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## **Development of a bivariate mathematical model to characterize simultaneously the dose-time-responses of pro-oxidant agents**

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**Abstract.** *The available data about the interference of oxidation compounds in the oxidation kinetics of process such as lipid oxidation chain reactions, the resistance of pharmaceutical drugs, the effects of free radical agents in cell tissue, the damage caused in DNA, etc, are examples of the many applications for in vivo and in vitro assays. However, often in these bio-assays, only semi-quantitative conclusions can be obtained, due to the use of quantification procedures disregarding kinetic considerations.*

*A pseudo-mechanistic model is proposed which is based on the accumulative Weibull's function, and represents a formal transfer from the field of the dose-response relationships. It allows researchers to obtain the simultaneous solution of a series of oxidation activities as a function of concentration and time. It describes satisfactorily simulations in which reaction compounds interact through a second order kinetic scheme. Its application is simple: it provides parametric estimates, which characterize the oxidative process; facilitates rigorous comparisons between the effects of distinct compounds in different systems; reduces the sensitivity to the experimental error; and its mathematical form constitutes a useful orientation to prepare more economic and efficient trial designs. The model was assayed, firstly, using the kinetic simulation of the oxidative process, and finally, it was applied to a variety of experimental data from other authors in different systems and conditions, obtaining highly satisfactory results in all cases. 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 ( $\text{adj } R^2$ , adjusted coefficient of multiple determination) were up to 0.98.*

**Keywords.** *oxidation reactions; mathematical modeling; non-linear responses; modeling biological processes; process engineering.*

## Introduction

Pro-oxidants are chemicals that induce oxidative stress, either through creating reactive oxygen species or inhibiting antioxidant systems. The oxidative stress produced by these chemicals can damage cells and tissues, for example an overdose of the analgesic paracetamol (acetaminophen) can cause fatal damage to the liver, partly through its production of reactive oxygen species. Some substances can act as either antioxidants, or pro-oxidants, depending on the specific set of conditions. Antioxidants and pro-oxidants are compounds that can retard 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), essential for a healthy life, to counteract various harmful (Hussain et al., 2003) pro-oxidants or reactive species (i.e.  $O_2$ ,  $H_2O_2$ ,  $ROO^\bullet$ ,  $OH^\bullet$ ). 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. Thus, recently the analysis of pro-oxidants activity for the identification of possible pro-oxidant substances that could damage the organism or inhibit the antioxidant effect of compounds have become topics of increasing interest and has become an active field.

Several *in vivo* and *in vitro* methods have been developed for determining the total pro-oxidant capacity of compounds. The capacity of pro-oxidant is frequently determined in competition assays, in which the pro-oxidant and indicators of the reaction (in general an antioxidant) compete for the reactive species. Competition assays are performed to describe pro-oxidant or antioxidant capacity and to rank the affinity of them 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. The current methods to test the pro-oxidant capacity still have left many open questions (Frankel and Meyer, 2000). The *in vitro* assays can only rank pro-oxidant 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 further developed the approaches and have tended to standardize the protocols to increase the effectiveness of methods for *in vitro* and *in vivo* responses.

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 significant controversy (Frankel, 1993; Frankel, 1994; Huang et al., 2005; Koleva et al., 2002; Laguerre et al., 2007; Naguib, 2000; Roginsky and Lissi, 2005). Commonly, the mathematical determinations of the OM capacity are based on a fixed endpoint without proper considerations of the kinetic behavior. The most typical and incorrect practice is to use the single-time dose-response of a well-known pro-oxidant 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 aspects as true. Their use today can be considered unreasonable, given the availability of computational applications and new instrumentation equipment that, when combined, provide the adequate tools to work with different variables.

In the current work, a simple non-linear mathematical application for competitive pro-oxidant assays, in which the responses have one common asymptote (the majority of them) is presented. It helps to describe accurately the response as a function of time and dose by two criteria values and allows one to easily perform 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.

## Material and Methods

### Equipment and reagents

- *Equipment:* Multiskan Spectrum Microplate Photometers from Thermo Fisher Scientific; Thermo Scientific Nunc 96-Well Polypropylene MicroWell Plate with flat bottom.
- *Main reagents of bleaching  $\beta$ -caroteno method:* cis, cis-9,12-octadecadienoic acid (Linoleic acid);  $\beta$ -caroteno; Polyoxyethylenesorbitan monopalmitate (Tween 40).
- *Main reagents for the bleaching crocin method:* crocin and 2,2'-azobis-(2-amidinopropane)dihydrochloride (AAPH or ABAP).

### $\beta$ -carotene bleaching method

The method described by Marco (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  $\beta$ 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, improved and applied to pro-oxidant compounds (Prieto et al., 2012).

The reagent is prepared by dissolving 4 mg of  $\beta$ -carotene ( $\beta$ C), 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<sub>2</sub> 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 final concentration of  $\beta$ C in the final solution of the reaction is 1  $\mu$ M.

The procedure is performed by adding 50  $\mu$ l of sample and 250  $\mu$ l 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 pro-oxidants standards and samples are analyzed kinetically for different doses.

### **Crocin bleaching method**

Crocin reagent was prepared by dissolving 4 mg of crocin (100  $\mu$ M in the final reaction) in 30 mL of 100 mM Briton buffer (pH=5.5) in Mili-Q water at 40 °C. The absorbance thus prepared at 450 nm should be ~1.400, but is very dependent on the origin and conservation state of crocin.

The procedure to assess the action of a pro-oxidant is performed in a preheated (37°C) microplate (96 wells, 350  $\mu$ l) containing 250  $\mu$ l of crocin reagent in each well and 50  $\mu$ l of sample solution in water:ethanol (9:1) which was added in triplicate at the start. 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). In addition to the sample set in study, the microplate must contain:

- a) A series (calibration) in which sample is replaced by a standard pro-oxidant, in water:ethanol (9:1), at the concentrations necessary to obtain at least a maximum bleaching of 50%.
- b) Three wells (blank) in which the sample is replaced by solvent.
- c) Three wells (control) with a reagent without AAPH and sample replaced by solvent. Thus, spontaneous bleaching of crocin is quantified for correction purposes.

### **Standard pro-oxidant compounds for an illustrative analysis**

Lipophilic and hydrophilic pro-oxidants are presented here for an illustrative analysis of the full capabilities of the developed quantification criteria for competitive assays. 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<sup>+2</sup>): 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) Porcine haemoglobin (Hb): is 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<sup>+2</sup>): 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.

## Numerical and statistical methods

Fitting of 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-squares (quasi-Newton) method provided by the macro *Solver* in *Microsoft Excel* 2003. It allows quick testing of hypotheses and display of its consequences. Subsequently, 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' macro, previously used (Prieto et al., 2011) and is freely available from de Levie's Excellaneous website:

<http://www.bowdoin.edu/~rdelevie/excellaneous/>

## Results

At first, for example, experimental data are used to illustrate the capabilities of the method, and afterwards, the quantification and comparative method was applied to different combinations of pro-oxidants compounds in two competition assays (the  $\beta$ C and Cr bleaching reactions).

### Illustration of the mathematical procedure and simple analytical criteria to compare the time-dose-response of compounds

In competitive assays, performed in an open system, 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 pro-oxidants. 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.

As an example, data obtained in the  $\beta$ C bleaching reaction are used to illustrate the methodological procedure to compute the capacity of the pro-oxidant  $\text{Fe}^{+2}$  as a function of time and dose.

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

$$RD(t) = P_t - C_t \quad (1)$$

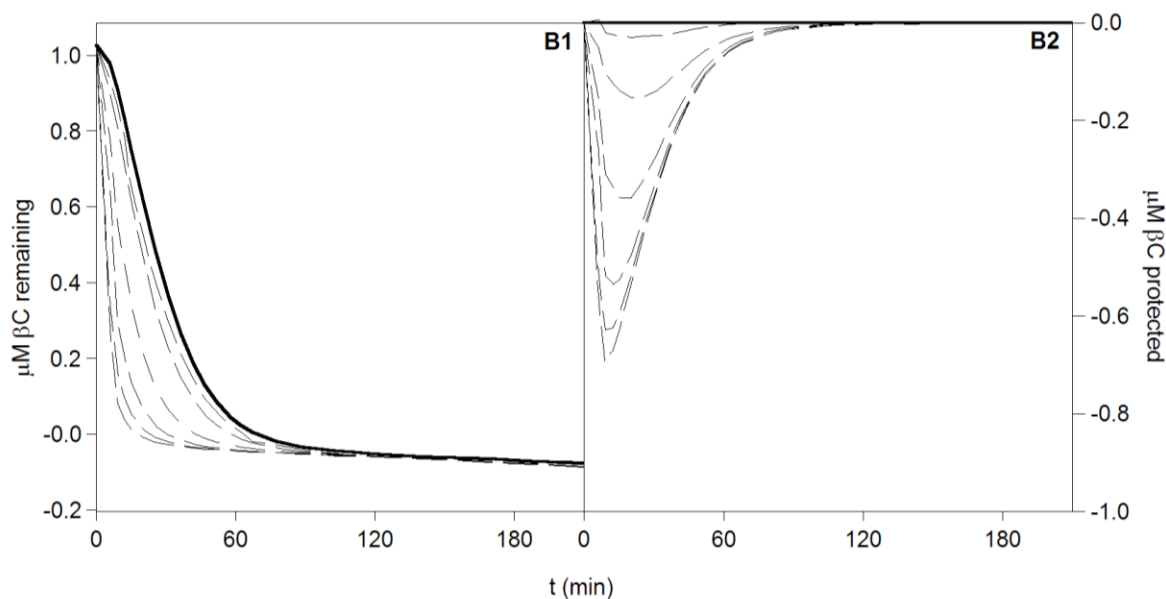
in which *P* and *C* are the kinetic response in the presence and absence (control) of an oxidation modifier concentration, respectively. *RD* is the relative difference found at any given time (*t*), which in this case accounts for the amount of  $\mu\text{M}$  of  $\beta\text{C}$  oxidized by the *P* agent.

In Figure 1, an illustrative representation of the characteristic profiles obtained by  $\beta\text{C}$  bleaching reaction using a time-dose-response of the antioxidant of the pro-oxidant  $\text{Fe}^{+2}$  is presented. Observing the response (Figure 1B1), it is obvious to mention 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 pro-oxidant, depending on the time selected. Alternatively, as in different fields of study, the traditional option is the analysis with S-shaped equations, producing several parameters that characterize in detail the remaining  $\beta\text{C}$  molecules response through the lag-time period, the time required for reaching half maximum response, the maximum bleaching velocity, etc. However, in this proposal, the kinetic relative difference response, exhibits an asymmetric bell profile (see Figure 1B2), which is equivalent to the substrate molecules oxidized. Such profiles show many different physical kinetic properties that could characterize the response. Among these physical properties, the maximum oxidized molecules of  $\beta\text{C}$  ( $P_m$ ) and the time at which it takes place ( $t_m$ ) are the most characterizing parameters that cannot be found through traditional equations.

This bell profile can be easily described by many bell functions (Di Marco and Bombi, 2001). After an extensive evaluation, in which several equations were tested, the generalized exponential function without intercept (also called the modified weibull distribution function) was found to be the most satisfactory one with less number of parameters and higher accuracy:

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

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 oxidized molecules of the substrate used in the reaction (in our experimental examples  $\beta\text{C}$  and Cr) and  $t_m$  the time at which  $P_m$  takes place.



**Figure 1. Illustrative representation of the characteristic profiles obtained for pro-oxidant responses using equation [1] to standardize in the  $\beta$ -carotene ( $\beta$ C) bleaching reaction as examples. B1 shows the raw responses of the  $\beta$ C reaction as function of time and B2 the asymmetric bell profile of the kinetic relative difference response.**

This model provides explicitly 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 2A shows the application of this model to predict the effect of  $\text{Fe}^{+2}$  in the  $\beta$ C reaction. All the parametric values are presented as part of Table 2, showing lower confidence intervals ( $\alpha=0.05$ ) and higher correlation coefficients in all cases ( $r^2>0.98$ ), 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 combined variations have a relevant characterizing value.

On one hand, plotting the  $P_m$  parameter against pro-oxidant concentration shows an asymptotic trend, suggesting that some radical-generating property of the system can be saturated (Gieseg and Esterbauer, 1994). This type of dose-response patterns, in general, can be adjusted to the following asymptotic function:

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

where  $[P]$  is the concentration of the  $P$  agent under study in  $\mu\text{M}$ ,  $P_m(P)$  is the response behavior of the parameters  $P_m$  as a function of  $[P]$ ,  $K$  is the asymptotic value of the parameter obtained ( $\mu\text{M}$  of the substrate oxidized) and  $r$  is the specific dose-rate ( $\mu\text{M}^{-1}$  of  $P$ ).

On the other hand, the  $t_m$  parameter shows a linear dose-response trend with an intercept that can be easily adjusted to:

$$t_m(P) = t_0 + b[P] \quad (4)$$

where  $b$  is the slope ( $\text{min}/\mu\text{M}$  of  $P$ ) 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  $P$ , in other words the extension time produced by  $1 \mu\text{M}$  of  $\beta$ C.

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  $[P]$ , obtaining in all cases highly consistent results with satisfactory confidence intervals ( $\alpha=0.05$ ), thus demonstrating the reliability of this second approach.

Therefore, these values can be used to compare the activities of different  $P$  agents. The information provided by the combination of both values represents an especially robust tool to compare the activities of different pro-oxidant agents based on the parametric estimations time-dose-dependent. With both values, an intuitive solution to compare  $P$  activities of compounds by a mathematical analysis is obtained, offering researchers an alternative robust solution based on parametric non-linear values to assess  $P$  action and compare their capacity rigorously.

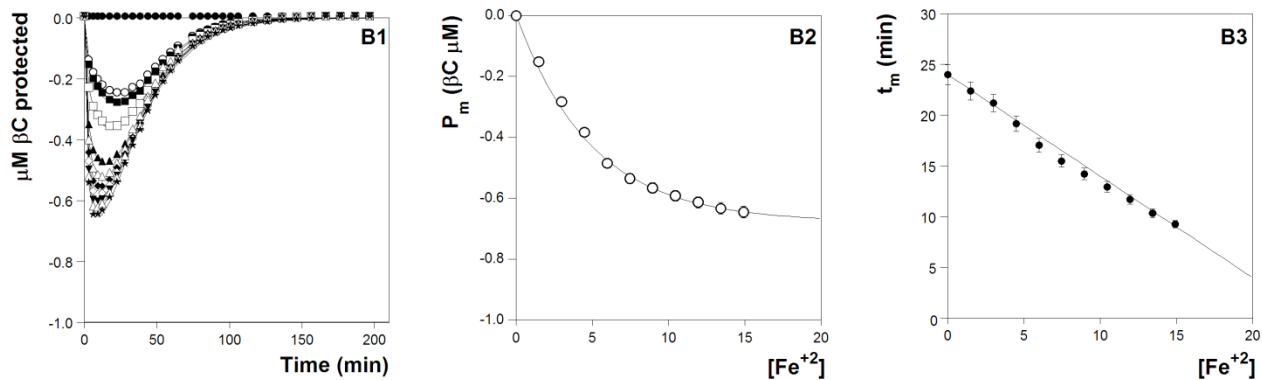


Figure 2. The kinetic parameters that could characterize the response the maximum oxidized / protected molecules of  $\beta\text{C}$  ( $P_m$ ) and the time at which it takes place ( $t_m$ ) are displayed. B1 shows the fittings to the asymmetric bell profile of the kinetic relative difference dose-response of the examples presented in Figure 1 to the model [2]. B2 shows the maximum protected molecules of  $\beta\text{C}$  ( $P_m$ ) fitted to the equation [3]. B3 displays 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 are in Table 1 and Table 2.

Table 1. Parametric estimates and confidence intervals ( $\alpha=0.05$ ) in percentage of the crocin bleaching kinetics as affected by the specified agents, according to model [2]. All the [P] are in  $\mu\text{M}$ .

[P]	BELL FUNCTION PARAMETRES				
	$P_m$	$d$	$t_m$	$l$	$r^2$
<b><i><math>\beta</math>-Carotene reaction</i></b>					
<b><math>\text{Fe}^{+2}</math></b>					
1.5	$-0.25 \pm 1.6$	$1.76 \pm 8.1$	$21.98 \pm 3.2$	$0.42 \pm 11.6$	0.9983
3.0	$-0.28 \pm 16.1$	$1.77 \pm 57.9$	$21.81 \pm 22.1$	$0.43 \pm 93.4$	0.9981
4.5	$-0.36 \pm 11.1$	$1.57 \pm 55.1$	$18.09 \pm 22.8$	$0.39 \pm 75.1$	0.9989
6.0	$-0.49 \pm 1.2$	$1.33 \pm 6.6$	$14.06 \pm 2.9$	$0.38 \pm 11.1$	0.9992
7.5	$-0.54 \pm 1.7$	$1.20 \pm 9.9$	$12.33 \pm 4.1$	$0.40 \pm 17.0$	0.9983
9.0	$-0.57 \pm 1.3$	$1.18 \pm 7.6$	$11.77 \pm 3.5$	$0.37 \pm 13.4$	0.9990
10.5	$-0.59 \pm 1.4$	$1.15 \pm 8.2$	$11.19 \pm 3.9$	$0.37 \pm 14.6$	0.9989
12.0	$-0.61 \pm 1.4$	$1.10 \pm 8.5$	$10.65 \pm 4.1$	$0.38 \pm 15.2$	0.9988
13.5	$-0.63 \pm 1.7$	$1.05 \pm 11.3$	$9.72 \pm 5.0$	$0.42 \pm 20.2$	0.9983
15.0	$-0.65 \pm 1.5$	$1.02 \pm 9.0$	$9.91 \pm 4.3$	$0.39 \pm 16.3$	0.9987
<b><math>\text{Cu}^{+2}</math></b>					
15.0	$-0.12 \pm 3.4$	$0.92 \pm 29.4$	$39.12 \pm 6.1$	$1.25 \pm 31.3$	0.9840
30.0	$-0.20 \pm 2.5$	$1.06 \pm 19.3$	$36.68 \pm 4.4$	$1.11 \pm 20.7$	0.9922
60.0	$-0.28 \pm 2.0$	$0.96 \pm 16.1$	$32.01 \pm 3.6$	$1.16 \pm 17.7$	0.9955
90.0	$-0.32 \pm 2.0$	$0.89 \pm 16.6$	$29.67 \pm 3.6$	$1.21 \pm 18.5$	0.9958
120.0	$-0.35 \pm 1.9$	$0.86 \pm 15.6$	$28.12 \pm 3.3$	$1.23 \pm 17.6$	0.9965
150.0	$-0.36 \pm 2.0$	$0.81 \pm 17.2$	$26.80 \pm 3.5$	$1.27 \pm 19.4$	0.9962
180.0	$-0.38 \pm 1.5$	$0.83 \pm 12.4$	$26.58 \pm 2.6$	$1.22 \pm 14.1$	0.9979
210.0	$-0.39 \pm 1.2$	$0.73 \pm 11.1$	$25.38 \pm 2.1$	$1.36 \pm 12.6$	0.9986
240.0	$-0.40 \pm 1.5$	$0.75 \pm 13.6$	$25.18 \pm 2.7$	$1.32 \pm 15.5$	0.9979

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**Hb**

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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

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**Crocin reaction**

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**AAPH**

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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

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**Verification of the quantification procedure when applied to assess and compare several pro-oxidant agents in two different competitive assays**

The capabilities of the developed model was tested by evaluating the dose-time-dependency of several agents on two of the best known competition assays responses, the  $\beta$ C (Marco, 1968) and Cr bleaching (Bors et al., 1984) reactions. 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.  $\beta$ C is a lipophilic oxidizable substrate, which is able to join the system of lipid micelles and the corresponding oxidation reaction is accomplished in a lipid environment. On the contrary, Cr is a hydrophilic oxidizable substrate.

Figure 3B1 shows the results of the proposed approach for the  $\beta$ C bleaching reaction applied to three common lipid pro-oxidants. Figure 3B2 shows the parametric estimates of the kinetic fittings the results of the proposed approach for the Cr bleaching reaction applied to AAPH pro-oxidant compound. Table 1 shows the parametric estimates of the time-dose fittings of equation [2]. In Table 2 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 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, the correlation coefficient ( $r^2$ ) between predicted and observed values was always > 0.98, with the vast majority of the fittings superior at 0.99.

Table 2. Parametric estimates of equations [3] and [4] obtained after fitting the parametric results ( $P_m$  and  $t_m$  parameters) of equation [2] for the crocin and  $\beta$ -Carotene bleaching kinetics as affected by the specified agents respectively

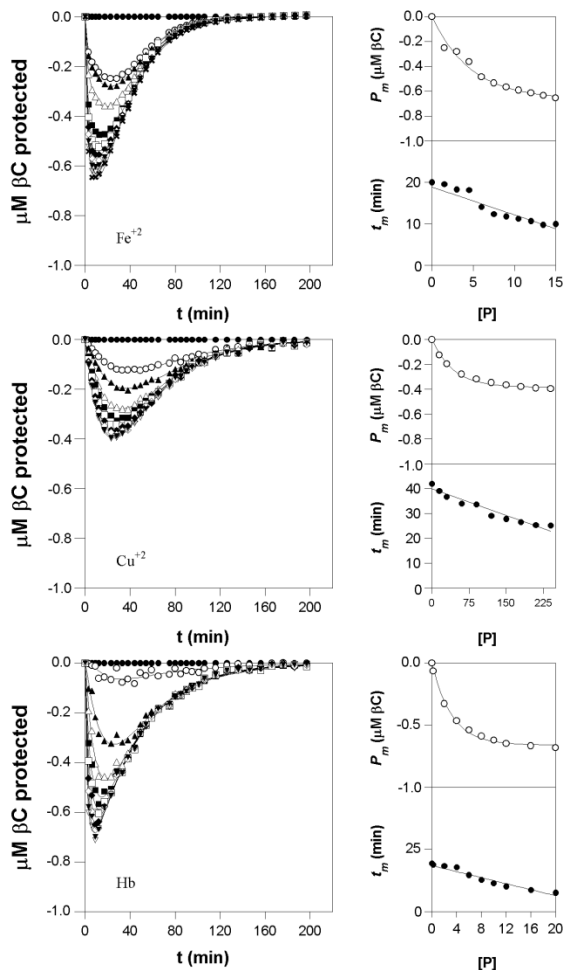
[P]	Parameters of $P_m$ (P)					Parameters of $t_m$ (P)				
	$K$		$r$		$r^2$	$t_0$		$B$		$r^2$
<b>B-CAROTENE ASSAY</b>										
<b>Fe<sup>+2</sup></b>	-0,668	$\pm 2,3$	0,212	$\pm 1,5$	0,9880	18,87	$\pm 2,8$	-0,671	$\pm 3,1$	0,9991
<b>Hb</b>	-0,659	$\pm 1,3$	0,313	$\pm 2,2$	0,9921	18,55	$\pm 4,5$	-0,61	$\pm 4,4$	0,9955
<b>Cu<sup>+2</sup></b>	-0,383	$\pm 7,8$	0,022	$\pm 3,4$	0,9902	41,10	$\pm 2,6$	-0,071	$\pm 3,6$	0,9976
<b>CROCIN ASSAY</b>										
<b>AAPH</b>	-77,82	$\pm 9,1$	0,002	$\pm 8,2$	0,9991	436,3	$\pm 4,2$	-0,123	$\pm 3,6$	0,9970

## Discussion

The reduction of a complex process to linear forms has little justification today, given the availability of computational resources for calculation, and microplate readers that provide adequate data to work with non-linear models. On the other hand, the use of rate equations and mass balances do not solve the problem because it does not directly provide values of practical interest, and the absence of analytical expressions makes it very difficult to calculate.

The equations [3] and [4] and the obtained parametric structures that precisely describe all the studied kinetic profiles, can characterize the pro-oxidant activity more reliably, consistently and in detail compared to the literature outlined in the references. The approach proposed here represents an intermediate position which can be reliable and easily managed: 1) to describe with precision the kinetic profiles detected; 2) to obtain reproducible characterizing values of practical interest, 3) to incorporate consistently, if necessary, environment variables that modify the process, 4) to deduce mechanistic details that can be verified by other methods.

### B1: TIME-DOSE ANALYSIS OF $\beta$ C ASSAY



### B2: TIME-DOSE ANALYSIS OF CROCIN ASSAY

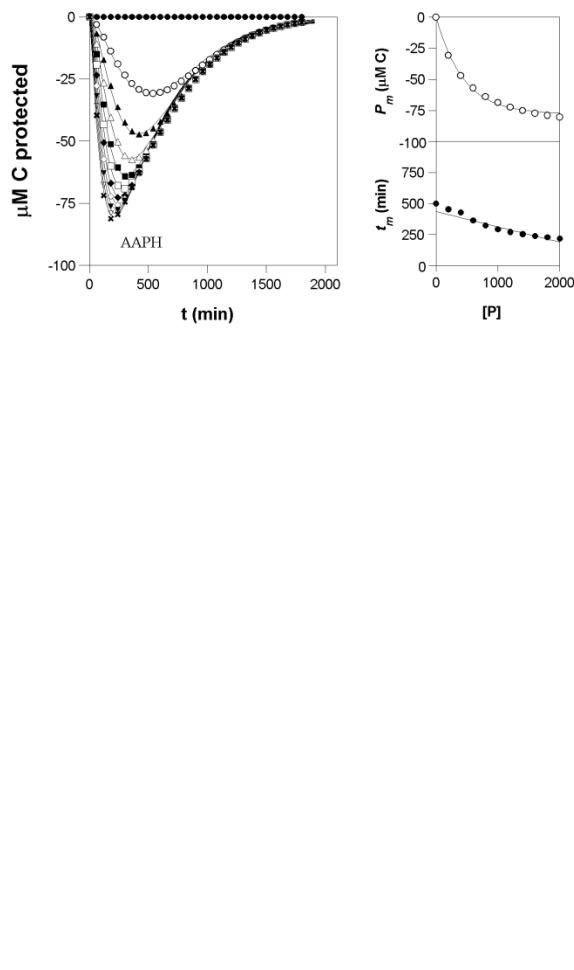


Figure 3. Experimental results for the  $\beta$ -carotene and crocin bleaching reaction. Each figure is divided as follows: on the left side, the time protection profiles drop orderly with the increase of the agent concentrations and are fitted to equation [2] and on the right side, the  $P_m$  and  $t_m$  parameters pattern are shown and fitted to the equations [3] and [4] respectively. All numerical results are in Table 1 and Table 2.

## Conclusion

The complexity of the topic of pro-oxidants plus the confusion introduced by improper use of questionable methods can lead to disarray in the research community and in 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 pro-oxidants 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 their behavior and a more efficient way to determine the total capacities than those techniques at a fixed point.

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