L. Prior, Wu, & Schaich, 2005; R. L. Prior & Cao, 1999; Sharma & Bhat, 2009; Sánchez-Moreno, 2002); and 2) unified criteria for data

processing (Frankel & Meyer, 2000; Frankel & Finley, 2008; Hamilton, Kalu, Prisk, Padley, & Pierce, 1997; Huang, Boxin, & Prior, 2005; Karen Schaich, 2004; Murado & Vázquez, 2010a; Niki, 2010; Roginsky & Lissi, 2005). In addition, variables such as the environmental polarity of protocols applied to test the activity of *OM* has led to questionable conclusions. The so called “polar paradox” (Frankel, Huang, Kanner, & Bruce German, 1994; Koleva, Van Beek, Linssen, De Groot, & Evstatieva, 2002) and the "apolar paradox" causes different behavior of an *OM* in different environments.

β -carotene (Marco, 1968) and crocin bleaching (Bors, Michel, & Saran, 1984) reactions are the basis of two methods extensively used to quantify antioxidant and pro-oxidant activities. The respective protocols have been repeatedly revised and improved. At present, they are well optimized (Prieto, Rodriguez-Amado, Vazquez, & Murado, 2012; Prieto, Vazquez, & Murado, 2013). Their execution shares the main conditions and operative requirements, which make their simultaneous achievement in a microplate reader possible. They are appropriate for lipophilic and hydrophilic matrices, respectively, and can provide useful complementary information in the study of complex natural extracts containing components with variable degrees of polarity. β -carotene is a lipophilic oxidizable substrate, especially sensitive to *OM*. By means of hydrophobic repulsion, it is able to join the system of lipidic micelles and the corresponding oxidation reaction is accomplished in a lipidic environment. Hydrophilic antioxidants, even powerful ones, produce a very low response in such systems (polar paradox). On the contrary, crocin is a hydrophilic oxidizable substrate, and lipophilic *OM*, even powerful ones, produces very low responses in the aqueous systems (apolar paradox).

The goal of this document is to report a new combined procedure for microplate readers, called the Carotene Combined Bleaching (CCB) assay, to assess the antioxidant and pro-

oxidant activity in lipophilic and hydrophilic environments in one single procedure. Therefore, in the assessment of any complex matrix containing an *OM*, the new combined procedure, represents a powerful informative tool, defining the basic characteristics and compound activity in two equal, but different systems in which the polar properties can be effectively revealed. The new combined microplate method of β -carotene and crocin bleaching reactions in one single assay, facilitates a less expensive procedure which is also less time consuming. Furthermore, the application of robust mathematical modeling produces consistent and meaningful criteria for the comparative characterization and activity quantification of any *OM*, taking into account dose-time-dependent behavior with a low experimental error. The method was tested, in an illustrative model of lipophilic and hydrophilic commercial antioxidants and some predictable pro-oxidant agents.

Tea is an aromatic beverage frequently prepared by pouring boiling hot water over cured leaves of the evergreen tea bush *Camellia sinensis* (Perva-Uzunalić, Škerget, Knez, Weinreich, Otto, & Grüner, 2006). The thousands of different varieties of teas available around the world only vary in their region of growth, the time of year picked, and the processing method. Tea is the second most consumed beverage after water and its water extracted compounds present different degrees of polarity (Chan, Lim, Chong, Tan, & Wong, 2010), therefore, it was considered as an excellent food case study. It has been argued that the consumption of herbal teas is beneficial for health; among others, for its antioxidant activity, mainly due to the presence of natural antioxidants such as vitamins (mainly A, B6, C and E), polyphenols (xanonoids, xavanols, xavonols, isoxavones, quercetin, catechin, epicatechin, etc.), co-enzyme Q10, carotenoids, selenium, zinc and phytochemicals (Abdullin, Turova, & Budnikov, 2001; Sakanaka, Tachibana, & Okada, 2005; Vinson & Dabbagh, 1998). In this article, the activity of five of the most common tea varieties were characterized and their

equivalent potential activity with commercial antioxidants were rigorously calculated using the carotene combined bleaching assay (CCB) developed in this research.

2. MATERIAL AND METHODS

2.1. Brief introduction of the kinetic methods merged to develop the carotene combine bleaching method

2.1.1. β -Carotene bleaching method

The method described by Marco (1968) is the reference for many subsequent modifications that simplified the operation (Miller, 1971) or transferred the procedure to microplate (Koleva et al., 2002). The method works in an aqueous emulsion of linoleic acid and β C, which is discolored by the radicals generated by the spontaneous oxidation of the fatty acids (Huang et al., 2005), promoted by thermal induction, typically at 45°C, and spectrophotometrically followed at 470 nm. The protocol has been recently revised and improved (Prieto, Rodriguez-Amado, Vazquez, & Murado, 2012).

2.1.2. Crocin bleaching assay

The assay, proposed by Bors et al. (1984), uses crocin as an oxidizable substrate and AAPH (2,2'-azobis-2-amidinopropane) as the source of radicals. The antioxidant to be tested competes with crocin for the produced radicals, and the bleaching rate of crocin is spectrophotometrically followed at 450 nm at 37°C. This assay can be classified within those assays that interfere with the transfer of one hydrogen atom. It is a suitable method for

aqueous systems, producing very consistent results. The original method has been modified several times by simplifying its protocol (Tubaro, Micossi, & Ursini, 1996), transferring it to microplate assay (Lussignoli, Fraccaroli, Andrioli, Brocco, & Bellavite, 1999), applying it in lipophilic environments (in this case using AMVN: 2,2'-azobis-2,4-dimethylvaleronitrile as a radical source), and adapting it to the measure of pro-oxidant activities (Manzocco, Calligaris, & Nicoli, 2002). Recently, the protocol has been revised and its quantification procedure improved (Prieto, Vazquez, & Murado, 2013).

2.2. Standard agents for an illustrative analysis

Lipophilic and hydrophilic commercial antioxidants and some predictable pro-oxidant agents are presented here for an illustrative analysis of the full capabilities of the developed method.

2.2.1. Commercial antioxidants

Four of the most common antioxidants are used as an example including natural and synthetic antioxidants:

- (a) butyl-hydroxyanisole (BHA): a synthetic food additive (E320) mainly used as an antioxidant and preservative. Its known activity 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) 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox): a synthetic water-soluble antioxidant analog of α -tocopherol, used in biological or biochemical applications to reduce oxidative stress or damage. It is also one of the most common standards used to quantify the equivalent potential activity of samples in many *in vitro* tests.
- (d) (2R)-2,5,7,8-tetramethyl-2-[(4R,8R)-(4,8,12-trimethyltridecyl)]-6-chromanol (α -tocopherol): 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.

2.2.2. Potential pro-oxidant agents

On the other hand, numerous agents such as transition metals can directly or indirectly catalyze the oxidative mechanisms in both lipophilic and hydrophilic environments. As a possible example of pro-oxidant activity, some transition metals are selected to test the method proposed. The effects on different systems is not less relevant than those of commercial antioxidants, since they can be present, either as constituents or contaminants, in many extract materials and as traces in buffer salts, distorting the results:

- (a) Iron (II) sulfide (Fe^{+2}): much attention has been paid to its oxygen complexes (ferryl and perferryl radical) in the food industry as they are considered as primary catalysts (initiators) of lipid peroxidation in meat products and others that contain lipids. They are generally present in crude biological extracts and traces in buffer salts.
- (b) Manganese sulfate (Mn^{+2}): a required trace mineral for all known living organisms, also extensively present as possible interference in salts.

- (c) Copper (II) sulfate (Cu^{+2}): an essential trace nutrient to all higher plant and animal life, also widely present in biological extracts, water and as possible interference in salts.
- (d) AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride): a hydrophilic chemical compound used to study the chemistry of the oxidation of drugs or the capabilities of antioxidants in different methods to counteract the two radicals produced after its degradation by thermal induction.

All commercial antioxidants and chemicals reagents in this study were purchased from Sigma S.A. (St. Louis, MO, USA).

2.3. Food agents as an antioxidant case study - Tea extracts

2.3.1. Compound extraction and preservation of tea extracts

A set of five loose unblended tea samples, free of additives (especially the antioxidant ones), were purchased from local retail shops (all of them from China and harvested in 2011): (A) Green tea (China Sencha); (B) Blue tea (China Oolong Natural); (C) White tea (Silver Needles); (D) Black tea (Darjeeling Margaret's Hopè); and (E) Red tea (Natural red tea).

Leaf teas were ground to obtain a homogeneous fine powder. Four hot-water consecutive extractions with 250 mL of distilled water at 85 °C for 30 min were applied to 20 g of each tea (obtaining a final volume 1L). The extracted material was centrifuged several times and the supernatant was filtered through Whatman paper filters, lyophilized and preserved at -16°C (Almajano, Carbó, Jiménez, & Gordon, 2008; Perva-Uzunalić, Škerget, Knez, Weinreich, Otto, & Grüner, 2006).

2.3.2. Common compositional analysis

- Total sugars (T_S): Phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956; Strickland, J.D.H., Parsons, T.R., 1968), with glucose as standard.
- Reducing sugars (R_S): were calculated following the 3,5-Dinitrosalicylic acid (DNS) reaction (Bernfeld P, 1951), with glucose as standard.
- Total protein content (Prot): measured as a function of the total nitrogen (Havilah, E.J., Wallis, D.M., Morris, R., Woolnough, J.A., 1977) present multiplied by 6.25.
- Suspended solids (S_S) and ash (Ash): Their determination were performed gravimetrically in crucibles; first a water evaporation at 60°C, followed by temperature treatment at 105 °C (24 h) in an oven (Suspended solids). Then, the same crucibles were transferred to a furnace and treated at 550 °C for at least 24 h (Ash).
- Determination of total phenolic (TP) content: TP content of tea extracts was determined using the Folin–Ciocalteu reagent according to the modified method of Singleton & Rossi (1965) using gallic acid as standard. The tea solution (1 mL, ~0.06 mg/mL) was mixed vigorously with 0.1 mL of Folin–Ciocalteu reagent, 1.0 mL of an aqueous solution of 7% Na_2CO_3 and 30 min after the absorbance was measured at 765 nm. The results are expressed as mM gallic acid/g dry matter of extract.
- Determination of the total flavonoid (TF) content: TF content was determined following the procedure described by Zhishen et al. (1999). A solution of tea samples (1 mL, ~0.06 mg/mL) was added to a $NaNO_2$ solution (0.5 mL, 2.5%). After 5 min, an $AlCl_3$ solution (0.5 mL, 5%) was added, after another 5 min a NaOH solution (0.5 mL, 1 M) was added and the absorbance was measured at 510 nm. TF amounts were expressed as μg catequin/g dry matter of extract.

All tests were performed in triplicate and the results of extraction percentages and compositional analysis are presented in Table 1.

2.4. Numerical and statistical methods

Fitting the experimental results to the proposed equations was carried out in two phases. First, parametric estimates were obtained by minimization of the sum of quadratic differences between observed and model-predicted values, using the nonlinear least-square (quasi-Newton) method provided by the macro *Solver* in *Microsoft Excel 2003*, which allows quick testing of hypotheses and display of its consequences. Next, the determination of the parametric confidence intervals and model consistency (Student's *t* and Fisher's *F* tests, respectively, in both cases with $\alpha=0.05$) were calculated using the '*SolverAid*' (Prikler, 2009) macro previously used (M. A. Prieto, Vázquez, & Murado, 2011). The '*SolverStat*' macro (Comuzzi, Polese, Melchior, Portanova, & Tolazzi, 2003) was used for detecting possible anomalies in the distribution of parametric estimates and residuals. Bias (B_f) and accuracy (A_f) factors of all equations were calculated (Ross, 1996; Vázquez, Durán, Rodríguez-Amado, Prieto, Rial, & Murado, 2011):

$$A_f = \frac{\sum \log\left(\frac{pred}{obs}\right)}{n} \quad \text{and} \quad B_f = \frac{\sum \log\left(\frac{obs}{pred}\right)}{n} \quad [1]$$

where *pred* and *obs* are the predicted and experimental values, respectively, and *n* is the number of observations. The closer the values of B_f and A_f are to 1, the better is the fitting of the experimental data to the model.

3. RESULTS

3.1. Development of the carotene combined bleaching method

The carotene combine bleaching method is a new microplate procedure for the simultaneous assessment of lipophilic and hydrophilic antioxidants and pro-oxidants, using the crocin and β -carotene bleaching methods in a single combined assay. The execution protocols share the main conditions and operative requirements, which make their simultaneous achievement in a microplate reader possible. The main conditions, operative requirements and quantification procedure for the carotene combined bleaching method (CCB) in a microplate reader are described next.

3.1.1. Reagents

- a) *Crocin bleaching reagent*: Crocin (4 mg; 100 μ M in the reaction mixture) and AAPH (75 mg; 7.68 mM in mixture) were dissolved in 25 mL and 5 mL of Mili-Q water, respectively. To avoid any initial degradation, both solutions must be prepared and mixed just before use. When testing pro-oxidants, AAPH must be omitted, because the crocin reaction in the absence of AAPH is itself an appropriate method for assessing pro-oxidant activities.
- b) *β -Carotene bleaching reagent*: 4 mg of β -Carotene, 0.5 mL of linoleic acid and 4 g of Tween-40 were dissolved in 20 mL of chloroform. The solution was distributed in aliquots of 1 mL in 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 N₂ and stored at -18°C (stable for one week). A single tube provides sufficient reagent for a microplate by adding 30 mL of Mili-Q water.

3.1.2. Assay conditions

The assays, routinely applied in the determination of the *OM* activities, are normally conducted with minimal calculation requirements leading to the misinterpretation of the effect of some factors such as oxidant concentration (linoleic acid or AAPH), pH, and temperature, among others; in some cases, leading to an over-standardization of the protocol or in other situations overlooking the important aspects that need to be standardized. However, in both cases, these factors have been rigorously revised and the protocols improved, and at present, they can be considered as highly optimized (Prieto, Rodriguez-Amado, Vazquez, & Murado, 2012; Prieto, Vazquez, & Murado, 2013). The information provided, made it possible to find the experimental conditions suitable to perform both methods in one single assay. However, a compromise is needed between the optimum conditions for both methods to obtain reliable results in a single microplate, in such a way that the respective sensitivities are not significantly affected. A satisfactory agreement is reached by working with the following conditions:

1. Temperature: 40°C.
2. Wavelength: 460 nm.
3. pH of both reagents: 5.5 (100 mM citrate buffer).
4. AAPH concentration in crocin reagent: 4.42 mM (the value is adjusted to achieve a radical release rate equivalent to that which takes place at the usual working temperatures: 35-37°C).

In both reactions, the final absorbance (at their respective wave length) prepared was ~1.4 and this value in any case must not be corrected by dilution.

3.1.3. Microplate procedure

- The experimental procedure is carried out using a preheated (37°C) plate (350 µl in a 96-Well polypropylene microwell plate with flat bottom) containing 50 µl of a sample and 250 µl of the appropriate reagent in each well. The reagent solutions must be dispensed just before use. The use of multi-channel pipettes is recommended.
- In the experience of the authors, the analytical time of 200 min always provided highly reproducible results without undesirable consequences such as evaporation and bleaching processes. Short analysis times (~50 min) lead to experimental error, while longer times (~500 min) favor solvent evaporation and natural thermal discoloration of crocin.
- The reaction was maintained with constant shaking at 660 cycles/min (1 mm amplitude), only interrupted for readings at 3, 5 and 10 min intervals (initial, propagation and asymptotic phases).
- For each sample tested, a dose response of a minimum of 8 concentrations must be freshly prepared. Water:ethanol (9:1) solutions help to dissolve different components.
- Besides the dilution series of the assayed samples, the microplate must include: 1) a calibration series for each method, in which sample dilutions are replaced by those of reference OM; 2) two or three wells (blank) in which samples are replaced by their solvents.
- A typical distribution of a microplate with each series in duplication, enabling the simultaneous analysis of two samples by both methods, is exemplified in Figure 1. Note, in order to avoid as much as possible the error produced by the temperature gradient in the

microplate reader, almost all the rows and columns around the plate were used as the control.

3.1.4. Quantification

Until recently, in both methods, the quantification procedure was simply assessed once each time, and often the conditions were forced to assume a linear kinetic, causing an important loss of information and the risk of erroneous conclusions.

However, recently (Murado & Vázquez, 2010) and subsequently (Prieto, Rodriguez-Amado, Vazquez, & Murado, 2012) proposed a more robust approach to quantify the results. In which it is assumed that S_0 and S_t are the concentrations of oxidizable substrate (β -carotene or crocin) at 0 and t times, the time-course of the oxidative response, defined as $R=1-(S_t/S_0)$, can be described with the Weibull equation (Weibull, 1951), reparametrized in the following form:

$$R(t) = K \left[1 - \exp\left(-\left(\frac{t}{\tau}\right)^\alpha\right)\right]; \text{ briefly: } R(t) = K \left(1 - e^{-\left(\frac{t}{\tau}\right)^\alpha}\right) \quad [2]$$

where K is the asymptote, τ the substrate half-life, or time when 50% of oxidation is achieved, and α a shape parameter related to the maximum slope of the response (r_m) that can be computed through:

$$r_m = \frac{K}{\tau} \left(\frac{1}{\tau}\right)^{\alpha-1} e^{-\left(\frac{1}{\tau}\right)^\alpha}; \text{ being: } G = \frac{\alpha-1}{\alpha} \quad [3]$$

In an open system, it can be accepted that exhaustive substrate oxidation is reached at sufficient time, and therefore $K=1$. The other two parameters (α and τ) will vary in the presence of any OM and, given their well-defined factual meanings regarding the oxidation kinetics, their variations have a relevant characterizing value. In general, the variation of any parameter θ as a function of the OM concentration can be adjusted to a hyperbolic function:

$$\tau_{\theta} = \frac{1+a_{\theta} \cdot OM}{1+b_{\theta} \cdot OM} \quad [4]$$

where a_{θ} and b_{θ} are fitting coefficients. Often one of them is zero, indicating that the parametric value is directly or inversely proportional to the concentration of the modifier.

Thus, the entire set of kinetic profiles can be determined simultaneously by including [4] into [2], obtaining the following bivariate equation:

$$R_{CO_2} = \frac{1+a_{\theta} \cdot OM}{1+b_{\theta} \cdot OM} \cdot \frac{1}{1+K \cdot OM} \quad [5]$$

This enables a robust characterization in which the effects of the experimental error are minimized as stated previously by many authors (De Lean, Munson, & Rodbard, 1978; M. A. Prieto, Vázquez, & Murado, 2012).

3.1.5. Comparison criteria and mechanistic inferences

The equation [4] used to insert the action of OM into [2], lacks individual meaning of the coefficients a_{θ} and b_{θ} , but jointly they define a term as a function of the concentration of a given OM. In fact, it is able to describe accurately increasing and decreasing tendencies, with

linear and –asymptotic or not– hyperbolic shape. It allows quantification of the variations of the kinetic profiles which characterize the different OM types in a useful way that can provide even indications concerning modes of action. On the other hand, the combination of equations [2] and [4] represents an especially robust tool. The use of other two parameter functions (Giese and Esterbauer, 1994), in which their parametric values will immediately explain intrinsic consequences, is also achievable with the model equation [4]. When using these other equations, whose parametric values are more informative, if any of them is not statistically significant, –because, as example, for the lack of information on the profile obtained (normally related with the asymptote)– will lead to reject the model and therefore, it would be needed to select a different approach. However, when using equation [4], simply if any of its parameters (a or b) is not statistically significant, by rejecting it, the analysis and fitting procedure could be continuous without altering equation [5]. Indeed, if $a=0$ or $b=0$, the equation would be a linear one (increasing or decreasing respectively) and if $a \neq b \neq 0$, the function would be a hyperbolic one. Thus, with the same expression one can focus on the profile defined by the specific action of the *OM* and not on its parameters.

Consequently, a meaningful way to compare *OM* activities consists of plotting the specific variation of the half-life ($H\tau$), given by equation [4] (using the parameters obtained in equation [5]) as a function of the agent concentration. This can provide fixed values, as concentration that doubles the half-life (antioxidants) or reduces it by half (pro-oxidants), and more interestingly when $H\tau$ is a non-linear term, allows the visualization of the agent-specific dynamics of these effects (see figure 1B). Therefore, by using this specific half-life extension (for antioxidants) or reduction (for pro-oxidants), non-linear characterization by the parameter behavior of $H\tau$ describing the oxidation process in three different effects as a function of the sample concentration can be found: Antioxidant (A), when the parameter increases; Pro-

oxidant (P), when the parameter decreases; and Null (N), when the parameter keeps constant. Afterwards, the antioxidant and pro-oxidant effects (see figure 1B) can be divided into three different types of mechanistic behavior as a function of the relative changes of $H\tau$ at as function of the slope variation, being: constant (A1 and P1), decreasing (A2 and P2) and increasing (A3 and P3).

3.2. Illustrative application to assess lipophilic and hydrophilic commercial antioxidants and some potential pro-oxidant agents

As a first general result, none of the bleaching kinetics of the tested compounds promoted, in the absence of linoleic acid or AAPH, significantly differ from the control. This suggests that in all cases the activity (anti or pro-oxidant) was not related to βC or crocin bleaching, but to the radical production.

In Table 2 and Figure 2, the results of the proposed approach are presented, applied to four well-known antioxidants (BHA, BHT, α -tocopherol and Trolox), and four potential pro-oxidant agents (Fe^{+2} , Mn^{+2} and Cu^{+2}).

For all the assayed agents, statistically significant descriptions, with very accurate predictions, were provided by the model [5]. Given the relationship [3], a variation of the half-life (τ) implies a variation with opposite trend in the maximum slope (r_m), when K and α remain constant. In the presence of antioxidants, τ increases and r_m decreases accordingly, the opposite occurs in the presence of pro-oxidants (see also Figure 1B). This variation is general enough to explain the alteration of the kinetic profile due to OM activity. However, changes in α can be found as well, modifying the relationship between τ and r_m . In fact, if an

antioxidant has an affinity to oxygen or radicals much higher than to the substrate, the propagation phase begins with a certain delay, resulting in an increase of α . In addition, a pro-oxidant action can be evidenced with a similar delay if the affinity of the substrate by oxygen or radicals is very high even in the absence of the pro-oxidant agents.

Kinetics of the eight studied agents illustrated, sometimes in a rather surprising way, diverse degrees of these behaviors. Polar and apolar paradoxes are shown. BHT (apolar) and Trolox (polar) showed detectable antioxidant activity only in lipophilic and hydrophilic environments, respectively, whereas BHA and α -tocopherol, with polar groups on apolar molecular bulks, were much more active in lipophilic medium (~180 times in the case of BHA), but maintained a low activity in a hydrophilic environment. Amongst the predictable pro-oxidant agents, similar, although less expected, differences took place. Fe^{2+} acted as a strong pro-oxidant in the lipophilic environment and was inactive at 10 times higher concentrations in the hydrophilic medium. Mn^{2+} behaved in an opposite way, but in both cases showing antioxidant activity (strong in a hydrophilic environment), and Cu^{2+} showed a medium-dependent inversion of its activity, antioxidant and pro-oxidant in lipophilic and hydrophilic environments, respectively.

This illustrative example, clearly demonstrates the capabilities of the CCB assay to discern the lipophilic and hydrophilic activities of a variety of *OM* agents providing useful information in the study of complex natural extracts containing components with variable degrees of polarity. Next, the CCB assay proposed was used for testing the activity of tea extracts.

3.3. Application to assess the antioxidant activity of natural agents - Tea extracts as a case study

As in the previous example, none of the bleaching kinetics of the tested compounds promoted, in the absence of linoleic acid or AAPH, significantly differ from the control. In Table 3 and Figure 3, the results of the proposed approach are presented, as applied to the five most common tea types (A: Green, B: Blue, C: White, D: Black and E: Red tea).

The fitting of results was always satisfactory. The mathematical equations were robust and consistent (p-values < 0.001 from Fisher's F test), the residuals were randomly distributed and autocorrelations were not observed by Durbin-Watson test (data not shown). The statistical analysis, parameter assessment tools and model prediction uncertainties provided by the 'SolverStat' macro agreed accordingly. Furthermore, all the adjusted coefficients of determination R_{adj}^2 between predicted and observed values were always greater than 0.95, with a wide majority of the fittings superior at 0.99. Accuracy and bias factors (A_f and B_f) also indicated the high accuracy and lack of bias of equations used to describe experimental effects of *OM* agents (data not shown).

Beyond quantitative differences, all the tea extracts promote the antioxidant activity in both lipophilic and hydrophilic environments. Based on the behavior of $H\tau$ (Table 3), the activity of the tea extracts decreased in the following order: (a) In a predominantly lipophilic environment: White > Black > Red > Blue > Green tea extracts; and (b) In a predominantly hydrophilic environment: Green > Red > White > Black > Blue tea extracts. The differences were much higher in a lipophilic environment than in a hydrophilic one.

Additionally, no clear links were found with the compositional analysis (Table 1). Although a relationship between antioxidant activity and total polyphenols and flavonoids would not be rare, its absence is not surprising either, since these chemical families can include particular compounds with very different specific powers and capabilities to act in lipophilic or hydrophilic environments.

Finally, the antioxidant effects, from tea extracts and commercial antioxidants tested, show different mechanistic behavior as a function of the relative changes of $H\tau$ being (see Figure 1B and Figure 4) and can be summarized as follow:

-In lipophilic environments: BHA and α -tocopherol proportionally constant (A1); BHA and all tea extracts behave as decreasing (A2); and trolox as a null effect.

-In hydrophilic environments: Trolox and α -tocopherol proportionally constant (A1); BHA and all tea extracts behave as decreasing (A2); and BHT as a null effect.

3.4. Potential equivalent activity of Tea extracts

Several studies have demonstrated that many herbs and medicinal plants have potential preventative effects against oxidative stress (Yusri, Chan, Iqbal, & Ismail, 2012), and a significant number of herbs, spices, cereals, and legumes have been explored as potential sources of antioxidants. Besides the disease-preventative and health-promoting effects of these natural sources of antioxidants, they have profound effects on food preservation, counteracting most common oxidative processes.

Antioxidants are used as food additives to prevent food from deterioration (oxidation) by oxygen and sunlight exposure mainly. Oxidative changes in foods is considered a major

concern for the food industry because it leads to flavor deterioration and loss of nutritional value. Commercial antioxidants such as BHA and BHT are repeatedly used as additives, being an effective strategy for preventing and reducing oxidative changes in foods. Some controversy surrounds the use of commercial antioxidants such as BHA or BHT (among others) in foods (Ito et al., 1986). Carcinogenicity of these compounds has been suggested based on evidence in experimental animals. For example, when BHA is administered in high doses as part of the diet of rats and hamsters, it has caused papillomas and squamous cell carcinomas of the forestomach (Moch, 1986). On the other hand, BHT is not without controversy as there are claimed links to child hyperactivity as well as some specific types of cancer. However, the evidence is unclear and links to such effects have not been demonstrated in humans (Hocman, 1988). Despite their potential risk associated with their use, these antioxidants and others have currently many food industrial uses worldwide.

Consequently, a search for naturally occurring compounds with antioxidant activity has increased dramatically in the past years. However, on many occasions the way of computing the equivalent activity is based on a single concentration, expecting that those single values will be equal at any lower or higher concentration (as a linear behavior). Unfortunately, this pattern only takes place in particular cases, and in the majority of the compounds and especially in mixed samples of natural extracts, the response is non-linear.

In Figure 4, an intuitive solution to compare antioxidant activities of tea extracts versus the commercial antioxidants has been proposed, by plotting the specific extension of the half-life ($H\tau$), given by the expression [4], as a function of their concentration. This graphical representation (the most simple and visual way) to analyze the parametric non-linear response of the antioxidant equivalent action and compare their activity rigorously, provides an easy

tool that facilitates the selection of appropriate concentrations of natural products to replace commercial antioxidants.

Therefore, the nonlinear equivalent responses of natural antioxidant compounds are characterized and compared with commercial substances within the concentration range tested. The activity of tea extracts are presented as a function of the powder material extracted (g/L of the final analytical solution) and the commercial antioxidants are presented as μM and nM for both responses, lipophilic and hydrophilic environments. Thus, the potential equivalent activity can be computed easily and the different mechanistic behavior as a function of the relative changes of $H\tau$ can also be easily identified (see Figure 1B and Figure 4). For example, the following *in vitro* results can be concluded:

- In lipophilic environments: ~ 1 g/L of green tea (A) is equivalent to ~ 150 μM of BHT
- In hydrophilic environments: ~ 0.65 g/L of green tea (A) is equivalent to ~ 265 μM of Trolox.

It is particularly noteworthy to point out that the equivalent potential activity of tea extracts reported in this study is only uncountable for *in vitro* responses. Thus, if any of these natural extracts were required to replace commercial antioxidants, the *in vitro* responses found, almost certainly, only serves as guiding values of the real responses that may be found for "*in vivo*" assessments.

4. DISCUSSION

The CCB assay developed here provides a standard procedure that: 1) is more informative than those commonly used to analyze antioxidant activities in media with different degrees of

polarity, and enables the assessment of any complex matrix; 2) facilitates a less expensive and and less time-consuming operation through the application of a robust mathematical model that produces consistent and meaningful criteria for comparative characterization and quantification of any *OM*.

4.1. Standard method to study complex natural extracts containing components with variable degrees of polarity

Often when describing the antioxidant activity of components of a complex natural extract as a function of the degree of polarity, the study involves a first extraction step with solvents with different polarity index such as water, dimethyl sulfoxide, methanol, dichloromethane, chloroform, acetone or hexane. Afterwards, these extracts are tested by different analytical procedures including DPPH, ABTS, β -carotene and crocin bleaching methods, among others. On other occasions, extracts or purified compounds are tested by the same methods using different solvents. The results are used to quantify the capabilities to counteract the oxidation as a function of the degree of polarity. However, the final activity also depends on the degree of polarity of the intermediate components of the reaction, such as the radicals, which in some cases could lead to erroneous interpretation of the results.

The CCB assay developed in this study presents a technique that analyzes the behavior of complex natural extracts in two opposite and common biological environments (lipophilic and hydrophilic), by combining two procedures into one single method. If the carotenoid of β -carotene is replaced with crocin in the β -carotene bleaching method, the lipidic radicals produced by the thermal induction of linoleic acid will not affect the bleaching kinetics of crocin at any point. Similar results are found by replacing crocin with β -carotene in the crocin

bleaching method, in which the hydrophilic radicals produced by the thermal breakdown of AAPH will not affect the bleaching kinetics of β -carotene. The intermediate components of both reactions are well defined and therefore its results can be interpreted adequately. An illustrative set of well known commercial compounds were used to prove that the results obtained after applying the CCB assay were in accordance with the degree of polarity of each *OM*.

4.2. *OM* characterization and robust quantification criteria

The kinetic oxidative resistance of hydrosoluble (crocin) and liposoluble (β C) carotenes can be experimentally described in anti- and pro-oxidant environments. The period of oxidation inhibition is characterized by the specific behavior of the half-life parameter (the term H_τ in [4] or [5]) in the presence of a given concentration of the considered *OM* agent. This term describes (see Figure 1B): 1) for antioxidants, the half-life extension produced by the radical scavenging reactions of the agent added; 2) for pro-oxidants, the half-life reduction produced by an agent that initiates the radical reactions (as iron in lipidic environments) or that directly acts as a radical (as AAPH in hydrophilic environments). For both cases, the term H_τ behaves as a function of the *OM* concentration in three particular ways that provide intrinsic mechanistic information about the reaction:

(a) An increasing slope variation of H_τ (cases A3 and P3, which seem the less probable ones) would suggest that when a radical encounters an *OM* molecule, their interaction produces a powerful *OM*, or that the *OM* interacts synergistically with one or more components of the reaction. However, no evidence has been found of this pattern in this study.

(b) A decreasing slope variation of H_τ (A2 and P2: the most general case) would suggest: 1) specific and significant differences in the interactions of the radicals with the oxidizable substrate and with *OM*; 2) antagonistic interactions between *OM* and one or more components of the reaction. Independently of which case is true, the result is that *OM* shows a pronounced maximum capability to counteract the radicals at the initial state of the reaction.

(c) A constant slope variation of H_τ (A1 and P1) suggests that every *OM* molecule will be exclusively consumed by protecting the specific radicals that depletes the substrate. Sometimes this behavior is only apparent, as a consequence of testing any of the preceding cases at a single time or using extremely short intervals.

In this study, it is considered that in any complex matrix, such as the tea extracts, the behavior of the term H_τ always follows a slope-decreasing variation, due to the presence of many different *OM*, and, indeed, all the results found (Figure 4 and Table 3) showed this pattern. If, for example, one considers a matrix whose main components determine a constant-slope variation of H_τ (A1), a minor part revealing a decreasing-slope variation is enough for shifting the joint behavior to this result (A2 and P2). The presence of metal cations or other pro-oxidant agents is probably a frequent cause of these effects.

Independently of the mechanistic interpretation that can be inferred by analyzing the specific behavior of the half-life parameter, the robustness of the mathematical model applied produces consistent and meaningful criteria for comparative characterization and quantification of any *OM* activity, in a concentration-time frame which minimizes the effects of the experimental error.

CONCLUSIONS

The carotene combined bleaching (CCB) assay is a new microplate procedure for simultaneous assessment of lipophilic and hydrophilic antioxidants and pro-oxidants, using the crocin and β -carotene bleaching methods in a single combined assay. It can provide useful complementary information in the study of complex natural extracts containing components with variable degrees of polarity. As a food compound case study, the antioxidant activity of five types of tea extracts (Green, Blue, White, Black and Red) were characterized and their equivalent potential activity with commercial antioxidants was calculated.

HIGHLIGHTS

A new combined procedure for microplate was developed> Enables the analysis of anti/pro-oxidants for lipo/hydro-philic environs in one assay> A robust quantification procedure is used> A consistent routine for a microplate assay is proposed avoiding over-standardization> Equivalent potential activities of different tea extracts were provided.

AKNOWLEDGEMENTS

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FIGURE CAPTIONS

Figure 1. **A:** Typical microplate array for two methods/two samples, with two replicates per series. C_i : calibration series, method i ; $S_{i,j}$: sample series, method i , sample j . **B:** Nonlinear characterization by the parameter behavior of τ describing (expression [4]) the oxidation process in three different effects as a function of the sample concentration: Antioxidant (A), when the parameter increases; Pro-oxidant (P), when the parameter decreases; and Null (N), when the parameter keeps constant.

Figure 2. Effects of eight agents on crocin and β -carotene bleaching reactions in lipophilic (L) and hydrophilic (H) media; Experimental results (dots) and respective fittings to the model [5] (lines). A control series (\bullet) and seven dilutions (\circ : 1/7, \blacktriangle : 2/7, \triangle : 3/7, \blacksquare : 4/7, \square : 5/7, \blacklozenge : 6/7, \lozenge : 7/7) were included in each case. Notice the method-dependent differences in the concentration (in final solution of the reaction) ranges tested. Parametric estimates and confidence intervals are shown in Table 2.

Figure 3. Antioxidant activity of tea extracts in both crocin and β -carotene bleaching reactions (lipophilic and hydrophilic media respectively); Experimental results (dots) and respective fittings to the model [5] (lines). A control series (\bullet) and seven dilutions (\circ : 1/7, \blacktriangle : 2/7, \triangle : 3/7, \blacksquare : 4/7, \square : 5/7, \blacklozenge : 6/7, \lozenge : 7/7) were included in each case. The concentrations range tested for all extracts are 0-(0.1)-0.7 g/L in final solution of the reaction. Parametric estimates and confidence intervals are shown in Table 3.

Figure 4. Potential equivalent activity of the five different varieties of tea extracts against some commercial antioxidants using the specific half-life extensions [4] calculated with the

parameters obtained in Tables 2 and 3. Note concentration scales on lines and that in the figure on the left (for lipophilic environment) the scales for commercial antioxidants are in μM , except for the case of α -Tocopherol that are in nM.

TABLE CAPTIONS

Table 1. Moisture and ash percentage of the tea types obtained. Yield percentage of the extraction procedure for each type of tea assessed. Compositional analysis of the extracted powder, in all cases the percentage is referred to the total w/w of the tea extracted material (% EM).

Table 2. Numeric results corresponding to the kinetics of the bleaching reactions from Figure 2, as fitted to the model [5].

Table 3. Numeric results corresponding to the kinetics of the bleaching reactions from Figure 3, as fitted to the model [5].

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TABLES

Table 1: Humidity and Ash percentage of the of tea types obtained. Yield percentage of the extraction procedure for each type of tea assessed. Compositional analysis of the extracted powder, in all cases the percentage is referred to the total w/w of the tea extracted material (% EM).

Tea types	Main compositional analysis of tea extracts							
	Humidity %	Ash %	Yield %	TS % EM	RS % EM	PROT % EM	PT % EM	FT % EM
A	6.32	4.42	25.20	39.12	44.17	67.05	31.38	19.01
B	2.39	4.08	29.69	34.81	46.31	22.18	33.65	25.74
C	6.46	4.55	28.22	18.48	36.57	46.91	30.50	25.94
D	6.15	4.99	37.23	19.62	35.41	29.16	33.72	40.74
E	6.97	6.34	32.72	24.29	45.66	43.52	24.83	40.61

TS: total sugars, RS: reduce sugars, PROT: proteins, PT: total polyphenols and FT: total falconoids

Table 2. Numeric results corresponding to the kinetics of the bleaching reactions from Figure 2, as fitted to the model [5].

Oxidation modifier (μM)		parametric estimates							R_{adj}^2
		control			modifying coefficients				
		K	τ	α	a_τ	b_τ	a_α	b_α	
<i>β-carotene method</i>									
BHA	0-5	0.931 ± 0.001	58.37 ± 0.39	1.202 ± 0.035	1.640 ± 0.030	0.027 ± 0.003	-	-	0.9987
BHT	0-30	“	“	“	0.199 ± 0.003	-	0.011 ± 0.003	-	0.9969
α-Toc.	0-0.04	“	“	“	49.61 ± 1.54	-	-	16.27 ± 1.21	0.9990
Trolox	0-250	“	“	“	-	-	-	-	0.9976
Fe+2	0-7	“	“	“	0.028 ± 0.011	1.029 ± 0.047	0.220 ± 0.059	0.438 ± 0.104	0.9989
Mn+2	0-250	“	“	“	0.002 ± 0.000	0.001 ± 0.000	-	-	0.9988
Cu+2	0-300	“	“	“	0.009 ± 0.000	0.034 ± 0.001	-	-	0.9993
AAPH	0-30000	“	“	“	0.055 ± 0.004	0.009 ± 0.001	0.061 ± 0.015	0.0217 ± 0.006	0.9971
<i>crocin method</i>									
BHA	0-400	0.970 ± 0.001	45.93 ± 0.47	1.297 ± 0.036	0.009 ± 0.001	0.003 ± 0.000	-	-	0.9954
BHT	0-200	“	“	“	-	-	-	-	0.9988
α-Toc.	0-25	“	“	“	0.022 ± 0.009	-	-	-	0.9993
Trolox	0-150	“	“	“	0.043 ± 0.008	-	-	-	0.9975
Fe+2	0-50	“	“	“	-	-	-	-	0.9989
Mn+2	0-100	“	“	“	0.232 ± 0.007	0.011 ± 0.001	-	-	0.9988
Cu+2	0-200	“	“	“	0.016 ± 0.001	0.003 ± 0.000	0.003 ± 0.002	-	0.9957
AAPH	0-20000	“	“	“	0.001 ± 1x10 ⁻⁴	0.004 ± 1x10 ⁻⁴	1.5x10 ⁻⁵ ± 3x10 ⁻⁶	-	0.9984

Confidence intervals for $\alpha=0.05$. R_{adj}^2 : linear correlation coefficient adjusted between observed and predicted values.

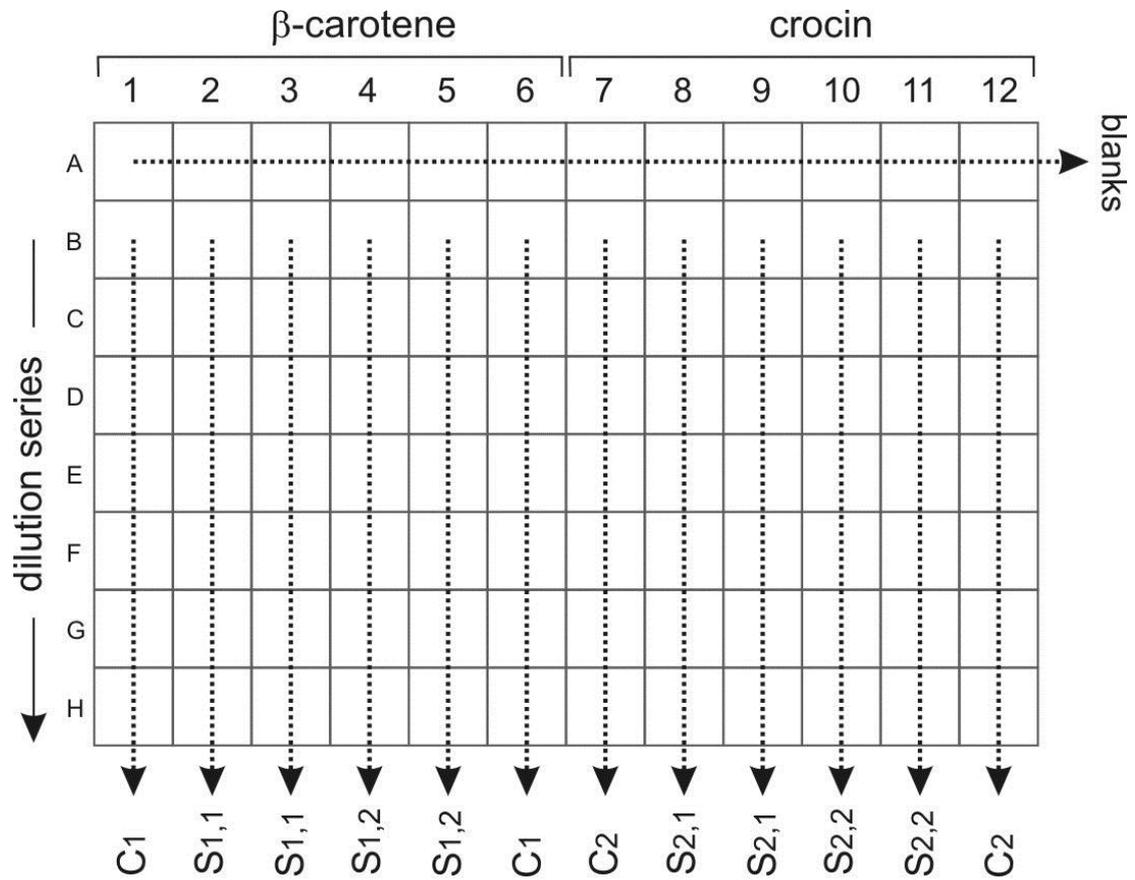
Table 3. Numeric results corresponding to the kinetics of the bleaching reactions from Figure 3, as fitted to the model [5].

tea types	parametric estimates							R_{adj}^2
	control			modifying coefficients				
	K	τ	α	a_τ	b_τ	a_α	b_α	
<i>β-carotene method</i>								
A	0.936 \pm 0.9	58.37 \pm 2.6	1.32 \pm 6.3	40.88 \pm 7.1	1.75 \pm 15.9	702 \pm 7.2	898 \pm 32.0	0.9967
B	"	"	"	32.44 \pm 8.0	2.14 \pm 16.4	758 \pm 23.5	1034 \pm 23.2	0.9929
C	"	"	"	44.36 \pm 8.9	2.96 \pm 15.2	757 \pm 5.3	960 \pm 15.5	0.9910
D	"	"	"	36.10 \pm 5.9	2.29 \pm 10.1	704 \pm 10.6	1001 \pm 10.8	0.9949
E	"	"	"	27.62 \pm 7.7	1.19 \pm 19.4	706 \pm 4.4	996 \pm 14.8	0.9974
<i>crocin method</i>								
A	0.832 \pm 1.3	45.93 \pm 2.6	1.28 \pm 3.7	81.46 \pm 4.6	1.79 \pm 34.5	17.63 \pm 60.7	26.19 \pm 54.7	0.9971
B	"	"	"	97.30 \pm 4.9	1.65 \pm 44.9	8.90 \pm 56.7	13.82 \pm 49.3	0.9969
C	"	"	"	442.30 \pm 2.8	9.12 \pm 18.2	104.22 \pm 52.7	140.18 \pm 48.8	0.9991
D	"	"	"	433.50 \pm 2.6	10.61 \pm 14.4	223.36 \pm 78.7	283.19 \pm 75.5	0.9991
E	"	"	"	222.69 \pm 2.1	4.92 \pm 15.3	64.78 \pm 34.4	90.77 \pm 31.6	0.9991

Confidence intervals for $\alpha=0.05$. R_{adj}^2 : linear correlation coefficient adjusted between observed and predicted values.

FIGURES

A: MICROPLATE DISTRIBUTION:



B: GENERAL OM AGENTS BEHAVIOR:

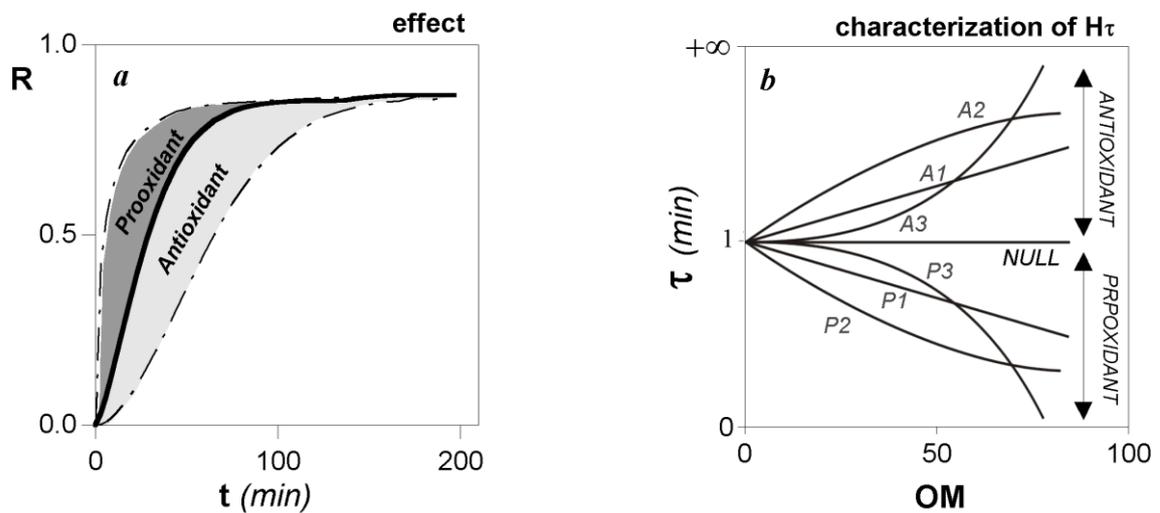


Figure 1. **A:** Typical microplate array for two methods/two samples, with two replicates per series. C_i : calibration series, method i ; $S_{i,j}$: sample series, method i , sample j . **B:** Nonlinear characterization by the parameter behavior of τ describing (expression [4]) the oxidation process in three different effects as a function of the sample concentration: Antioxidant (A), when the parameter increases; Pro-oxidant (P), when the parameter decreases; and Null (N), when the parameter keeps constant.

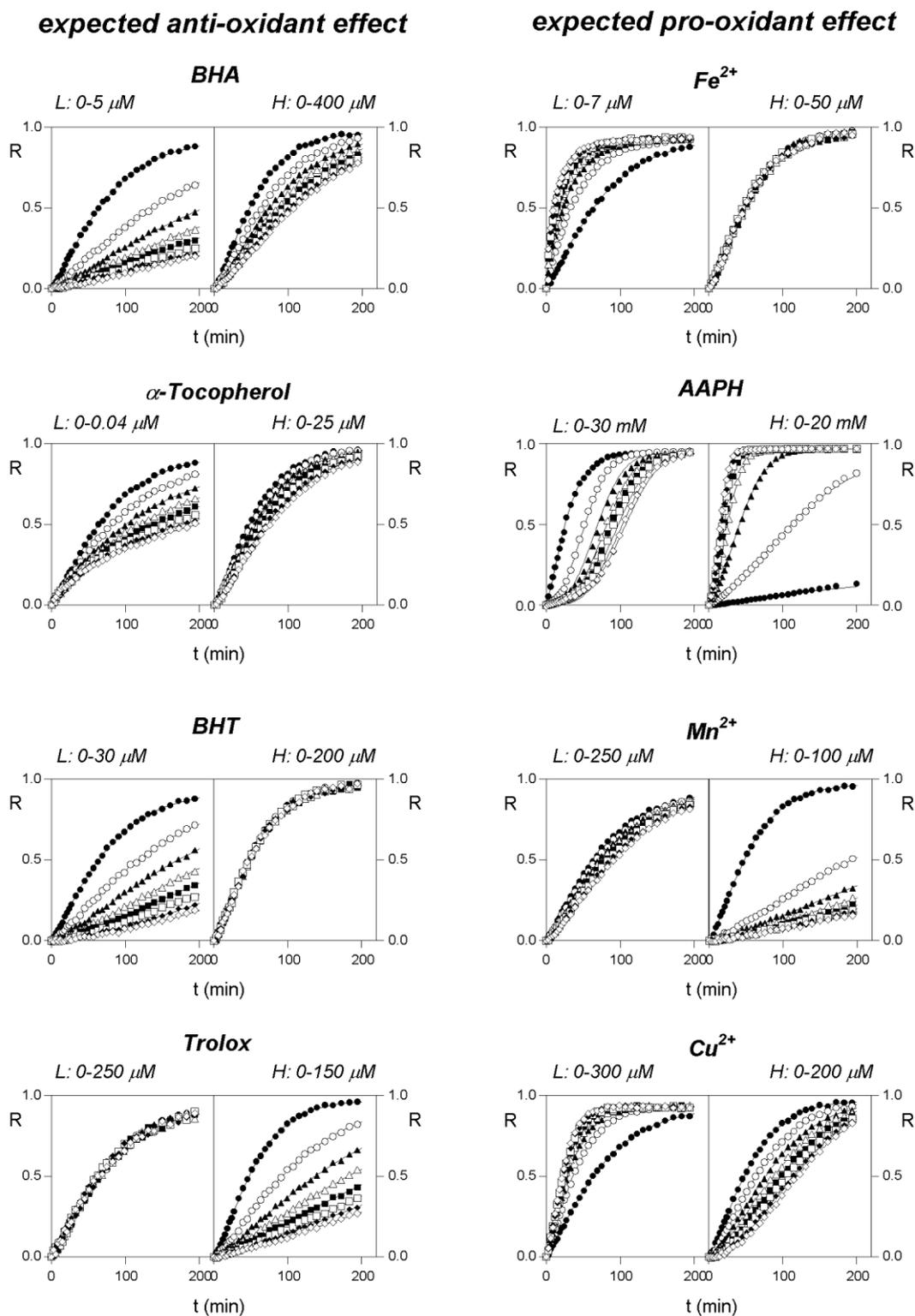
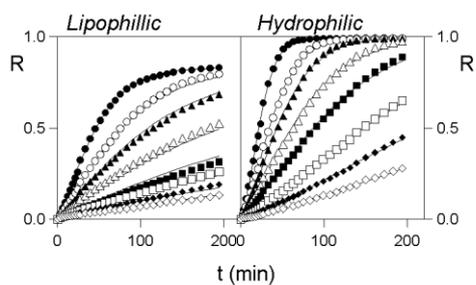
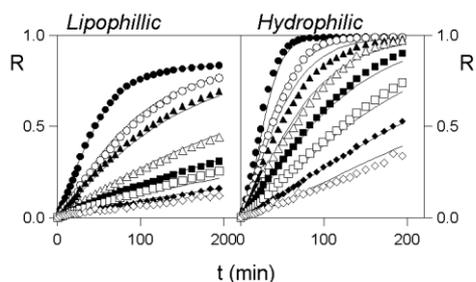


Figure 2. Effects of eight agents on crocin and β -carotene bleaching reactions in lipophilic (L) and hydrophilic (H) media; Experimental results (dots) and respective fittings to the model [5] (lines). A control series (\bullet) and seven dilutions (\circ : 1/7, \blacktriangle : 2/7, \triangle : 3/7, \blacksquare : 4/7, \square : 5/7, \blacklozenge : 6/7, \diamond : 7/7) were included in each case. Notice the method-dependent differences in the concentration (in final solution of the reaction) ranges tested. Parametric estimates and confidence intervals are shown in Table 2.

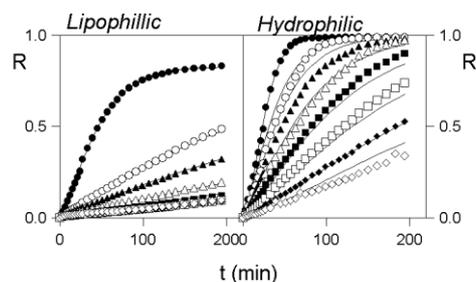
A: Green tea



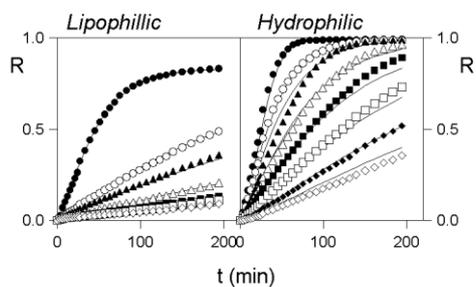
B: Blue tea



C: White tea



D: Black tea



E: Red tea

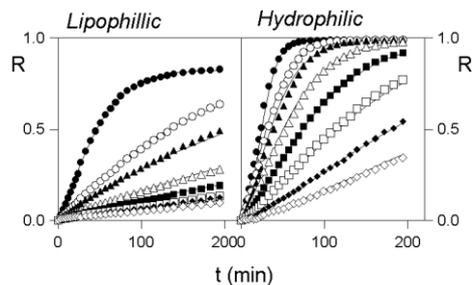


Figure 3. Antioxidant activity of tea extracts in both crocin and β -carotene bleaching reactions (lipophilic and hydrophilic media respectively); Experimental results (dots) and respective fittings to the model [5] (lines). A control series (●) and seven dilutions (○: 1/7, ▲: 2/7, △: 3/7, ■: 4/7, □: 5/7, ◆: 6/7, ◇: 7/7) were included in each case. The concentrations range tested for

all extracts are 0-(0.1)-0.7 g/L in final solution of the reaction. Parametric estimates and confidence intervals are shown in Table 3.

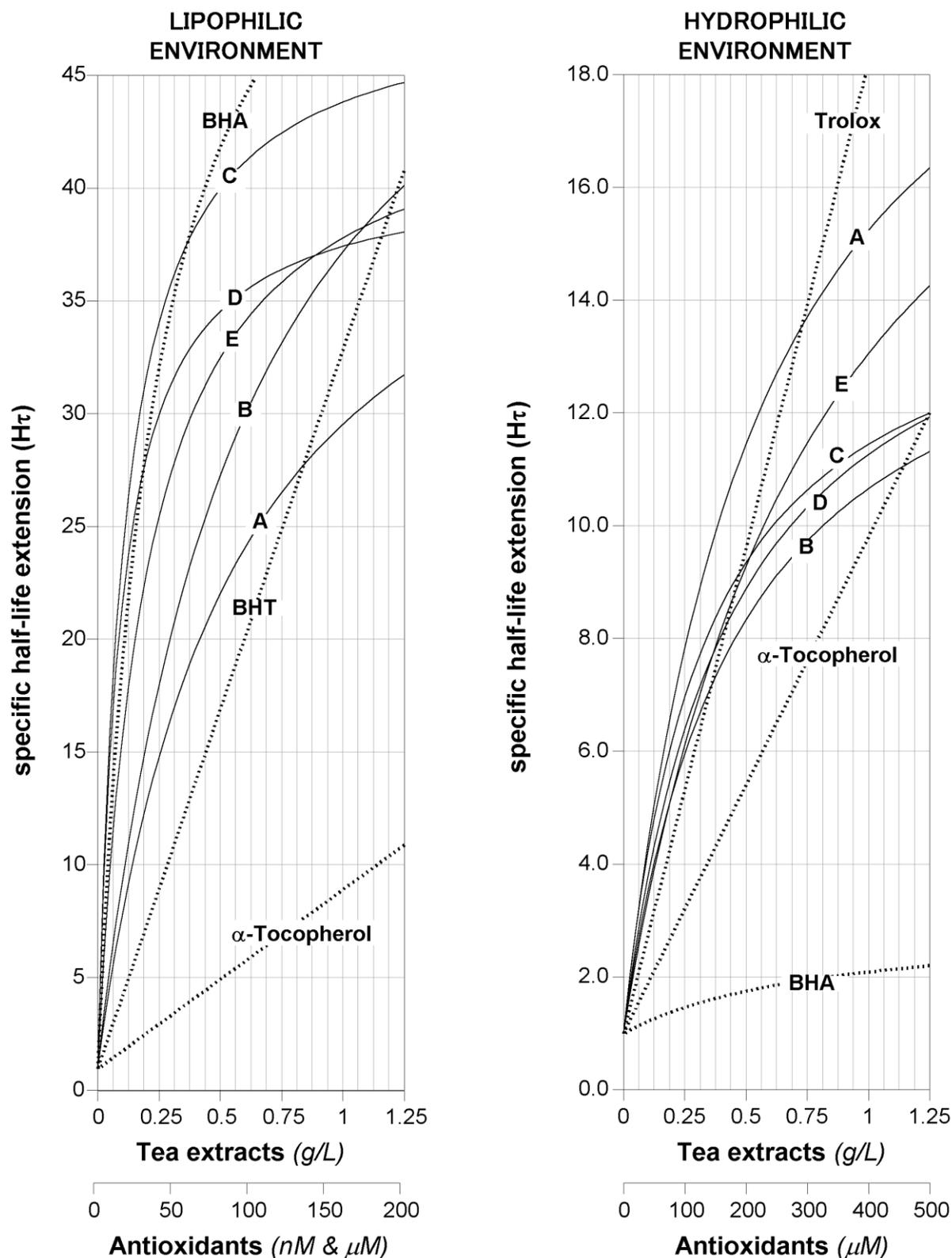


Figure 4. Potential equivalent activity of the five different varieties of tea extracts against some commercial antioxidants using the specific half-life extensions [4] calculated with the parameters obtained in Tables 2 and 3. Note concentration scales on lines and that in the figure on the left (for lipophilic environment) the scales for commercial antioxidants are in μ M, except for the case of α -Tocopherol that are in nM.