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.

Keywords: antioxidant activity; pro-oxidant activity; competition methods; mathematical modeling; time-dose response.

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 et al., 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 et al., 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 (Naguib, 2000; Tsuchihashi et al., & Niki, 1995; Halliwell, 2013) for different compounds. The current methods to test the OM capacity still have left many open questions (Frankel & Meyer, 2000; Halliwell, 2012). The i*n 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 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 et al., 2005; 1999).

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 et al., 2005; Koleva et al., 2002; Laguerre & Villeneuve, 2007; Naguib, 2000; Roginsky, 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 et al., 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 Mili-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 minutes (initiation, propagation and asymptotic phase), during a period of 200 minutes. 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 transfered to microplate readers (Prieto et al., 2013a; 2013b) 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 Mili-Q water at 40 ºC. 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, 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, 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, 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 radical) 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 cells. Hb can be found in many food compounds interfering with its antioxidant activity and also is a typical compound that caused rapid rancidity.
(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.

The concentration ranges in μM of the antioxidants used for the βC reaction are: Fe^{+2} 0-(1.5)-15; Cu^{+2} 0-(15)-240; Hb 0-(2)-20.0. For the Cr reaction AAPH 0-(12.5)-125 was used. All compounds were purchased from Sigma S.A. (St. Louis, MO, USA).

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 α=0.05) were calculated using the '*SolverAid*' (Prikler, 2009). The '*SolverStat*' macro (Comuzzi et al., 2003; Prieto et al, 2011) was used for detecting possible anomalies in the distribution of parametric estimates and residuals.

3. RESULTS

At first, as an example, experimental data values are used to illustrate the capabilities of the method, and afterwards, the quantification and comparative method was applied to different combinations of OM compounds in two competition assays (the βC and Cr bleaching reactions). Then, to illustrate its capabilities, the model was further extended to the analysis of the combine effect of an antioxidant and a pro-oxidant simultaneously. Finally, some methods in which the quantification and comparative method of the OM capacity could be potentially applied, are presented, and data from other authors was used to extend the validation of the procedure into another competitive assays.

3.1. Illustration of the bell protection function and simple analytical criteria to compare the time-dose response of compounds

In competitive assays, performed in systems without limitations of oxygen, it can be accepted that exhaustive substrate oxidation is reached at sufficient time, and therefore the final asymptotic value will be equal for all the kinetic responses in absence and presence of any type of OM. The method developed here can only be applied if this requirement is fulfilled, which is the case of the most common competitive assays in the oxidation field.

Data obtained in the βC bleaching reaction is used to illustrate the procedure to assess the capacity of OM. The antioxidant of BHT and the pro-oxidant Fe^{+2} as a function of time and dose are used as example.

3.1.1. Standardizations and fittings

The first step is to standardize the response, thus all kinetic profiles in the presence of a concentration of an OM are subtracted by the kinetic profile in its absence, as follows:

$$RD(t) = OM_t - C_t \qquad [1]$$

in which *OM* and *C* are the kinetic response in the presence and absence (control) of an oxidation modifier concentration, respectively. *RD* is the relative difference found at any given time (*t*), which in this case accounts for the amount of μM of βC or Cr protected by the OM agent. When the agent is a pro-oxidant the profile will be a negative bell function and when it is an antioxidant will be a positive bell profile.

In Figure 1, a representation of the characteristic profiles obtained by βC bleaching reaction using a time-dose response of the antioxidant of BHT and the pro-oxidant Fe^{+2} is presented.

Observing the response (Figure 1, top), it is clear that the analysis of this profile, with simple mathematical relations measured at one single time, will produce under- or over-estimations of the capacity of the antioxidant, depending on the time selected. Alternatively, the traditional option is the analysis with S-shaped equations, producing several parameters that characterize the response of the remaining βC molecules through the lag-time period, the time required for reaching half maximum response, the maximum bleaching rate, etc. However, our proposal, the kinetic relative difference response, exhibits an asymmetric bell profile (Figure 1, bottom), which is equivalent to the substrate molecules protected (positive for antioxidants and negative for pro-oxidants) by the OM molecules as a function of time. Such profiles show many different physical kinetic properties that could characterize the response. Among these physical properties, the maximum protected molecules of βC (P_m) and the time at which it takes place (t_m) are the most characterizing parameters that cannot be found through traditional equations. For example, in the food industry, the combinatory use of these parameters could provide the state of the oxidation of the reaction after the chain reaction will be inevitably affecting the taste, flavors and other properties of foods, because it focuses on analyzing the quantity of protection and the moment at which such protection would be lost.

This characteristic bell protection profile can be described by many bell functions (Di Marco, 2001). After testing several equations, the generalized exponential function without intercept (also called the modified Weibull distribution function) was found to be the most satisfactory one with least number of parameters and highest accuracy:

$$RD(t) = P_m \left\{ \frac{i}{d} \left[1 - \left(\frac{t}{t_m} \right)^d + \ln\left(\frac{t}{t_m} \right) d \right] \right\} \qquad [2]$$

in which the parameter d is related to the distance between the tails of the function, i a value related to the asymmetry of the bell profile, P_m the maximum protected molecules of the substrate used in the reaction (βC and Cr in this case) and t_m the time at which P_m takes place.

This model explicitly provides the characterizing parameters (P_m and t_m) of the RD response, and therefore their statistical significance can be tested through the determination of its confidence intervals. Figure 2 (A1 and B1 plots) shows the application of this model to predict the effect of BHT and Fe^{+2} in the βC reaction. All the parametric values are presented in appendix section (Table A1 and Table A2), showing lower confidence intervals (α=0.05) and higher correlation coefficients in all cases (r^2>0.99), thus demonstrating the reliability of this approach. The two characterizing parameters (P_m and t_m) will vary in the presence of any antioxidant and, given their well-defined factual meanings regarding the oxidation kinetics, their combine variations have a relevant characterizing value.

On the one hand, plotting the P_m parameter against OM concentration show an asymptotic trend (Figure 2, A2 and B2 plots), suggesting that some radical-generating property of the system can be saturated (Gieseg & Esterbauer, 1994). This type of dose-response patterns, in general, can be adjusted to the following asymptotic function:

$$P_m([OM]) = K\left[1 - \exp(-r[OM])\right] \qquad [3]$$

where [OM] is the concentration of the OM agent under study in μM, P_m (OM) is the response behavior of the parameters P_m as a function of [OM], K is the asymptotic value of the parameter

obtained (μM of the protected substrate) and r is the specific dose-rate (μM^{-1} of OM). If the OM agent is an antioxidant the response will be positive and negative for pro-oxidants.

On the other hand, the t_m parameter shows a linear dose-response trend (Figure 2, A3 and B3 plots) with an intercept that can be easily adjusted to:

$$t_m(OM) = t_0 + b[OM]\ Q = K \times r \qquad [4]$$

where b is the slope (min/μM of OM) of the dose-response trend and t_0 is the extension time (min) at which the lipid change oxidation reaction behaves in the absence of any OM, in other words the extension time produced by 1 μM of βC (the competitor antioxidant). If the OM agent is an antioxidant the linear response will be positive increasing and decreasing for pro-oxidants.

The resulting kinetic parameters, obtained after the fitting procedure to equation [2], are adjusted to their respective equations [3] and [4] as a function of [OM], obtaining in all cases highly consistent results with satisfactory confidence intervals (α=0.05).

3.1.2. Simple analytical criteria to compare the time-dose response of compounds

In addition, after obtaining the parametric estimates of equations [3] and [4], it is possible to summarize the time-dose response in two complementary single values (the Q and S values).

The Q value, which corresponds to the amount of molecules protected per unit of OM (μM of the protected substrate/μM of OM) at the moment of maximum predicted capacity, is calculated by multiplying both parameters (K and r) estimated by equation [3] as follows:

$$Q = K \times r \qquad [5]$$

In the case of S value, its determination is performed following the next procedure: First, to compute the OM concentration needed at any percentage of the response by equation [3], the P_m (OM) is considered to be P_m=K×n/100, in which n can be any value between 0-100%, consequently the corresponding $[OM]_n$ can be computed to obtain any n percentage of the maximum μM of the substrate protected P_m (OM) by the following expression:

$$[OM]_n = -\frac{Ln\left(1-\left(n/100\right)\right)}{r} \qquad [6]$$

Then, by inserting this $[OM]_n$ to reach n percentage of the protected substrate into equation [4], the protection time until the substrate reaches this n percentage can be obtained as:

$$S_n = t_0 + b[OM]_n \qquad [7]$$

in which t_0 and b are the parameter estimates previously computed by equation [4]. Even if the typical approach is to consider the half-life response or in this case n=50%, it would be appropriate to compute the S value for the concentration needed to reach the asymptotic value of equation [3] (K or 100% of the response), complementing accordingly the information provided by the Q value. However, when computing the S value for n=K, the $[OM]_{n=K}$ will be excessively high, in occasionally outside of the kinetic range capabilities or extending the assay

inappropriately. Therefore the 95% value was considered the more suitable response (Figure 2, A2 and B2 plots).

These values can be used to compare the activities of different OM agents. For example, the Q value of BHT showed that the maximum capability of one molecule is to protect 0.10 molecules of βC (0.10 μM βC protected/μM of BHT), on the other hand, the $S_{n=95\%}$ for BHT showed that at the 95% of its maximum capabilities the protection time was 89.23 min (knowing that $[BHT]_{n=95\%}$=20.51 μM). The information provided by the combination of both values represents a robust tool to compare the activities of different antioxidant agents based on the parametric estimations time-dose response. With both values, an intuitive solution to compare OM activities of compounds by a mathematical analysis is obtained, offering researchers an alternative solution based on parametric non-linear values to assess OM action and compare their capacity rigorously. Furthermore, the application may facilitate the ranking process and the selection of appropriate concentrations of natural products to replace commercial antioxidants.

3.2. Verification of the quantification procedure when applied to assess and compare several OM agents in two different competitive assays

3.2.1. Antioxidants

Figure 3A and Table A1 (appendix) show the graphic representations of the results and the parametric estimates of the time-dose fittings of equation [2] to the results of the proposed approach for the βC bleaching reaction applied to five common commercial antioxidants. Figure 3B and Table A3 (appendix) show the corresponding results of the proposed approach for the Cr bleaching reaction. Table 1, the parametric estimates of equations [3] and [4] obtained after fitting the parametric results (P_m and t_m parameters) from equation [2] are shown for both assessed reactions. It is particularly noteworthy to point out that for both reactions, only for the case of ETX in the βC system, the maximum substrate protected (P_m) reaches an asymptotic value (K) equal to the total amount of βC present in the final solution of the reaction.

Furthermore, the computed criteria values Q and S to compare the antioxidant capacity are presented in Table 1 and Figure 3 (A2 and B2 plots):

- In the βC reaction, the value Q for the compound ETX was found to protect 291.2 molecules of the substrate βC per molecule of antioxidant, which is by far the highest value reached, followed by TOC with 20.62. With regards to the time at which the maximum protection took place, the value S again show that ETX protected the oxidation of βC (139.9 min) for longer periods than the others, such as TOC with 121.9 min.
- In the Cr reaction the differences between the antioxidants assessed were less than in the βC reaction. Nevertheless, the antioxidant ETX showed the best criteria values than compared to any of the other compounds tested.

The combined criteria values, provide complementary information to compare the capacity of different compounds. Beyond quantitative differences, the following ranking of their capacity can be established:

- For the βC reaction: ETX >> TOC >> BHA > BHT > PG.
- For the Cr reaction: ETX > Mn^{+2} > Tr > AA > TBHQ.

3.2.2. Pro-oxidants

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, thus distorting the results. Figure 3C and Table A2 (appendix) show the graphic representations of the results and the parametric estimates of the time-dose fittings of equation [2] to the results of the proposed approach for the βC and Cr bleaching reactions applied to four common commercial antioxidants. In Table 1, the parametric estimates of equations [3] and [4] obtained after fitting the parametric results (P_m and t_m parameters) of equation [2] are shown for both assessed reactions.

The amount of reduced hemoglobin used, which refers to hemoglobin (considering an average of of 64,500 kDa per molecule) which contains iron in the Fe^{+2} oxidation state, had the approximately the same quantity of Fe^{+2} as the amount introduced directly as iron (II) sulfide. In fact, the parametric response (Table 1) as well as the graphical representation of the results (Figure 3C) are approximately equivalent, demonstrating the reliability of the tools here developed.

3.3. Extension of the model application to the combine effect of an antioxidant and a pro-oxidant agent

One of the additional features of the developed approach is that can be easily extended to a more complex situations, that occasionally are experimentally found. For instance, when testing the OM activity of natural compounds is likely to expect responses that can be a combination of some antioxidants and pro-oxidants. Such responses cannot be directly analyzed by the usual approaches, and therefore to identify the joint activity of each OM compound certain further steps need to be executed.

As example, the combinatory analysis of the antioxidant BHT and the pro-oxidant Fe^{+2} in the βC assay will be presented. A 6×10 arrays of an increasing concentrations of a mixture of an antioxidant and a pro-oxidant, in which 25 µL of each OM solution are added to each well containing 250 µL of the preheated reagent and the other conditions were kept. A total of 30 independent kinetic measures per each of the 60 concentration combinations were obtained and are displayed in Figure A1 (appendix section). It can be seen that as the concentration of pro-oxidant increases the oxidation of βC increases and the effect of the antioxidant becomes less effective. The temporal space of action (t_m) of the pro-oxidant compound is earlier than for the antioxidant, causing biphasic curves caused by its interaction. To analyze such a response additive equations must be used increasing the number of parameters, which makes more difficult the interpretation of the results.

When the effects are displayed in terms of RD (using equation [1]) in Figure 4A (BHT time-dose response for three pro-oxidant concentrations), depending on the range of concentrations used for each compound, only antioxidant activity is seen, which are the curves in the positive axes, only pro-oxidant activity (curves in the negative axis) or both actions when the curves goes from one axis (negative or positive) to the other. The application of the RD standardization allows to visually detect the opposite actions of both agents and provide a quick overall output of the final interaction. However, its analytical determination also requires the sum of two independent equations (one for each OM) as the one described in [2]. As well as if we applied other common resources to the raw data (Figure A1, appendix) a high number of parameters are needed, and depending on the profile of the curve some of them will be non-statistically significant due to the lack of effect. Therefore, the outputs obtained by modeling those types of profiles must be rejected.

However, since the RD is based on the subtraction of the control, it can be consider that the effect of one of the OM as a function as other as a type of control subtracting its effect, thus reducing the number of variables. In Figure 4B the effect of the each concentration of P is subtracted to the antioxidant time-dose response, allowing to analyze the entire set of responses by equation [2] producing statistically significant parametric results (Table A4). The subtraction of the effect of the P only simplifies the operational procedure, and still possible to quantify the interactive effects by determining the parametric values P_m and t_m. Since both values are affected by the interaction of two OM, the univariate equations [3] and [4] (P_m and t_m, respectively) can be expanded to perform a much consistent approach taking into account both effects simultaneously by the following bivariate analysis:

$$P_m\left(A,P\right) = K\left\{1-\exp\left[-r_A\left[A\right]\exp\left(-r_P\left[P\right]\right)\right]\right\} \quad [8]$$

$$t_m\left(A,P\right) = t_0 + b_A\left[A\right] + b_P\left[P\right] \quad [9]$$

Figure 4C shows the univariate results (points) and the fitting to the bivariate equations [8] and [9] (surface). The parametric results of the bivariate analysis of P_m are K=0.717 μM of the substrate protected, r_A=0.667 μM^{-1} of BHT and r_P=0.213 μM^{-1} of Fe^{+2} with a r^2=0.9927. On the other hand, the parametric results of t_m are t_0=37.10 min, b_A=8.001 min/μM of BHT and b_P=3.912 min/μM of Fe^{+2} with a r^2=0.9862.

3.4. Verification of the quantification procedure with experimental data from other competitive methods

The bibliographical abundance about antioxidant activity in a competitive reaction, in raw and purified extracts, makes it practically superfluous to extend the experimental work specifically devoted to validate the model proposed here. In this respect, its descriptive accuracy was verified using results from other authors (taken from the published figures by means of *GetData Graph Digitizer 2.24*), selected in such a way that they implied different methods, substrates and time domains.

3.4.1. Oxidative hemolysis inhibition assay (OxHLIA)

The method is based in the oxidation of erythrocyte membranes by AAPH-derived peroxyl radicals that induces oxidation of lipids and proteins and eventually causes hemolysis, and this hemolysis can be inhibited by antioxidants. OxHLIA is a good experimental model for free radical-induced biomembrane damage and its inhibition by antioxidants. Figure 5 (A1 plot) shows the typical time-dose response of hemolysis curves using the antioxidant Tr at various concentrations 0-(25)-125 mM. The results were obtained from the study of Takebayashi et al. (2010) who recently made a detail revision of the method. Figure 5 (A2 plot) shows the fittings (lines) of the equation [2] to the data in terms of RD (points). Figure 5 (A3 plot) and Table 1 shows the parametric results equations [3] and [4] (P_m and t_m, respectively). Furthermore the computed criteria values Q and S to compare the antioxidant capacity are presented in Table 1.

3.4.2. Oxygen radical absorbance capacity assay (ORAC)

Currently, this method has been automated and transferred to a microplate format producing a large amount of dose-time-data effortless. The assay depends on the free radical damage to the fluorescent compound of fluorescein, which acts as the indicator of the reaction, changing its

fluorescent intensity. It is assumed that the degree of change is indicative of the amount of radical damage. The addition of antioxidants results in a competitive inhibition in the free radical damage to the fluorescent compound. The data was obtained from the work of Ou et al. (2001) who developed and validated the assay. Figure 5 (B1 plot) shows the typical time-dose response fluorescein decay curves in the presence 0-(0.05)-0.2 mg/L of grape seed extract. Figure 5 (B2 plot) shows the fittings (lines) of the equation [2] to the data in terms of RD (points). Figure 5B3) and Table 1 shows the parametric results equations [3] and [4] (P_m and t_m, respectively). Furthermore the obtained values Q and S to compare the antioxidant capacity are also summarized in Table 1.

4. DISCUSSION

Perhaps, the biggest problem is related to the lack of a validated assay that can reliably measure the antioxidant and pro-oxidant capacity of samples, thus making it essential to test the capacity with different methods. As a result, authors tend to simplify the calculation method in order to amplify the number of testing procedures. However, the method used to measure and compute the antioxidant capacity has a major impact on the results, because in both *in vivo* and *in vitro*, the oxidation reactions are complex. The abbreviated approach to study the dose-response at one single-time expecting to find linear forms (as described by the non-kinetic approaches) frequently leads to unreliable results and misinterpretations, making it extremely difficult to compare the results from different assays. The preference of apparently simple assays, routinely applicable with minimal calculation requirements, is not very justifiable today, given the availability of computational applications and automatic equipment (such as microplate readers), whose combination provides adequate tools to work with data sets that allow accurate evaluations by the available non-linear modeling (Labuza & Dugan, 1971; Murado & Vázquez, 2010; Terpinc & Abramovič, 2010; Wardhani et al., 2013; Özilgen & Özilgen, 1990). Despite the advisability of using mechanistic or empiric kinetic models as indicated by different authors, researchers continue to use simple calculation alternative methods more often than necessary.

The detailed mechanistic description of lipid oxidation is complex and varies from one to the other systems, which has led to the search for empirical general models, able to describe the most common profiles. In this sense, among the available non-linear models to describe the time part of an oxidative reaction individually for increasing concentrations of the OM agent, may also be subjected to analysis. For example, the power function developed by Terpinc & Abramovič, (2010) is appropriate only to adjust fractional-order kinetic profiles, but fails in the description of first-order processes or sigmoidal profiles. Other empirical approaches such as the Logistic and Weibull equations, that have been transferred from other fields to describe the oxidation action (Murado & Vázquez, 2010; Özilgen & Özilgen, 1990), are more appropriate for modeling processes as the lipid oxidation. Those equation are able to produce key parameters to summarize the responses, such as the asymptote, maximum velocity or the lag-phase, they can characterize the response and help to quantify the effect of OM agents. In general, the three parameter sigmoidal group of functions (such as the Logistic, Weibull, Hill, Gompertz or Richards-Chapman) is the best solution to fit individually the kinetic profiles corresponding to a series of increasing levels of OM agents. Alike in many other complex systems, some authors (Murado & Vázquez, 2010; Prieto et al., 2013a; 2013b) have suggested directly or indirectly further analysis, in which the oxidative responses are described as a function of both the dose and the exposure time, in a bivariate form.

Our proposal represents an alternative for the dose-time-response behavior, based on two kinetic parameters of equation [2], which jointly defines the capacities of the OM to extend or shorten the maximum protection as a function of the concentration. In fact, it is able to describe

accurately different rate-dose tendencies. It allows quantification of the variations of the kinetic profiles which characterize the different types of antioxidants in a useful way that can provide even indications concerning modes of action. Independently of the mechanistic interpretation that can be inferred by analyzing the specific behavior of both characterizing parameters, in competitive assays, the time dependent bell protection function produces consistent and meaningful criteria for comparative characterization and quantification of any antioxidant, in a dose-time frame which minimizes the effects of the error produced by the experimental conditions.

Additionally, by standardizing the response using the equation [1] the results obtained do not depend on the experimental conditions, particularly on the initial concentration of the reactive species, which is in practice, one of the common problems when analyzing the efficacy of an antioxidant in competitive methods. In a competition assay, it has to be realized that during the assay the concentration of the antioxidant as well as that of the indicator of the reaction can be reduced to a considerable extent. The consumption of both during the experiment, as an inevitable consequence of the competition that has to take place, is a potential cause of inaccurate results (Balk et al., 2009).

In this work, we have clearly demonstrated the capabilities of the model to discern the effects of several commercial agents providing useful information in the study of complex natural extracts containing components with variable degrees of OM capacity. For all the assayed agents, statistically significant descriptions, with very accurate predictions, were provided by model [2]. In the presence of antioxidants or pro-oxidants, the molecules of the substrate protected and the time at what takes place increases according to equations [3] and [4]. This variation is general enough to explain the alteration of the kinetic profile due to the presence of an OM compound.

CONCLUSIONS

The complexity of the topic of antioxidants and pro-oxidants plus the confusion introduced by improper use of questionable methods leads to the disarray of the antioxidant research community and industry. In this paper, a quantification method was developed for competitive assays and tested by investigating the capacity of several antioxidants in different competitive systems. The analysis of the antioxidant capacity of commercial antioxidants reveals the lack of meaning of single-time criteria and the possibilities of the proposal presented. The model parameters obtained were used to compare the capacity, identifying complex trends and analyzing the dose-equivalent system response, providing more complete information about antioxidant behavior and a more efficient way to determine the total antioxidant capacities that those techniques at a fixed point.

AKNOWLEDGEMENTS

The authors wish to thank CSIC (Intramural project: 200930I183) and Ministerio de Ciencia e Innovación (project CTM2010-18411, co-financed with FEDER funds by European Union) for financial support. Miguel Ángel Prieto Lage was awarded one grant from the JAE predoctoral program co-financed by the CSIC and European Social Fund (ESF).

FIGURES

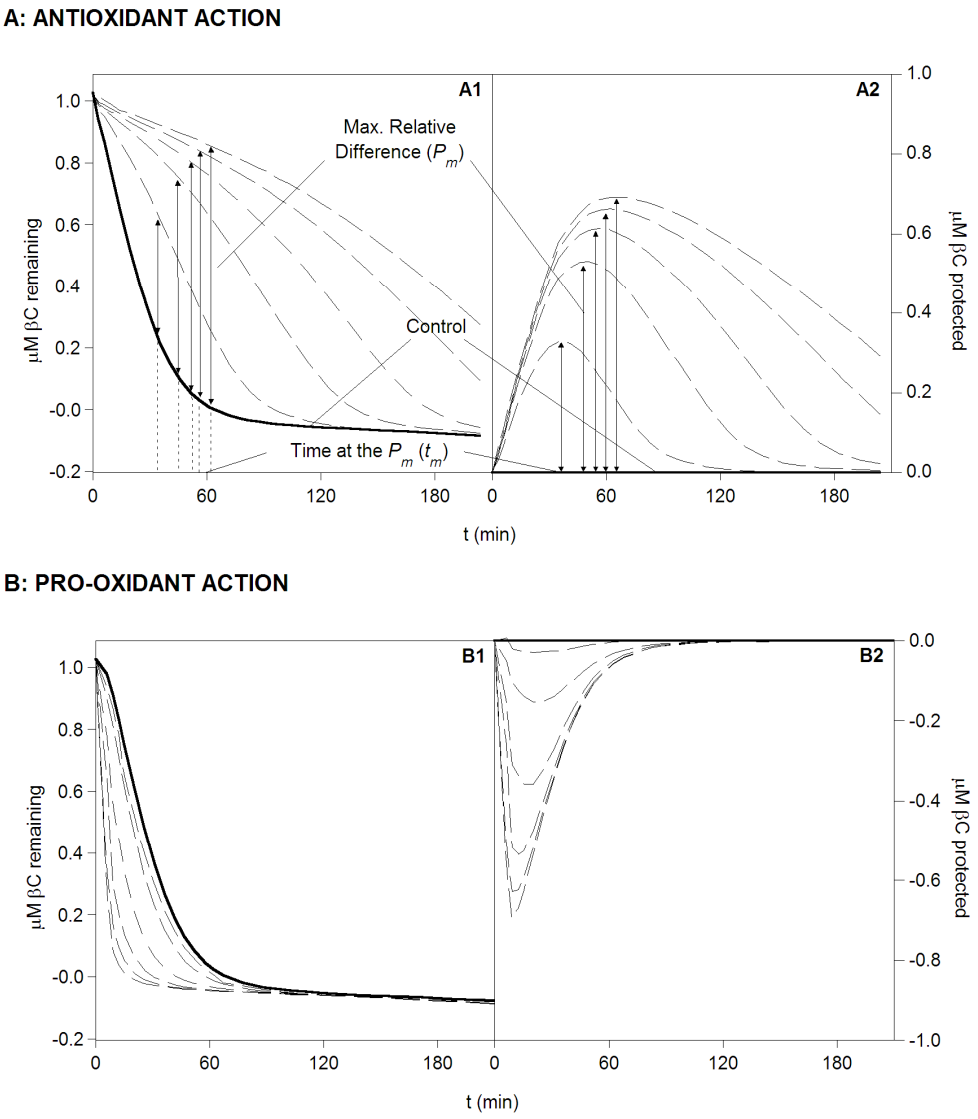

Figure 1: Illustrative representation of the characteristic profiles obtained for antioxidant (A) and pro-oxidant (B) responses using equation [1] to standardize in the β-carotene (βC) bleaching reaction as examples. A1 and B1 show the raw responses of the βC reaction as function of time and A2 and B2 the asymmetric bell profile of the kinetic relative difference response.

1

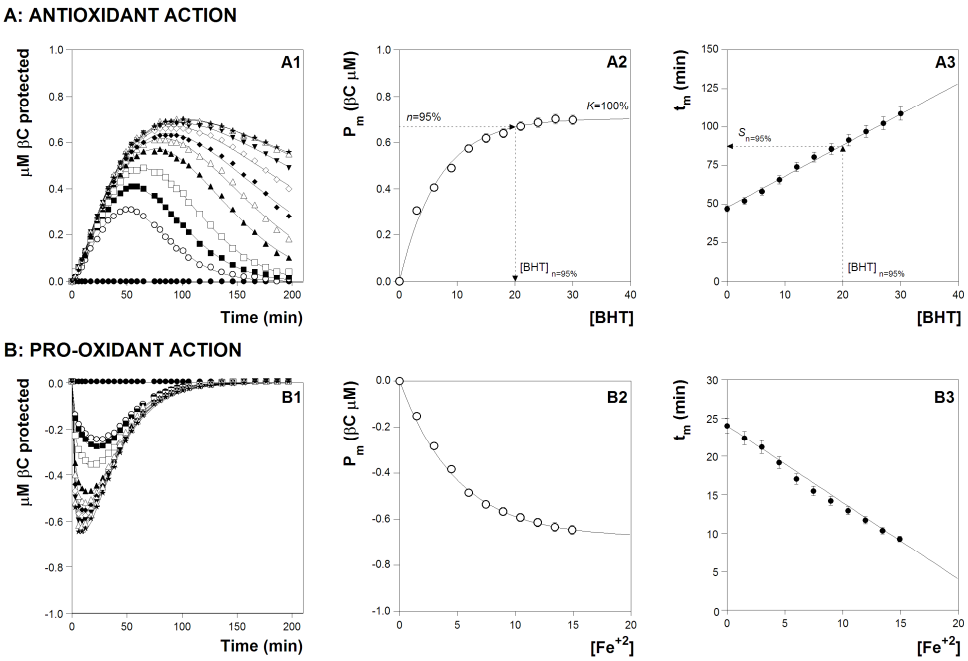

Figure 2: The kinetic parameters that could characterize the response the maximum protected molecules of βC (P_m) and the time at which it takes place (t_m) are displayed. A1 and B1 show the fittings to the asymmetric bell profile of the kinetic relative difference dose-response of the examples presented in Figure 1 to the model [2]. A2 and B2 show the maximum protected molecules of βC (P_m) fitted to the equation [3]. A3 and B3 display the time at which it takes place (t_m) the P_m fitted to the equation [4]. For all cases, the points are the findings and the lines are the fitted results to the corresponding model. All numerical results in Table 1, Table A1 and Table A2.

2

3

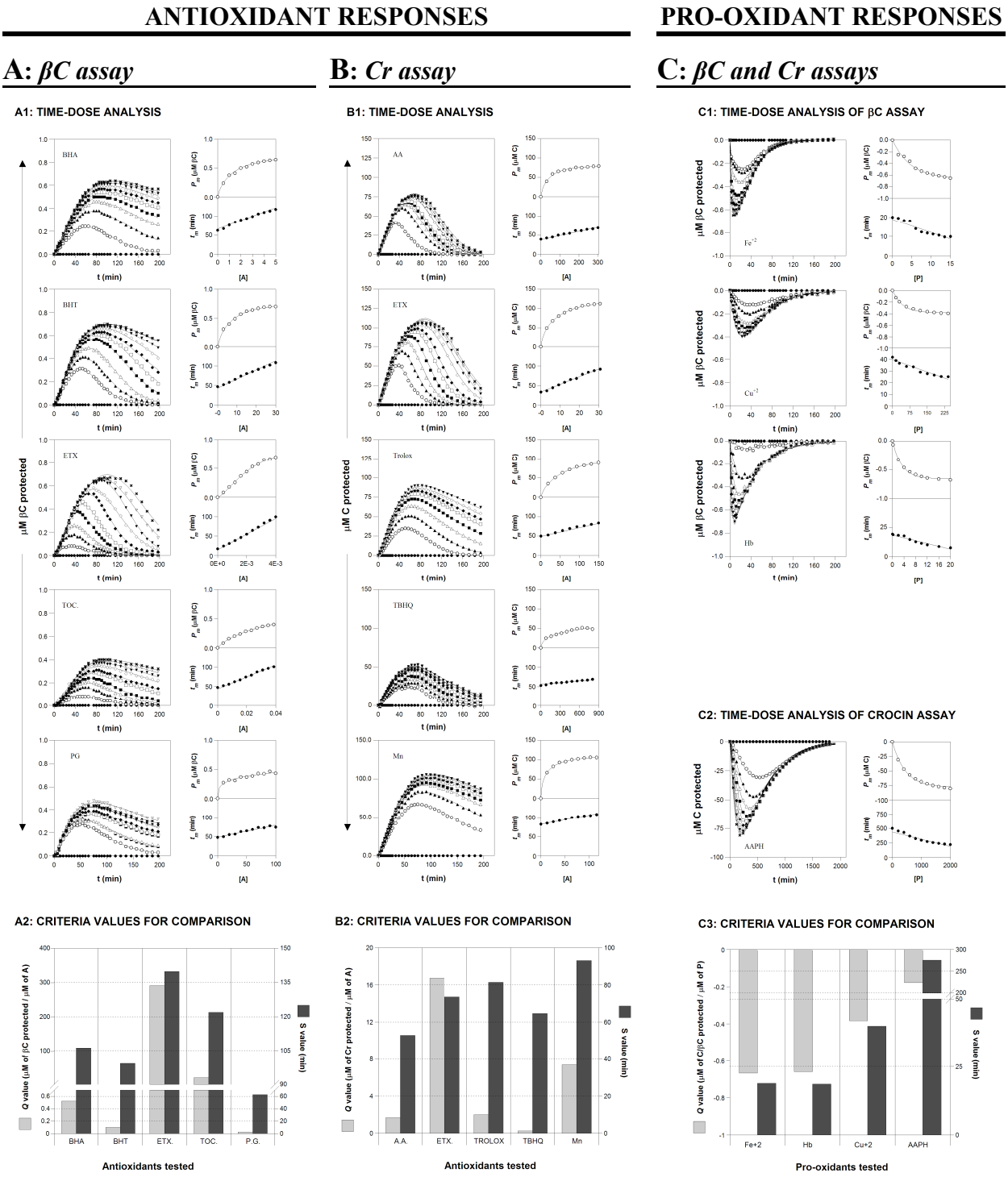

Figure 3: Experimental results for the β-carotene and crocin bleaching reaction. Each figure of the OM analysis is divided as follows: on the left side, the time protection profiles drop orderly with the increase of the agent concentrations and are fitted to equation [2] and on the right side, the P_m and t_m parameters pattern are shown and fitted to the equations [3] and [4] respectively. Figures in the sub-sections A1, B1, C1 and C2 show the effects of several antioxidants and pro-oxidants obtained in the βC and Cr bleaching assays. Sub-sections A2, B2 and C3 show the results of the analytical criteria values (Q and S) used to compare the capacity of OM.. Experimental results are points and fittings to the corresponding models are lines. All numerical results are in Table 1, Table A1, Table A3 and Table A2.

4

5

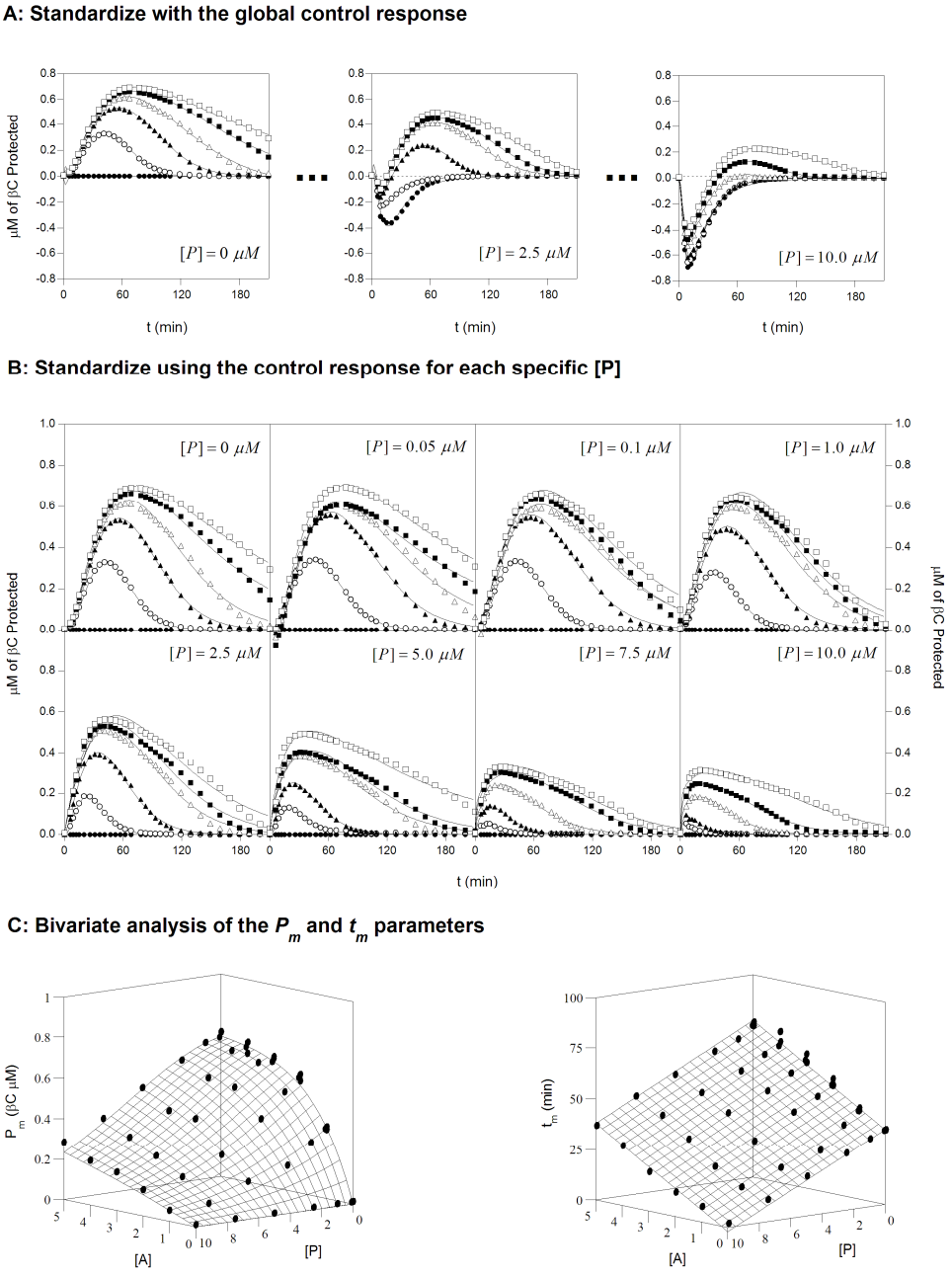

Figure 4: Analysis of the combine action of an antioxidant and a pro-oxidant. All numerical results are in Table A4. The parametric results of the bivariate analysis are described in the text.

6

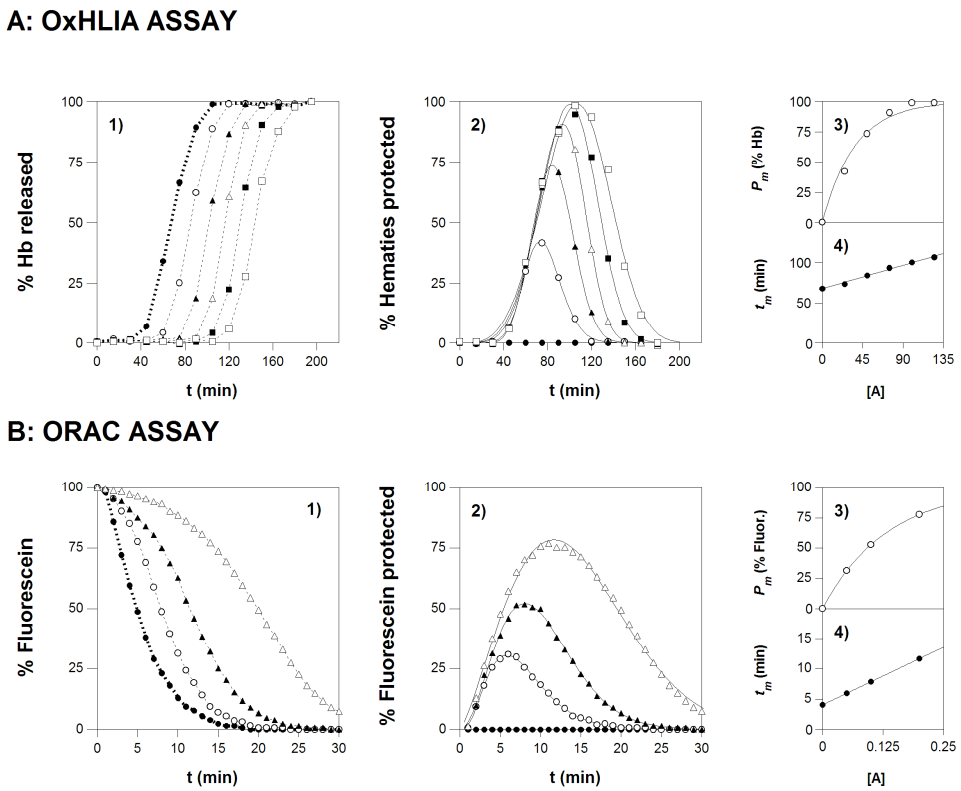

Figure 5: Analysis of other potential methodological applications. **A:** OxHLIA assay data, obtained from Takebayashi et al. (2010) that shows the typical time-dose response hemolysis curves of Trolox (0 ●, 25 ○, 50 ▲, 75 △, 100 ■ and 125 □ mM using sheep erythrocytes suspended at a concentration of 0.7% (v/v) in PBS incubated at 37°C with 40 mM of AAPH. **B:** ORAC assay data, obtained from Ou et al. (2001) showing the fluorescein decay curve induced by AAPH in the presence of 0 ●, 0.05 ○, 0.1 ▲ and 0.2 △ mg/L of grape seed extract. Analytical criteria values (*Q* and *S*) used to compare the capacity among several antioxidants are presented in Table 1. All numerical results are in Table A5 (appendix).

TABLES

Table 1: Parametric estimates of equations [3] and [4] obtained after fitting the parametric results (P_m and t_m parameters) from equation [2] for the crocin and β-Carotene bleaching kinetics as affected by the specified agents respectively. Also the analytical criteria values (Q and S) used to compare the capacity among several antioxidants are shown. The confidence intervals (α=0.05) are in percentages.

[OM]	Parameters of P_m (OM)			Parameters of t_m (OM)			Criteria values	
	K	***r***	r^2	t_0	***b***	r^2	***Q***	***S***
B-CAROTENE ASSAY								
BHA	0.635±*4.0*	0.827±*6.1*	0.9990	69.03±*3.0*	10.28±*6.1*	0.9992	0.525±8.4	106.2±2.2
BHT	0.700±*3.2*	0.146±*1.2*	0.9996	47.97±*1.1*	2.011±*5.6*	0.9994	0.102±3.8	89.23±0.7
ETX.	1.000±*3.2*	291.2±*2.3*	0.9988	47.28±*1.3*	9002±*3.4*	0.9991	291.2±7.4	139.8±3.2
TOC.	0.481±*2.7*	42.87±*3.1*	0.9981	58.15±*3.2*	912.4±*4.4*	0.9990	20.61±8.4	121.9±4.8
P.G.	0.421±*2.2*	0.064±*4.1*	0.9984	48.48±*5.3*	0.309±*3.5*	0.9981	0.027±9.0	62.94±4.1
Fe^{+2}	-0.668±*2.3*	0.212±*1.5*	0.9880	18.87±*2.8*	-0.671±*3.1*	0.9991	-0.668±*5.6*	18.87±*4.4*
Hb	-0.659±*1.3*	0.313±*2.2*	0.9921	18.55±*4.5*	-0.610±*4.4*	0.9955	-0.660±*3.1*	18.55±*6.6*
Cu^{+2}	-0.383±*7.8*	0.022±*3.4*	0.9902	41.10±*2.6*	-0.071±*3.6*	0.9976	-0.383±*7.8*	40.00±*5.1*
CROCIN ASSAY								
A.A.	76.62±*5.1*	0.022±*3.3*	0.9970	38.06±*3.1*	0.109±*4.4*	0.9989	1.685±6.8	52.90±2.2
ETX.	100.0±*3.4*	0.150±*2.2*	0.9997	33.59±*2.3*	2.001±*1.3*	0.9986	16.72±7.5	73.55±1.1
TROLOX	91.12±*1.7*	0.022±*1.3*	0.9977	51.69±*1.5*	0.218±*0.9*	0.9989	2.004±2.2	81.37±1.0
TBHQ	50.03±*2.8*	0.005±*1.7*	0.9976	56.32±*7.8*	0.014±*1.5*	0.9980	0.250±4.8	64.71±6.8
Mn^{+2}	100.0±*4.5*	0.072±*5.1*	0.9987	83.42±*6.6*	0.233±*2.6*	0.9985	7.358±3.0	93.11±5.9
AAPH	-77.82±*9.1*	0.002±*8.2*	0.9991	436.3±*4.2*	-0.123±*3.6*	0.9970	-0.178±*3.3*	275.18±*7.1*
OxHLIA ASSAY								
Trolox	100.0±*5.4*	0.0276±*20.3*	0.9911	68.04±*1.2*	0.320±*28.2*	0.9920	2.760±*31.2*	102.84±*36.1*
ORAC ASSAY								
Grape seed	100.0±*10.3*	7.522±*55.1*	0.9878	4.067±*12.2*	38.05±*33.1*	0.9841	752.2±*21.3*	19.22±*19.9*

APPENDIX SECTION

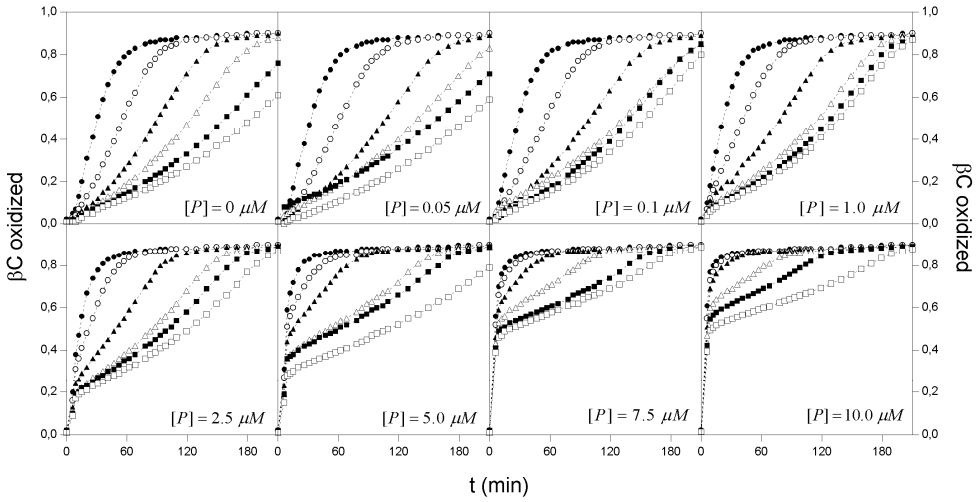

Figure A1: Raw kinetic responses of the combinatory analysis of the antioxidant BHT and the pro-oxidant Fe^{+2} in the βC assay. Each of the eight dose-response graphs corresponds to a different concentration of Fe^{+2} at six different concentrations of BHT.

Table A1: Parametric estimates and confidence intervals (α=0.05) in percentage of the β-Carotene bleaching kinetics as affected by the specified agents, according to model [2]. All the [A] are in μM.

[A]	BELL FUNCTION PARAMETERS				
	P_m	d	t_m	i	r^2
			BHA		
0.5	0.24±*1.9*	1.45±*14.4*	61.34±*2.6*	1.40±*14.5*	0.9947
1.0	0.37±*1.5*	1.07±*17.6*	74.94±*2.5*	1.53±*16.2*	0.9956
1.5	0.45±*1.0*	0.66±*22.3*	84.95±*1.9*	2.17±*19.8*	0.9981
2.0	0.50±*1.2*	0.64±*30.1*	92.55±*2.3*	2.06±*25.6*	0.9973
2.5	0.54±*1.2*	0.50±*38.9*	98.21±*2.2*	2.35±*33.0*	0.9977
3.0	0.57±*1.0*	0.45±*40.3*	101.94±*2.0*	2.54±*34.3*	0.9982
3.5	0.59±*1.3*	0.44±*51.7*	105.88±*2.5*	2.40±*43.2*	0.9973
4.0	0.62±*1.2*	0.46±*49.7*	110.51±*2.4*	2.31±*40.6*	0.9976
4.5	0.63±*1.3*	0.57±*43.6*	112.62±*2.6*	1.83±*33.4*	0.9971
5.0	0.64±*1.2*	0.63±*37.6*	115.33±*2.5*	1.60±*27.5*	0.9975
			BHT		
3.0	0.31±*1.7*	1.63±*11.5*	49.98±*2.1*	1.44±*11.3*	0.9975
6.0	0.40±*1.3*	1.68±*8.5*	56.20±*1.7*	1.27±*8.4*	0.9981
9.0	0.49±*1.0*	1.81±*6.2*	65.27±*1.3*	1.21±*6.1*	0.9987
12.0	0.57±*0.9*	1.72±*6.9*	75.83±*1.4*	1.16±*6.4*	0.9985
15.0	0.62±*1.0*	1.57±*9.4*	82.38±*1.7*	1.15±*8.0*	0.9979
18.0	0.64±*1.0*	1.15±*13.4*	86.64±*1.8*	1.37±*11.1*	0.9979
21.0	0.67±*0.8*	0.88±*14.3*	92.08±*1.5*	1.48±*11.4*	0.9987
24.0	0.69±*0.6*	0.63±*15.5*	97.21±*1.2*	1.77±*12.5*	0.9993
27.0	0.70±*0.7*	0.48±*23.6*	101.72±*1.3*	2.10±*19.4*	0.9992
30.0	0.70±*0.6*	0.37±*28.5*	102.35±*1.3*	2.62±*24.4*	0.9993
			ETX		
0.0004	0.08±*4.9*	1.21±*33.0*	29.03±*7.8*	1.10±*36.4*	0.9802
0.0008	0.18±*2.9*	0.92±*26.6*	32.90±*4.1*	1.92±*28.2*	0.9931
0.0012	0.25±*3.2*	0.92±*31.8*	37.06±*4.4*	2.16±*33.3*	0.9917
0.0016	0.37±*2.9*	1.14±*27.1*	44.53±*3.4*	2.30±*27.9*	0.9934
0.0020	0.44±*2.2*	1.62±*16.6*	53.18±*2.5*	1.75±*16.4*	0.9959
0.0024	0.53±*1.8*	1.97±*11.6*	64.39±*1.9*	1.50±*11.3*	0.9967
0.0028	0.57±*0.8*	1.87±*5.7*	72.90±*1.1*	1.34±*5.6*	0.9990
0.0032	0.65±*1.4*	2.56±*9.0*	85.84±*1.9*	1.09±*7.8*	0.9968
0.0036	0.66±*1.4*	2.21±*10.4*	91.13±*2.0*	1.09±*8.4*	0.9968
0.0040	0.66±*2.4*	2.36±*19.8*	100.54±*3.7*	0.98±*14.0*	0.9901
			TOC		
0.004	0.08±*3.7*	1.19±*32.3*	52.52±*4.4*	2.03±*33.0*	0.9853
0.008	0.16±*3.0*	0.94±*33.0*	56.42±*3.5*	2.59±*34.1*	0.9895
0.012	0.20±*3.7*	1.18±*33.9*	62.61±*4.9*	1.84±*34.4*	0.9805
0.016	0.24±*2.0*	0.95±*24.3*	67.60±*2.7*	2.30±*24.4*	0.9939
0.020	0.28±*1.5*	0.47±*43.6*	74.84±*2.5*	3.83±*42.4*	0.9959
0.024	0.30±*1.8*	0.36±*72.1*	79.77±*3.2*	4.50±*69.3*	0.9941
0.028	0.34±*2.1*	0.65±*48.6*	88.18±*4.0*	2.01±*41.9*	0.9915
0.032	0.36±*1.7*	0.24±*15.3*	92.97±*3.2*	5.28±*18.3*	0.9952
0.036	0.37±*4.2*	0.24±*28.5*	92.97±*8.0*	5.28±*21.1*	0.9891
0.040	0.38±*1.4*	0.18±*15.3*	102.99±*2.6*	6.88±*37.0*	0.9969
			PG		
10.0	0.27±*1.9*	0.86±*20.3*	49.89±*2.9*	1.93±*21.0*	0.9948
20.0	0.32±*1.7*	0.60±*28.2*	55.16±*3.0*	2.37±*28.3*	0.9945
30.0	0.31±*1.6*	0.68±*24.4*	56.51±*3.1*	1.90±*24.1*	0.9945
40.0	0.37±*1.4*	0.39±*40.4*	62.82±*3.0*	2.79±*38.8*	0.9955
50.0	0.37±*1.3*	0.27±*58.9*	64.90±*2.9*	4.08±*57.1*	0.9958
60.0	0.38±*1.5*	0.38±*47.9*	68.48±*3.3*	2.70±*45.0*	0.9948
70.0	0.43±*1.1*	0.43±*34.4*	73.65±*2.6*	2.36±*31.5*	0.9969
80.0	0.42±*1.0*	0.21±*66.1*	72.84±*2.4*	4.74±*63.4*	0.9975
90.0	0.47±*1.3*	0.29±*61.4*	77.83±*3.1*	3.05±*56.6*	0.9960
100.0	0.49±*1.3*	0.01±*33.9*	79.39±*3.0*	6.55±*29.7*	0.9965

Table A2: Parametric estimates and confidence intervals (α=0.05) in percentage of the crocin bleaching kinetics as affected by the specified agents, according to model [2]. All the [P] are in μM.

[P]	BELL FUNCTION PARAMETERS				
	P_m	d	t_m	i	r^2
β-Carotene reaction					
			Fe^{+2}		
1.5	-0.25±*1.6*	1.76±*8.1*	21.98±*3.2*	0.42±*11.6*	0.9983
3.0	-0.28±*16.1*	1.77±*57.9*	21.81±*22.1*	0.43±*93.4*	0.9981
4.5	-0.36±*11.1*	1.57±*55.1*	18.09±*22.8*	0.39±*75.1*	0.9989
6.0	-0.49±*1.2*	1.33±*6.6*	14.06±*2.9*	0.38±*11.1*	0.9992
7.5	-0.54±*1.7*	1.20±*9.9*	12.33±*4.1*	0.40±*17.0*	0.9983
9.0	-0.57±*1.3*	1.18±*7.6*	11.77±*3.5*	0.37±*13.4*	0.9990
10.5	-0.59±*1.4*	1.15±*8.2*	11.19±*3.9*	0.37±*14.6*	0.9989
12.0	-0.61±*1.4*	1.10±*8.5*	10.65±*4.1*	0.38±*15.2*	0.9988
13.5	-0.63±*1.7*	1.05±*11.3*	9.72±*5.0*	0.42±*20.2*	0.9983
15.0	-0.65±*1.5*	1.02±*9.0*	9.91±*4.3*	0.39±*16.3*	0.9987
			Cu^{+2}		
15.0	-0.12±*3.4*	0.92±*29.4*	39.12±*6.1*	1.25±*31.3*	0.9840
30.0	-0.20±*2.5*	1.06±*19.3*	36.68±*4.4*	1.11±*20.7*	0.9922
60.0	-0.28±*2.0*	0.96±*16.1*	32.01±*3.6*	1.16±*17.7*	0.9955
90.0	-0.32±*2.0*	0.89±*16.6*	29.67±*3.6*	1.21±*18.5*	0.9958
120.0	-0.35±*1.9*	0.86±*15.6*	28.12±*3.3*	1.23±*17.6*	0.9965
150.0	-0.36±*2.0*	0.81±*17.2*	26.80±*3.5*	1.27±*19.4*	0.9962
180.0	-0.38±*1.5*	0.83±*12.4*	26.58±*2.6*	1.22±*14.1*	0.9979
210.0	-0.39±*1.2*	0.73±*11.1*	25.38±*2.1*	1.36±*12.6*	0.9986
240.0	-0.40±*1.5*	0.75±*13.6*	25.18±*2.7*	1.32±*15.5*	0.9979
			Hb		
0.2	-0.06-±*25.8*	0.05±*41.4*	32.40±*39.1*	2.27±*47.1*	0.8617
2.0	-0.33-±*4.5*	0.80±*38.6*	25.35±*7.8*	1.27±*44.0*	0.9821
4.0	-0.46-±*3.1*	0.64±*29.7*	18.39±*5.5*	1.28±*35.5*	0.9927
6.0	-0.54-±*2.8*	0.71±*24.4*	14.62±*5.5*	0.88±*32.7*	0.9947
8.0	-0.59-±*2.7*	0.65±*25.9*	12.49±*5.7*	0.85±*36.0*	0.9952
10.0	-0.62-±*2.7*	0.66±*25.7*	10.98±*6.3*	0.72±*38.3*	0.9954
12.0	-0.65-±*2.4*	0.66±*22.9*	9.72±*6.2*	0.64±*36.3*	0.9965
16.0	-0.67-±*2.6*	0.71±*22.1*	8.65±*8.4*	0.51±*38.5*	0.9957
20.0	-0.68-±*2.5*	0.66±*21.6*	7.93±*8.7*	0.51±*38.0*	0.9961
Crocin reaction					
			AAPH		
200.0	-30.8±*0.2*	1.26±*1.5*	533.53±*0.2*	1.63±*1.7*	1.0000
400.0	-47.1±*0.3*	0.96±*2.9*	428.75±*0.4*	1.86±*3.3*	0.9999
600.0	-57.1±*0.4*	0.76±*4.0*	365.61±*0.5*	2.08±*4.6*	0.9999
800.0	-63.9±*0.6*	0.64±*6.5*	323.56±*0.8*	2.24±*7.4*	0.9997
1000.0	-68.7±*0.8*	0.56±*10.5*	293.59±*1.2*	2.34±*11.9*	0.9995
1200.0	-72.4±*1.1*	0.52±*14.9*	271.20±*1.7*	2.36±*17.0*	0.9991
1400.0	-75.2±*1.4*	0.49±*19.2*	253.88±*2.2*	2.31±*22.0*	0.9986
1600.0	-77.3±*1.7*	0.49±*22.7*	240.11±*2.6*	2.19±*26.4*	0.9981
1800.0	-79.1±*1.9*	0.49±*25.3*	228.91±*3.0*	2.03±*29.9*	0.9976
2000.0	-80.4±*2.1*	0.51±*26.8*	219.58±*3.4*	1.85±*32.2*	0.9972

Table A3: Parametric estimates and confidence intervals (α=0.05) in percentage of the crocin bleaching kinetics as affected by the specified agents, according to model [2]. All the [A] are in μM.

[A]	BELL FUNCTION PARAMETERS				
	P_m	d	t_m	i	r^2
			AA		
30.0	39.74±*1.8*	1.60±*11.7*	32.82±*2.2*	1.37±*11.7*	0.9977
60.0	58.75±*1.6*	1.85±*9.3*	44.77±*1.9*	1.25±*9.1*	0.9978
90.0	65.31±*1.4*	1.96±*8.1*	49.50±*1.7*	1.18±*7.7*	0.9982
120.0	68.21±*1.1*	2.07±*6.3*	52.54±*1.4*	1.10±*5.9*	0.9987
150.0	69.80±*1.0*	2.06±*5.8*	54.33±*1.3*	1.10±*5.4*	0.9989
180.0	75.45±*1.2*	2.42±*6.7*	61.41±*1.6*	0.96±*5.9*	0.9982
210.0	74.75±*1.2*	2.33±*7.0*	61.17±*1.7*	0.98±*6.2*	0.9981
240.0	76.89±*1.3*	2.42±*7.4*	64.45±*1.8*	0.93±*6.4*	0.9977
270.0	78.30±*1.4*	2.32±*7.7*	65.79±*1.9*	0.96±*6.7*	0.9975
300.0	79.21±*1.6*	2.46±*8.9*	68.56±*2.2*	0.89±*7.5*	0.9963
			ETX		
3.0	49.56±*2.6*	1.91±*15.9*	36.20±*2.8*	1.44±*15.3*	0.9958
6.0	67.86±*1.5*	2.06±*9.1*	44.16±*1.7*	1.36±*8.6*	0.9983
9.0	80.45±*1.5*	2.36±*8.5*	51.57±*1.8*	1.22±*7.7*	0.9981
12.0	89.36±*1.4*	2.51±*7.7*	58.26±*1.7*	1.13±*6.7*	0.9982
15.0	96.81±*1.4*	2.61±*7.8*	65.42±*1.8*	1.04±*6.6*	0.9976
18.0	101.15±*1.5*	2.63±*8.3*	70.15±*1.9*	0.98±*6.9*	0.9970
21.0	107.01±*1.8*	2.64±*9.9*	78.67±*2.4*	0.91±*8.0*	0.9953
24.0	109.60±*2.1*	2.62±*12.0*	83.55±*2.9*	0.86±*9.4*	0.9931
27.0	111.12±*2.2*	2.58±*13.7*	86.60±*3.3*	0.85±*10.4*	0.9915
30.0	112.40±*2.3*	2.57±*14.6*	87.92±*3.5*	0.83±*10.9*	0.9906
			TROLOX		
18.8	35.12±*1.0*	1.64±*6.6*	52.72±*1.2*	1.46±*6.4*	0.9990
37.5	50.58±*0.5*	1.42±*3.7*	58.65±*0.8*	1.27±*3.6*	0.9996
56.3	62.98±*0.8*	1.09±*8.4*	64.22±*1.4*	1.38±*7.7*	0.9988
75.0	71.96±*0.8*	0.82±*11.1*	69.48±*1.4*	1.64±*10.0*	0.9988
93.8	79.09±*0.9*	0.67±*17.0*	74.20±*1.8*	1.82±*14.9*	0.9984
112.5	82.57±*1.0*	0.60±*21.0*	76.71±*2.0*	1.91±*18.3*	0.9982
131.3	85.83±*0.9*	0.50±*24.9*	80.40±*1.9*	2.15±*21.7*	0.9984
150.0	89.52±*1.0*	0.40±*34.1*	83.91±*2.0*	2.50±*30.0*	0.9983
			TBHQ		
80.0	24.91±*4.0*	2.22±*22.7*	54.80±*5.2*	1.07±*20.7*	0.9831
160.0	29.68±*3.2*	1.98±*19.0*	58.50±*4.4*	1.07±*17.6*	0.9870
240.0	34.27±*3.2*	1.71±*20.9*	60.07±*4.6*	1.16±*19.6*	0.9855
320.0	37.58±*2.7*	1.30±*21.5*	60.48±*3.9*	1.47±*20.6*	0.9894
400.0	41.46±*2.2*	1.31±*17.8*	61.94±*3.4*	1.35±*16.8*	0.9921
480.0	45.75±*1.9*	1.27±*16.7*	63.73±*3.1*	1.31±*15.5*	0.9933
560.0	49.18±*2.1*	1.12±*20.4*	64.88±*3.5*	1.42±*18.9*	0.9919
640.0	51.52±*1.8*	1.18±*17.3*	66.18±*3.1*	1.29±*15.7*	0.9935
720.0	50.87±*2.0*	0.98±*22.9*	67.00±*3.4*	1.63±*21.1*	0.9927
800.0	50.96±*2.0*	1.17±*18.9*	65.09±*3.4*	1.30±*17.3*	0.9922
			Mn^{+2}		
12.5	66.30±*1.0*	0.85±*15.4*	78.43±*1.8*	1.64±*13.1*	0.9981
25.0	82.33±*1.1*	0.76±*21.6*	88.40±*2.1*	1.61±*17.2*	0.9978
37.5	92.88±*1.1*	0.65±*26.4*	93.58±*2.1*	1.74±*20.9*	0.9979
50.0	94.76±*0.7*	0.51±*22.3*	97.93±*1.4*	1.79±*18.1*	0.9992
62.5	99.71±*0.8*	0.60±*21.8*	100.81±*1.5*	1.74±*16.9*	0.9990
75.0	101.64±*0.8*	0.54±*23.2*	102.63±*1.5*	1.86±*18.1*	0.9991
87.5	104.17±*0.8*	0.60±*22.9*	104.04±*1.6*	1.69±*17.3*	0.9990
100.0	106.05±*0.9*	0.61±*24.3*	105.18±*1.7*	1.63±*18.2*	0.9988
112.5	105.36±*0.7*	0.59±*21.4*	105.69±*1.4*	1.69±*16.1*	0.9992

Table A4: Parametric estimates and confidence intervals (α=0.05) in percentage of the β-Carotene bleaching kinetics as affected by the specified agents, according to model [2]. All the [OM] are in μM.

[A]	BELL FUNCTION PARAMETERS				
	P_m	d	t_m	i	r^2
			[P] = 0 μM		
6.0	0.33±*32.9*	1.49±*4.5*	43.8±*7.3*	3.30±*3.4*	0.9994
12.0	0.54±*27.1*	1.50±*5.5*	53.4±*9.8*	2.81±*2.8*	0.9977
18.0	0.63±*21.1*	1.32±*6.2*	61.8±*10.1*	2.68±*2.1*	0.9930
24.0	0.68±*16.9*	0.69±*4.1*	69.3±*16.6*	3.36±*1.0*	0.9933
30.0	0.70±*14.0*	0.31±*2.2*	75.6±*32.0*	5.22±*0.4*	0.9975
			[P] = 0.05 μM		
6.0	0.34±*34.4*	1.42±*4.1*	45.0±*8.3*	3.40±*3.2*	0.9986
12.0	0.57±*28.6*	1.35±*4.7*	56.6±*12.2*	2.92±*2.4*	0.9948
18.0	0.62±*20.8*	0.79±*3.8*	66.0±*16.4*	3.61±*1.2*	0.9896
24.0	0.62±*15.6*	0.11±*0.7*	74.3±*87.8*	16.64±*0.1*	0.9907
30.0	0.70±*14.1*	0.16±*1.2*	77.3±*59.9*	9.06±*0.2*	0.9977
			[P] = 0.1 μM		
6.0	0.34±*33.5*	1.45±*4.3*	44.0±*7.7*	3.19±*3.3*	0.9993
12.0	0.56±*28.0*	1.43±*5.1*	53.8±*11.0*	2.65±*2.7*	0.9960
18.0	0.61±*20.4*	0.83±*4.1*	63.2±*15.0*	3.34±*1.3*	0.9870
24.0	0.66±*16.4*	1.14±*6.9*	68.6±*9.5*	2.61±*1.7*	0.9891
30.0	0.68±*13.5*	0.88±*6.5*	75.9±*10.4*	2.89±*1.2*	0.9883
			[P] = 1.0 μM		
6.0	0.28±*27.6*	1.29±*4.7*	37.7±*5.9*	3.24±*3.4*	0.9996
12.0	0.50±*25.0*	1.36±*5.4*	49.2±*9.2*	2.63±*2.8*	0.9949
18.0	0.62±*20.5*	1.39±*6.8*	57.8±*9.1*	2.27±*2.4*	0.9880
24.0	0.65±*16.2*	1.35±*8.3*	64.2±*7.7*	2.21±*2.1*	0.9849
30.0	0.66±*13.2*	1.29±*9.8*	70.2±*6.7*	2.20±*1.8*	0.9828
			[P] = 2.5 μM		
6.0	0.18±*18.0*	1.21±*6.7*	27.0±*2.7*	3.25±*4.5*	0.9993
12.0	0.38±*19.2*	1.37±*7.1*	43.0±*5.4*	2.36±*3.2*	0.9969
18.0	0.51±*17.1*	1.48±*8.7*	50.8±*5.9*	1.90±*2.9*	0.9867
24.0	0.53±*13.3*	1.40±*10.6*	57.9±*5.0*	1.87±*2.4*	0.9804
30.0	0.59±*11.8*	1.18±*10.0*	65.8±*5.9*	1.90±*1.8*	0.9735
			[P] = 5.0 μM		
6.0	0.13±*12.9*	0.43±*3.4*	21.2±*3.8*	5.80±*2.0*	0.9930
12.0	0.23±*11.7*	1.06±*9.0*	31.6±*2.6*	2.53±*3.3*	0.9962
18.0	0.39±*12.9*	1.36±*10.6*	42.8±*3.7*	1.67±*3.2*	0.9796
24.0	0.40±*10.0*	1.31±*13.1*	50.1±*3.0*	1.63±*2.6*	0.9730
30.0	0.49±*9.7*	0.62±*6.4*	57.3±*7.6*	1.98±*1.1*	0.9661
			[P] = 7.5 μM		
6.0	0.05±*5.0*	1.56±*31.2*	14.3±*0.2*	1.84±*10.8*	0.9859
12.0	0.15±*7.6*	1.30±*17.0*	21.9±*0.9*	1.86±*5.9*	0.9960
18.0	0.23±*7.7*	1.43±*18.6*	32.7±*1.2*	1.62±*4.4*	0.9933
24.0	0.30±*7.4*	1.72±*23.3*	41.8±*1.3*	1.36±*4.1*	0.9824
30.0	0.36±*7.2*	1.76±*24.3*	49.7±*1.5*	1.31±*3.5*	0.9762
			[P] = 10.0 μM		
6.0	0.04±*4.3*	0.22±*5.1*	7.7±*0.8*	3.77±*2.9*	0.9888
12.0	0.12±*5.9*	0.27±*4.6*	12.0±*2.6*	3.96±*2.2*	0.9868
18.0	0.18±*6.0*	1.69±*28.3*	19.5±*0.6*	1.37±*8.7*	0.9966
24.0	0.21±*5.2*	1.68±*32.3*	29.6±*0.6*	1.26±*5.7*	0.9927
30.0	0.27±*5.5*	1.65±*30.3*	38.0±*0.9*	1.21±*4.4*	0.9788

Table A5: Parametric estimates and confidence intervals (α=0.05) in percentage of the crocin bleaching kinetics as affected by the specified agents, according to model [2]. All the [A] are in μM.

[A]	BELL FUNCTION PARAMETERS				
	P_m	d	t_m	i	r^2
OxHLIA ASSAY					
25.0	42.11±*2.1*	1.36±*16.9*	73.37±*2.4*	14.33±*1.6*	0.9980
50.0	73.57±*1.4*	2.85±*7.3*	84.25±*2.5*	7.01±*3.9*	0.9991
75.0	90.82±*2.1*	4.25±*3.8*	93.62±*1.9*	4.37±*10.8*	0.9977
100.0	98.92±*3.0*	3.89±*5.4*	100.40±*2.3*	3.87±*15.0*	0.9948
125.0	99.00±*3.6*	3.49±*6.2*	106.92±*2.2*	3.35±*25.5*	0.9920
ORAC ASSAY					
0.05	30.71±*1.6*	0.95±*17.1*	5.81±*1.6*	3.15±*18.6*	0.9984
0.10	52.16±*1.3*	1.51±*9.9*	8.03±*1.4*	1.98±*10.7*	0.9986
0.20	78.49±*1.9*	1.89±*13.3*	11.62±*2.5*	1.26±*13.7*	0.9946

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