

## $\beta$ -Carotene Assay Revisited. Application To Characterize and Quantify Antioxidant and Prooxidant Activities in a Microplate

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**S** Supporting Information

**ABSTRACT:** The  $\beta$ -carotene bleaching assay, a common method for evaluating antioxidant activity, has been widely criticized due to its low reproducibility, problematic quantification, complex reagent preparation, and interference of different factors (temperature, pH, solvents, and metals). In this work we have examined the effects of these factors and developed a highly reproducible procedure for microplate assay, evaluated the critical points of the method, and proposed a kinetic model for quantifying both antioxidant and prooxidant activities. The application of these tools produced very consistent results, which provide robust and meaningful criteria to compare in detail the characteristics of several well-known commercial antioxidants, as well as several predictable prooxidants, and can be easily applied to natural extracts, food samples, and many other type of compounds. As an example, we have tested a set of commercial antioxidants and some typical lipophilic prooxidants. The activity of the tested antioxidants decreased in the following order: ethoxyquin  $\gg$   $\alpha$ -tocopherol > butylhydroxyanisole > butylhydroxytoluene  $\gg$  propyl gallate. On the other hand, hemoglobin and  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Co}^{2+}$ , and  $\text{Cu}^{2+}$  showed a strong prooxidant effect, and the activity was null in  $\text{Cd}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Sr}^{2+}$ , slightly antioxidant in  $\text{Mg}^{2+}$ , and strongly antioxidant in  $\text{Zn}^{2+}$  and  $\text{Mn}^{2+}$ .

**KEYWORDS:** antioxidant activity,  $\beta$ -carotene method, mathematical modeling, Weibull equation

### INTRODUCTION

Methods to quantify the antioxidant or prooxidant activity of a given agent (hereafter oxidation modifier, OM) are numerous, diverse, and dependent on the oxidizable substrate, the OM, and the whole system (aqueous, lipidic, emulsion, multiphase). Under these conditions, the effort has been focused on multiplying assessment methods (it is common to apply 3–6 different procedures in each evaluation), and less attention has been paid to their meaning, optimization, and standardization. Moreover, the same method is frequently performed through different experimental protocols (see Table 1) and calculation procedures. Consequently, in the past decade many authors have claimed to unify the approaches<sup>1–10</sup> and have proposed standardized criteria to assess the real effectiveness of an antioxidant, both in vitro and in vivo.<sup>11–13</sup>

The main antioxidants used in the food industry inhibit lipid oxidation by acting as metal chelators (such as ethylenediaminetetraacetic acid) or hydrogen donors (such as butylhydroxyanisole (BHA)). In the latter case, one of the most common methods for assessing antioxidant activity is the  $\beta$ -carotene ( $\beta$ C) bleaching assay.<sup>14,15</sup> 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 acid,<sup>7</sup> promoted by thermal induction, typically at 50 °C. Quantification is based on varying the rate at which  $\beta$ C absorbance decays ( $\sim$ 470 nm) in the presence of increasing concentrations of the antioxidant under evaluation. Although this method is widely used, it has been criticized for many different reasons.

First, it has been argued that the  $\beta$ C reaction is not representative of the lipid oxidation in foods, where the fatty

acids are mainly present as triglycerides<sup>1,7</sup> and whose crude extracts can contain interfering materials<sup>1</sup> and even other OM agents, potentially causing synergistic or antagonistic interactions, which have not been studied yet in depth.<sup>16,17</sup>

Second,  $\beta$ C is sensitive to oxygen and temperature even in the absence of linoleic acid.<sup>18,19</sup> Moreover, in a lipid emulsion, the hydrophobic repulsion phenomena favor the activity of the apolar OMs against the polar ones (polar paradox<sup>20</sup>).

Finally, the assay reproducibility can be low due to (a) the reagent complexity,<sup>4</sup> (b) the nonspecific conditions of heat induction,<sup>5,8</sup> which has led to suggest the use of free radicals (e.g., 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) or  $\text{Fe}^{2+}$ ) as initiators, (c) the frequent use of a single reaction time, which does not guarantee the reliability of the results,<sup>5,9</sup> and (d) the effects on the reaction of factors such as pH, solvent ratios, and the presence of metals,<sup>21</sup> which can be especially relevant in complex systems.

Some of these criticisms are simply due to the impossibility of defining a universal method that would be suitable for any antioxidant in any system. Others are related to the lack of attention paid to the variables affecting the process. Finally, other problems derive from formal inadequacies. Often the convenience of simple assessment routines has taken priority over other considerations, despite the advisability of using mechanistic or empiric kinetic models as indicated by different authors.<sup>22–25</sup> MO agents interfere with a process which is

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Table 1. Main Conditions Commonly Used in the  $\beta$ C Method<sup>a</sup>

[ $\beta$ C] ( $\mu\text{g}\cdot\text{mL}^{-1}$ )	component relationships			T ( $^{\circ}\text{C}$ )	R:S (v/v, mL)	source
	$\beta$ C	LA	Tw			
12.00	1	150.00	333.3	50	50:2	Marco (1968) <sup>14</sup>
40.00	1	0.09	1000	50	5:0.2	Miller (1971) <sup>15,b</sup>
4.00	1	0.11	1000	55	0.25:0.03	Dapkevicius et al. (2010) <sup>21</sup>
5.00	1	36.00	200	50	0.25:0.03	Mikami et al. (2009) <sup>39</sup>
6.67	1	0.11	1000	45	0.25:0.05	present work

<sup>a</sup>The ratios between reagent components ( $\beta$ C =  $\beta$ -carotene, LA = linoleic acid, Tw = Tween-40, R = reagent, and S = sample) are established on the  $\beta$ C basis, whose concentration is specified in the first column. <sup>b</sup>We have assumed that the original work contains a typo in the units which requires dividing by 1000 the concentrations of  $\beta$ C and LA specified in that report.

necessarily asymptotic and generally sigmoidal. In this context, one can look for conditions that hide this fact and make acceptable linear approximations based on activities measured at a single time, but even if such a case is possible, useful information is lost and a relevant characterization of the studied phenomenon cannot be claimed. In fact, it has been repeatedly pointed out that the use of simple quantification criteria (general linear responses) not only generates serious reproducibility problems, preventing meaningful comparisons<sup>1,4,5,7,8,16,26</sup> between the samples under evaluation, but also makes the identification of the key critical points and the standardization of the assessment conditions<sup>21</sup> difficult.

This work starts from accepting that the oxidation and its inhibitions are only accurately described by sigmoidal profiles. Therefore, one should expect that the usual working conditions would not be necessarily coincident with those used when the implicit hypothesis is a linear process. The present revision of the critical points of the method, the proposed microplate assay, and the use of a robust mathematical tool allow the response to be studied in detail, providing parameters whose factual meanings are able to characterize antioxidant and prooxidant activities in a consistent, practical, useful, and reproducible way.

## MATERIALS AND METHODS

**Equipment and Reagents.** *Equipment.* Multiskan Spectrum microplate photometers from Thermo Fisher Scientific and a 96-well polypropylene microwell plate with a flat bottom were used.

*Main Reagents.* Linoleic acid,  $\beta$ C, and Tween 40 were the main reagents.

*Antioxidant Agents.* BHA, butylhydroxytoluene (BHT), 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline (ethoxyquin), propyl 3,4,5-trihydroxybenzoate (propyl gallate), and (2R)-2,5,7,8-tetramethyl-2-[(4R,8R)-(4,8,12-trimethyltridecyl)]-6-chromanol ( $\alpha$ -tocopherol) were the antioxidants.

*Predictably Prooxidant Agents.* Iron(II) and iron(III) sulfides, copper(II) sulfate, magnesium sulfate, manganese sulfate, cadmium nitrate, nickel nitrate, zinc chloride, and hemoglobin were the prooxidants.

All reagents and chemicals were purchased from Sigma S.A. (St. Louis, MO).

**$\beta$ -Carotene Bleaching Method.** *Reagent and Procedure Conditions.* The method described by Marco<sup>14</sup> is the reference for many subsequent modifications that simplified the operative<sup>15</sup> or transferred the procedure to a microplate.<sup>4</sup> Some of these modifications involved differences in the preparation and preservation of the reagents, pH, temperature, and sometimes solvents, which prevent direct comparison between the experimental results (see Table 1).

*Quantification.* According to Marco,<sup>14</sup> when the absorbance at 470 nm is measured at increasing times in the presence of increasing concentrations of an antioxidant, the response can be assessed through the percentage of the extended induction time ( $E_t$ ) defined as

$$E_t (\%) = \frac{t_n - t_0}{t_{\text{ref}} - t_0} \times 100 \quad (1)$$

where  $t_0$ ,  $t_n$  and  $t_{\text{ref}}$  are the times at which the  $\beta$ C concentration is  $p\%$  of the initial one in the absence of antioxidant, in the presence of a given concentration of the tested antioxidant, and in the presence of a reference antioxidant, respectively. The author averaged out the values for  $p = 50$  and  $p = 70$  and recommended to linearize (using logit paper) the sigmoidal response obtained. He also noted that for some common antioxidants the relationship between antioxidant concentration and its effect on the  $\beta$ C extended induction time was close to linear.

**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 of the Microsoft Excel 2003 spreadsheet. 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,<sup>27</sup> freely available from de Levie's Excellaneous Web site (<http://www.bowdoin.edu/~rdelevie/excellaneous/>). The SolverStat macro<sup>28</sup> 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 as<sup>29,30</sup>

$$A_f = 10^{\sum \log(\text{pred}/\text{obsd})/n} \quad \text{and} \quad B_f = 10^{\sum \log(\text{pred}/\text{obsd})/n} \quad (2)$$

where "pred" and "obsd" are the predicted and experimental values, respectively, and  $n$  is the number of observations. The nearer the values of  $B_f$  and  $A_f$  are to 1, the better the fitting of the experimental data to the model.

## RESULTS AND DISCUSSION

The oxidant action implies interfering in an autocatalytic process in which no less than five chemical species are present (oxygen, oxidizable substrate, antioxidants, and oxidation products). Additionally, reactions of first and second order can take place, and interactions can occur at several levels of the sequence. Under these conditions, the apparently simple assays, routinely applicable with minimal calculation requirements, can misunderstand the effects of some factors that modify the response (linoleic acid, antioxidant concentration, pH, and temperature, among others). These effects have induced in some cases overstandardization of the protocol and in some others overlooking of aspects that need to be standardized.

These factors shall be revised in conditions that were selected with three purposes: to obtain well-defined kinetic profiles, to avoid excessive differences with regard to the conditions reported by other authors (see Table 1), and to define a method able to analyze both antioxidant and prooxidant

responses. Although the revised factors will be independently considered, it should be kept in mind that the existence of interactions among them implies that, in practice, a given domain in one of them can restrict the viable domain of some others. Next the assay performances used in this work are described, and the differences from other alternatives are discussed and justified.

**Assay Performances: Reagent, Procedure, Analytical Time, and Quantification Criterion.** *Reagent.* A common criticism of the  $\beta$ C method is the lack of reproducibility due to the complexity and instability of the reagent. Some authors<sup>31</sup> store a stock solution of  $\beta$ C and Tween in chloroform at  $-16\text{ }^{\circ}\text{C}$  and add linoleic acid at the time of use. Under these conditions the stock solution becomes concentrated by evaporation and significantly bleached in  $\sim 40$  h. The daily preparation of the reagent corrects this problem, but causes variations in the initial conditions.<sup>4</sup> The procedure described next will provide enough reagent for testing 2400 samples and should be stable at least for one week, without significant changes in the initial conditions.

$\beta$ -Carotene (4 mg), linoleic acid (0.5 mL), and Tween-40 (4 g) were mixed in 20 mL of chloroform in a round-bottomed flask, with vigorous shaking of the suspension. The chloroform was evaporated in a rotary evaporator at a temperature lower than  $50\text{ }^{\circ}\text{C}$  in a short period of time to avoid the beginning of the lipid oxidation process as much as possible. Inefficient chloroform evaporation leads to high turbidity values that cause confusing results. The oily residue was distributed in aliquots of 1 mL in 30 mL tubes, which were preserved in the dark at  $-18\text{ }^{\circ}\text{C}$  under nitrogen. A single tube provides sufficient reagent to fill a microplate (120 wells), which is obtained by adding 30 mL of 100 mM Britton buffer, pH 6.5, in preheated Milli-Q water at the assay temperature ( $45\text{ }^{\circ}\text{C}$ ). The absorbance at 470 nm of the reagent thus prepared is  $\sim 1.4$ , a value that remains stable for a week. Incidental higher values should not be corrected by dilution, to avoid changes in the proportions of the emulsion.

*Procedure.* The procedure was performed in a 96-well microplate with flat-bottom wells (well capacity 330  $\mu\text{L}$ ) by combining 50  $\mu\text{L}$  of the sample to be tested and 250  $\mu\text{L}$  of reagent. The reader device was programmed to  $45\text{ }^{\circ}\text{C}$  with agitation (660 sections/min and 2 mm amplitude). In addition to the sample under evaluation, the microplate must contain the following.

- A series (calibration) in which the sample is replaced by a reference OM in the selected solvent (water:ethanol = 9:1) at increasing concentrations.
- Two or three wells (control) in which the sample is replaced by solvent.
- If the effect of  $\beta$ C bleaching in the absence of linoleic acid should be taken into account, two or three wells (blank) in which linoleic acid and sample are replaced by solvent.
- If sample or calibration antioxidant absorbs at 470 nm, an additional series (correction) in which the reagent is replaced by solvent.

*Analytical Time.* The analytical time is a nontrivial aspect. Short analysis times ( $\sim 50$  min) prevent the kinetic definition of the process, whereas longer times ( $\sim 500$  min) enhance the effect of factors such as solvent evaporation and thermal discoloration of  $\beta$ C. When the microplate reader was configured to measure the absorbance at intervals of 3, 5, and 10 min (initiation, propagation, and asymptotic phase) for 200

min, highly consistent and reproducible data sets were systematically obtained.

On the other hand, as we will see later, the theoretical limit of the half-life extension promoted by an antioxidant is  $+\infty$ , but the limit of half-life contraction promoted by a prooxidant is zero. Therefore, a progressive packing of the kinetic profiles takes place in this last case, which can make accurate evaluations difficult. Conditions that allow a control half-life of  $\sim 35$  min were found as optimal for analyzing both antioxidant and prooxidants. If especially efficient prooxidant determination is necessary, a simple way to achieve it consists of increasing the half-life of the control by reducing the temperature of the assay (at  $35\text{ }^{\circ}\text{C}$ , the half-life is  $\sim 115$  min).

*Quantification.* Quantification was carried out using as a basis the Murado and Vázquez model,<sup>25</sup> which describes the whole kinetic profile by applying the Weibull mass function.<sup>32</sup> When  $S_0$  and  $S_t$  are the substrate ( $\beta$ C) concentrations at times 0 and  $t$ , the time course of the oxidative response, defined as  $R = 1 - (S_t/S_0)$ , can be adjusted to the equation

$$R = K\{1 - \exp[-(\ln 2)(t/\tau)^a]\} \quad (3)$$

where  $K$  is the asymptote,  $\tau$  the substrate half-life or time when 50% oxidation is achieved, and  $a$  a shape parameter associated with the maximum slope of the response ( $v_{\max}$ ):

$$v_{\max} = \frac{Ka}{\tau} (\ln 2)^{1/a} G^G \exp(-G), \quad \text{where } G = \frac{a-1}{a} \quad (4)$$

Given the form of the  $G$  term, when  $a \leq 1$ ,  $v_{\max}$  is indeterminate or negative. For practical purposes, if functions with  $a$  values higher and lower than 1 are found, the  $v_{\text{med}}$  (corresponding to the median abscissa  $\tau$ ) may be used instead of  $v_{\max}$ :

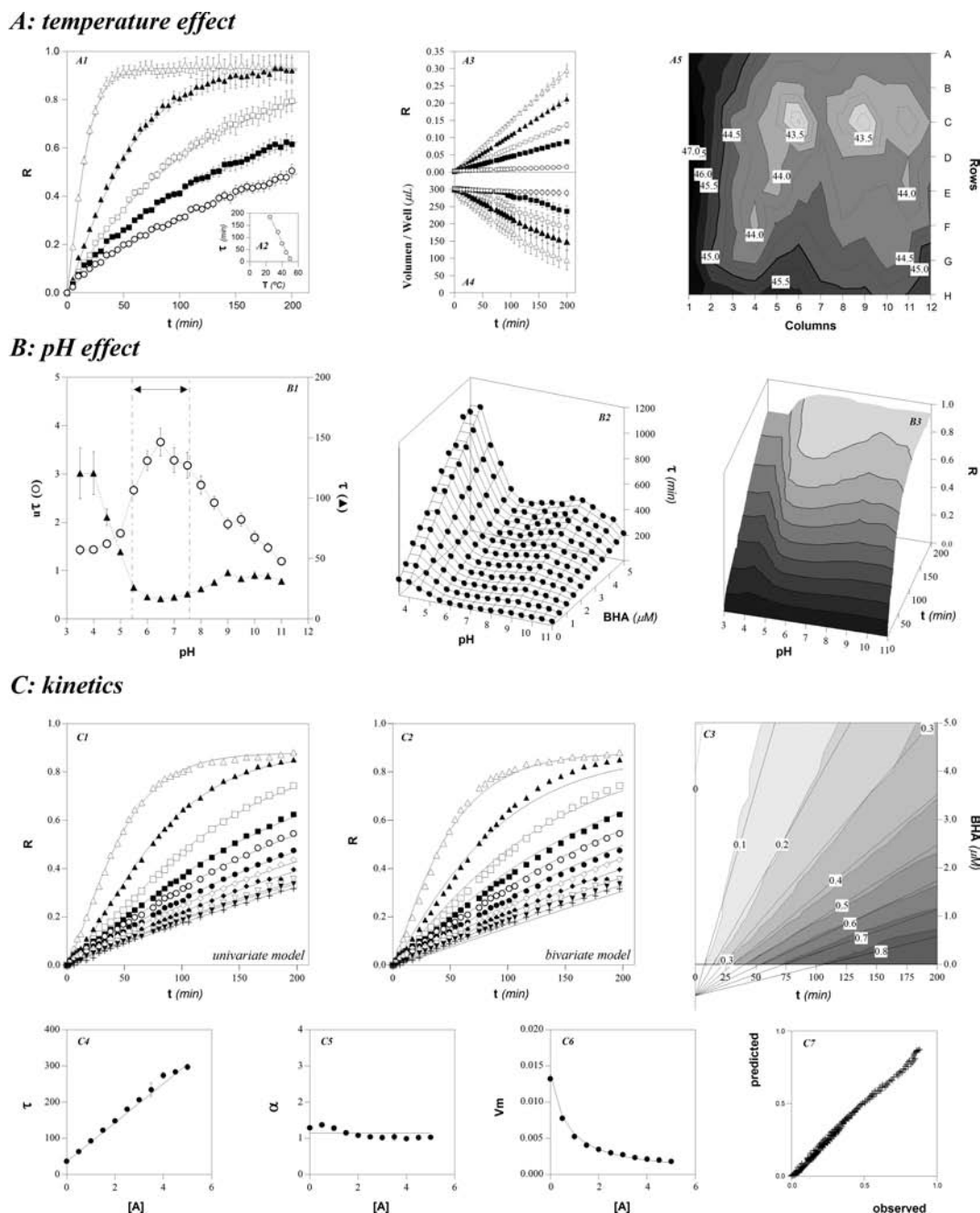
$$v_{\text{med}} = \frac{Ka \ln 2}{2\tau} \quad (5)$$

Equation 3 is very versatile: if  $a < 1$ , it can describe the profiles generated by the model developed by Terpinč and Abramovič,<sup>24</sup> if  $a = 1$ , it describes a first-order kinetic, and if  $a > 1$ , a large variety of sigmoidal profiles are produced.

In an open system, it is accepted that the substrate oxidation is exhaustive at one point, which implies a constant asymptote. Under this condition, any alteration of the oxidative kinetics will modify at least one of the other two parameters. The half-life ( $\tau$ ) is always increased by the presence of an antioxidant and because of eq 4, the maximum slope ( $v_{\max}$ ) decreases if  $a$  remains constant. Additionally,  $a$  could vary as well, modifying the relationship between  $\tau$  and  $v_{\max}$ . If the affinity of the antioxidant by oxygen or radicals is much higher than that of the substrate, the propagation phase shall begin with a certain delay, which shall be translated into an increase of  $a$ . A prooxidant shall promote the opposite effects: half-life decrease (with corresponding  $v_{\max}$  increase) and  $a$  decrease if the effect on  $\tau$  is not enough to explain the earlier beginning of the propagation phase.

Therefore, the model in eq 3 can be applied to fit individually the kinetic profiles corresponding to a series of increasing levels of an OM agent, thus providing the values of  $\tau$  and  $a$ , whose variations characterize and quantify the effect of the OM agent.

An alternative and preferable option is to consider that any modification of any parameter  $\theta$ , due to an OM agent, can be described by means of a hyperbolic factor  $H_{\theta}$  as



**Figure 1.** Factors affecting  $\beta$ C bleaching in microplate assay. (A) Temperature ( $^{\circ}\text{C}$ ) effects (30,  $\circ$ ; 35,  $\blacksquare$ ; 40,  $\square$ ; 45,  $\blacktriangle$ ; 50,  $\triangle$ ) on the bleaching kinetics in the presence (A1) and in the absence (A2) of linoleic acid, evaporation (A3, A4), and the thermal gradient in the microplate (A5). (B) pH effects on the parameters of eq 3 in the absence of BHA (B1) and on the half-life (B2) and the time course of the response (B3) in the presence of different concentrations ( $\mu\text{M}$ ) of BHA (0,  $\Delta$ ; 0.5,  $\blacktriangle$ ; 1.0,  $\square$ ; 1.5,  $\blacksquare$ ; 2.0,  $\circ$ ; 2.5,  $\bullet$ ; 3.0,  $\diamond$ ; 3.5,  $\blacklozenge$ ; 4.0,  $\nabla$ ; 4.5,  $\blacktriangledown$ ; 5.0,  $+$ ). (C) Kinetics of  $\beta$ C bleaching, at  $45^{\circ}\text{C}$  and pH 6.5, in the presence of the same concentrations of BHA, according to the univariate (C1) and bivariate (C2) models in eqs 3 and 7. Isoboles of the response surface from the model in eq 7 are also represented (C3), as well as the effects of the BHA concentration on  $\tau$  (C4),  $a$  (C5), and  $v_{\max}$  (C6) and correlation between observations and predictions (C7). In all cases, dots are the experimental results and lines the corresponding fittings to the specified models. See also Table 2.

$$H_{\theta} = \frac{1 + u_{\theta}[\text{OM}]}{1 + v_{\theta}[\text{OM}]} \quad (\theta = \tau, a) \quad (6)$$

where  $[\text{OM}]$  is the agent concentration and  $u_{\theta}$  and  $v_{\theta}$  are fitting coefficients (when  $v_{\theta} = 0$ , the dependence is linear). This leads to formulation of a bivariate equation, as a function of time and the agent concentration, in terms of

$$R(t, [\text{OM}]) = K\{1 - \exp[(-\ln 2)(t/H_{\tau}\tau)^{H_{\tau}a}]\} \quad (7)$$

Thus, the entire set of kinetic profiles can be simultaneously described, in the most complex case, by the seven parameters of the model in eq 7. A useful characterization of OM activity is now provided by the term  $H_{\tau}$ , which represents the increase (antioxidants) or decrease (prooxidants) of the half-life in the presence of a given concentration of the considered agent (Figure 4). Such a characterization is robust, minimizing the effects of the experimental error, and as stated by other authors,<sup>33,34</sup> the simultaneous description of all curves is

**Table 2.** Parametric Estimates and Confidence Intervals ( $\alpha = 0.05$ ) of the  $\beta$ C Bleaching Kinetics As Affected by: (A) Temperature, According to the Model in Eq 3; (B) pH, According to the Model in Eq 7 Applied, at Each pH Value, to the Series of BHA Concentrations Tested in (C); and (C) BHA Concentration, at  $T = 45^\circ\text{C}$  and pH 6.5, According to the Models in Eqs 3 and 7<sup>a</sup>

	$K$	$\tau$	$a$	$u_\tau$	$R_{\text{adj}}^2$
(A) Temperature ( $^\circ\text{C}$ )					
30	0.940 $\pm$ 0.01	181.61 $\pm$ 0.91	0.86 $\pm$ 0.03		0.9983
35	0.961 $\pm$ 0.02	116.04 $\pm$ 2.05	0.86 $\pm$ 0.02		0.9993
40	0.945 $\pm$ 0.01	73.66 $\pm$ 6.83	2.03 $\pm$ 0.04		0.9992
45	0.932 $\pm$ 0.03	38.61 $\pm$ 17.6	1.15 $\pm$ 0.04		0.9987
50	0.930 $\pm$ 0.02	12.05 $\pm$ 5.01	1.22 $\pm$ 0.06		0.9977
(B) pH					
3.5	1.000 $\pm$ 0.23	120.46 $\pm$ 51.0	0.78 $\pm$ 0.06	1.43 $\pm$ 0.10	0.9682
4.0	1.000 $\pm$ 0.10	120.59 $\pm$ 17.6	1.04 $\pm$ 0.04	1.43 $\pm$ 0.05	0.9885
4.5	1.000 $\pm$ 0.02	84.08 $\pm$ 6.83	1.10 $\pm$ 0.03	1.56 $\pm$ 0.06	0.9918
5.0	1.000 $\pm$ 0.02	55.45 $\pm$ 2.0	1.09 $\pm$ 0.02	1.77 $\pm$ 0.05	0.9958
5.5	0.962 $\pm$ 0.01	25.95 $\pm$ 0.91	1.03 $\pm$ 0.03	2.67 $\pm$ 0.10	0.9942
6.0	0.963 $\pm$ 0.02	18.14 $\pm$ 1.05	1.00 $\pm$ 0.04	3.27 $\pm$ 0.20	0.9863
6.5	0.954 $\pm$ 0.02	16.67 $\pm$ 1.20	1.06 $\pm$ 0.05	3.66 $\pm$ 0.29	0.9779
7.0	0.956 $\pm$ 0.02	17.89 $\pm$ 1.27	1.09 $\pm$ 0.06	3.28 $\pm$ 0.26	0.9778
7.5	0.932 $\pm$ 0.02	20.46 $\pm$ 1.61	1.03 $\pm$ 0.06	3.18 $\pm$ 0.27	0.9714
8.0	0.929 $\pm$ 0.02	24.73 $\pm$ 1.51	1.12 $\pm$ 0.05	2.77 $\pm$ 0.19	0.9765
8.5	0.945 $\pm$ 0.02	30.16 $\pm$ 1.57	1.09 $\pm$ 0.04	2.41 $\pm$ 0.13	0.9819
9.0	0.998 $\pm$ 0.03	38.14 $\pm$ 2.66	1.02 $\pm$ 0.05	1.96 $\pm$ 0.12	0.9784
9.5	0.957 $\pm$ 0.03	33.22 $\pm$ 2.26	1.07 $\pm$ 0.05	2.06 $\pm$ 0.14	0.9749
10.0	1.000 $\pm$ 0.04	35.94 $\pm$ 3.02	1.02 $\pm$ 0.06	1.68 $\pm$ 0.13	0.9711
10.5	1.000 $\pm$ 0.03	35.37 $\pm$ 2.64	1.03 $\pm$ 0.06	1.47 $\pm$ 0.10	0.9771
11.0	0.999 $\pm$ 0.02	31.09 $\pm$ 1.99	1.11 $\pm$ 0.06	1.19 $\pm$ 0.07	0.9747
(C) [BHA] ( $\mu\text{M}$ )					
Univariate Model (Eq 3)					
0.0	0.869 $\pm$ 0.01	36.83 $\pm$ 0.70	1.29 $\pm$ 0.04		0.9993
0.5	0.908 $\pm$ 0.01	63.56 $\pm$ 0.71	1.37 $\pm$ 0.03		0.9993
1.0	0.898 $\pm$ 0.01	92.89 $\pm$ 0.91	1.28 $\pm$ 0.03		0.9991
1.5	0.923 $\pm$ 0.02	122.27 $\pm$ 1.67	1.15 $\pm$ 0.03		0.9981
2.0	0.895 $\pm$ 0.01	148.31 $\pm$ 2.09	1.08 $\pm$ 0.03		0.9982
2.5	0.885 $\pm$ 0.03	180.38 $\pm$ 3.03	1.04 $\pm$ 0.03		0.9981
3.0	0.884 $\pm$ 0.02	206.49 $\pm$ 4.86	1.02 $\pm$ 0.03		0.9973
3.5	0.880 $\pm$ 0.03	233.90 $\pm$ 5.13	1.04 $\pm$ 0.03		0.9979
4.0	0.881 $\pm$ 0.05	273.46 $\pm$ 8.09	1.02 $\pm$ 0.03		0.9979
4.5	0.879 $\pm$ 0.09	283.22 $\pm$ 5.77	1.02 $\pm$ 0.02		0.9986
5.0	0.878 $\pm$ 0.11	296.95 $\pm$ 7.71	1.05 $\pm$ 0.03		0.9981
Bivariate Model (Eq 7)					
BHA	0.878 $\pm$ 0.01	35.88 $\pm$ 0.57	1.14 $\pm$ 0.01	1.49 $\pm$ 0.02	0.9952

<sup>a</sup>Not statistically significant parameters of the model in eq 7 were omitted. See also Figure 1.

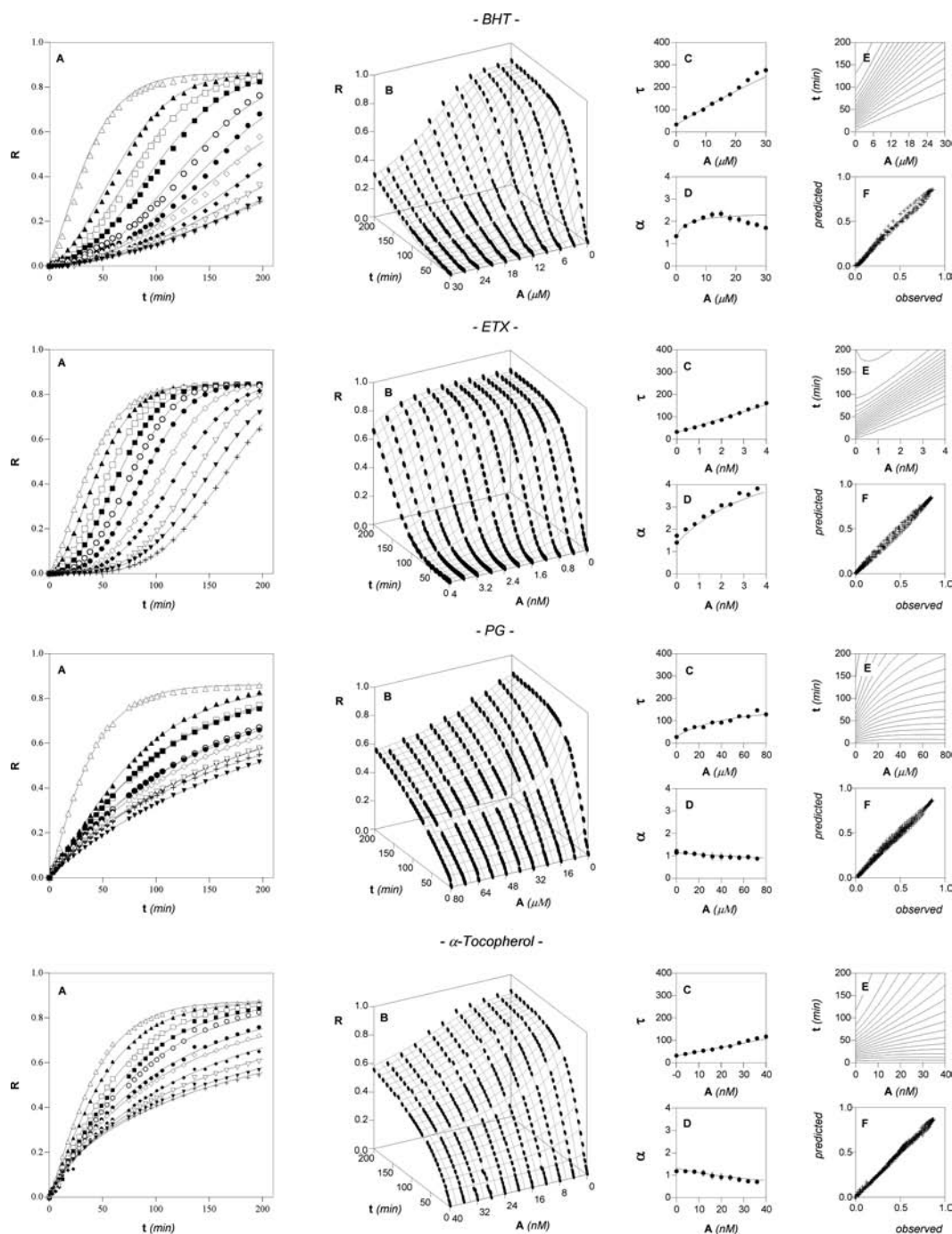
preferable to the individual fittings. Indeed, if some kinetic series does not span the full range of the response, it does not provide sufficient information for ensuring the parametric estimates in the individual option, but it contributes to the system definition in the simultaneous analysis.

**Analysis of the Main Critical Points.** *Temperature Effect.* Temperature affects the evaluation of the OM activity by enhancing the  $\beta$ C bleaching rate (both in the presence and in the absence of linoleic acid), the solvent evaporation, and the microplate thermal gradient, the last a usual problem in such devices.<sup>35</sup> In fact, the working temperature is appropriate when it satisfies the following conditions: (a) the oxidation profile of the control is sigmoidal with a well-defined asymptote, and its maximum slope enables the detection of lower (antioxidant) and higher (prooxidant) values in the same assay; (b)  $\beta$ C bleaching in the absence of linoleic acid, evaporation, and a thermal gradient is minimized in a reasonable reaction time

(Figure 1 and Table 2). All these effects were examined by studying the time course of the control response at different temperatures (30, 35, 40, 45, and 50  $^\circ\text{C}$ ) and wavelengths (470, 900, and 975 nm), with results that are summarized next.

The bleaching rate was fast in the presence of linoleic acid and slow in its absence, in both cases increasing with temperature (Figure 1 and Table 2A). Thus, the  $\beta$ C bleaching includes the effect of temperature and  $\text{O}_2$  and that due to the radicals released by linoleic acid oxidation. Although it is not the usual practice, the first can be corrected by using the blank described in the methodological section.

Evaporative effects in microplate readers, even during periods longer than 12 h, can be neglected by using transparent, oxygen-permeable films.<sup>36</sup> In this case, it was not even necessary. The time course of the evaporation at different temperatures was quantified through the liquid level (average of the 96 wells), in turn determined through the difference

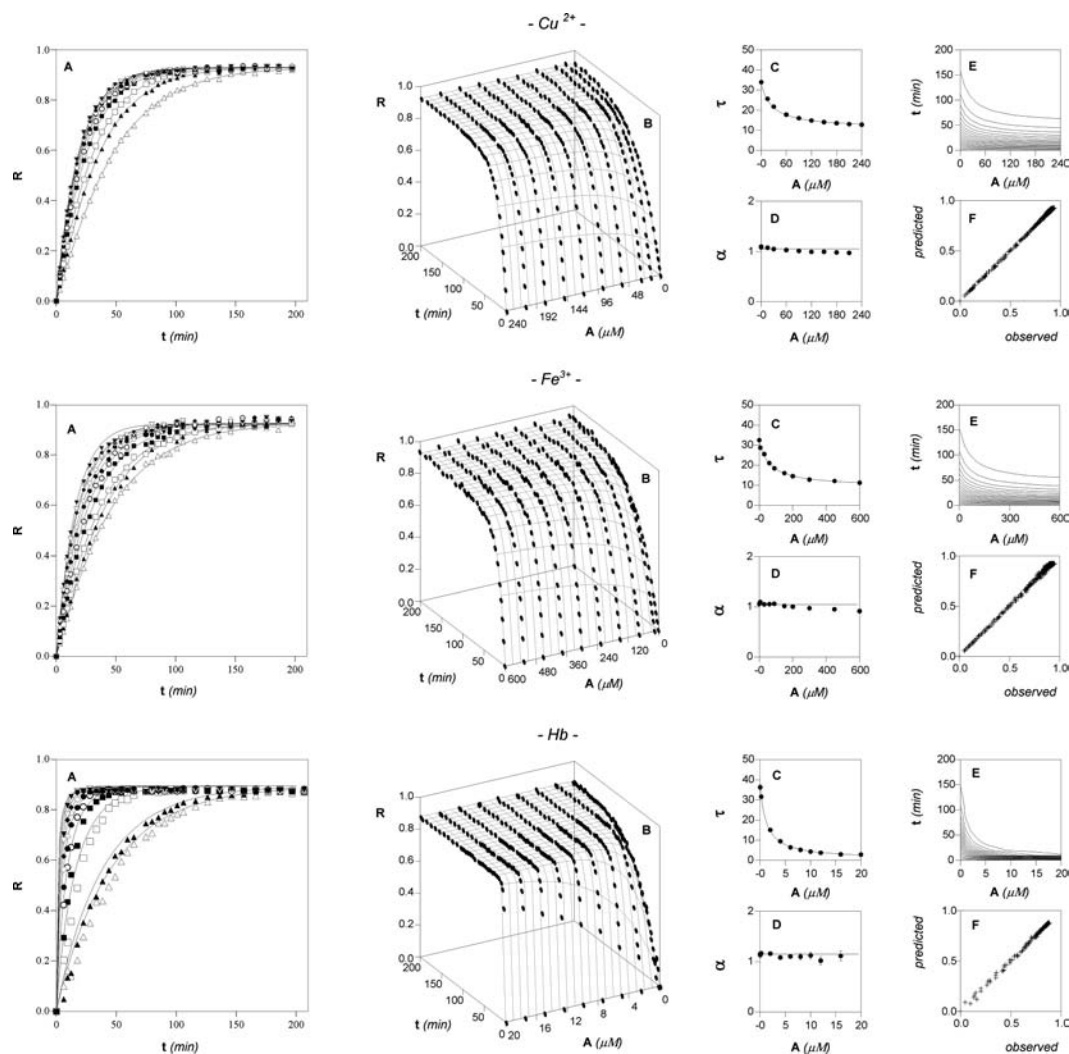


**Figure 2.** Effects of the specified antioxidants (ranges in Table 3) on the  $\beta C$  bleaching reaction: (A) experimental results (dots) and fittings (lines) to the univariate model in eq 3; (B) experimental results (dots) and fittings (grid line) to the dose–time-dependent model 7; (C) parametric variations as a function of the antioxidant concentration, obtained from the models in eqs 3 (dots) and 7 (lines); (D) correlations between observations and predictions and isobolograms. In all cases a control series ( $\Delta$ ) and 10 dilutions of the maximum concentration were tested ( $\blacktriangle$ , 1/10;  $\square$ , 2/10;  $\blacksquare$ , 3/10;  $\circ$ , 4/10;  $\bullet$ , 5/10;  $\diamond$ , 6/10;  $\blacklozenge$ , 7/10;  $\nabla$ , 8/10;  $\blacktriangledown$ , 9/10;  $+$ , 10/10). See also Table 3.

between the absorbances at 975 and 900 nm (Figure 1A4). Although over 35 °C a significant volume is lost, the simultaneous variation in path length and concentration cancels this effect. Thus, evaporation is a troubling fact only if it causes precipitation of solutes or when the well volume drops to a level ( $\sim 120 \mu\text{L}$ ) at which the meniscus corrupts the measure.

The thermal gradient is a more problematic issue, as illustrated in Figure 1A5. The temperature distribution in our device was studied by a two-step process. First, using the average values of four central wells, we verified the linear

increase of the  $\tau$  parameter from eq 3 with temperature, within the range 30–50 °C (see Table A in the Supporting Information for the parametric stimulations), both in the presence and in the absence of linoleic acid. Second, using this linear relationship, the values of  $\tau$  provided in the absence of linoleic acid, in each of 96 wells, enabled calculation of the corresponding temperatures. The thermal gradient thus revealed allows the conclusion that (1) a temperature of 45 °C, close to values used by other authors, was a less risky condition (see Figure 1A5), (2) the gradient cannot be



**Figure 3.** Effects of the specified prooxidants (ranges in Table 3) on the  $\beta C$  bleaching reaction. Graphic criteria and keys as in Figure 2. See also Table 3.

detected at  $\sim 32$  °C and is very strong over 55 °C, disabling the possibility of an accurate analysis, and (3) at 45 °C, the first three columns on the left and the two bottom rows of the microplate should be excluded.

Both the original method<sup>14</sup> and its adaptation to a microplate<sup>21</sup> operate between 55 and 50 °C. Our results, however, showed that, although eqs 3 and 7 always provided statistically significant descriptions, lower temperatures enabled better fittings in terms of correlation between observations and predictions and confidence intervals of parametric estimates. We selected a temperature of 45 °C because (a) it significantly reduces the spontaneous oxidation of  $\beta C$  (b), it leads to better discrimination between different levels of antioxidant, (c) it is significantly more sensitive to prooxidant effects (in fact, these effects cannot be accurately detected at 50 °C (see Figure 1), and their determination would improve even at 40 °C), (d) it significantly reduces the temperature gradients, and (e) although it extends the analytical time, it is not problematic from the point of view of any collateral effect.

Lower temperatures produce even statistically better results, but unnecessarily extend the analysis time. Higher temperatures reduce this time, but tend to infringe on conditions a and b established above, and they move the reaction conditions away from those in which antioxidants have a practical interest. This

does not seem important when the evaluation is based on measures at a single time, but in such a case, the characterization loses just what characterizes the assessed phenomenon, that is, the key factual meanings of the parameters  $\tau$  and  $a$ . The comparison of the cases depicted in Figures 2 and 3 makes evident the difficulties that an operation at a single time would have to translate these results into useful and representative assessments.

**Kinetics.** A common—but questionable—practice is to use the values obtained at a single time, both in the sample under evaluation and in a calibration antioxidant, assuming too many aspects as true. Such a practice is hardly justifiable today, given the availability of computational applications and microplate readers, whose combination provides an adequate tool to work with different variables in nonlinear models. In our opinion, any criterion that does not take into account the kinetics of the process can be considered as a poor simplification which produces an incorrect result and can lead to serious misinterpretations when one works with natural extracts containing complex mixtures of antioxidant and prooxidants.

The time-dependent response in the  $\beta C$  method is inherently sigmoidal, and it represents its most important factor. The reduction of the study at one single time and expectation to find linear forms often lead to unreliable values

hiding the real aspects of the response. Next we will examine in detail the kinetic aspects of the  $\beta C$  bleaching reaction, by combining the use of eq 7 with a high amount of data with low experimental error. As a reference, the case of BHA will be discussed first.

The individual fitting to eq 3 (Figure 1C1) of the kinetic profiles obtained in the presence of BHA showed that the antioxidant determines (a) an increase of the half-life ( $\tau$ ) following a linear relation ( $\tau = 54.58[\text{BHA}] + 39.73$ ;  $r^2 = 0.993$ ) and (b) a slight variation of  $a$  that reaches a stable minimum from  $2.0 \mu\text{M}$  BHA, which is anyway scarcely relevant. The simultaneous fitting (Figure 1C2) of all profiles to the bivariate eq 7 confirmed the linear effect on  $\tau$  and the lack of statistical significance for  $a$  variation. Although this last model showed deviations in some profiles (compare parts C1 and C2 of Figure 1), it is preferable because it treats the system as a whole, enabling verification of the statistical consistence of all the sides involved in the resulting characterization. The asymptotic drop of  $v_{\text{med}}$  was consistently described by both approaches (dots and lines for uni- and bivariate models, respectively, in Figure 1C4,C5,C6).

Moreover, the isoboles (projections on the plane  $A-t$  of the lines of equal response) of the surface from eq 7 formed a straight beam converging at one point (Figure 1C3). In other words, the relationship between the BHA concentration and time required to achieve a given response was linear, with the same intercept at any response level. This allows the antioxidant to be characterized by a nomogram, which facilitates prediction of its effects. Since  $a$  does not vary with the BHT concentration, and  $\tau$  varies linearly, the mean value of the half-life concentration ratio ( $55.81 \pm 1.25 \text{ min} \cdot \mu\text{M}^{-1}$  in this case) can provide a specific half-life extension as a characterizing index. Unfortunately, this behavior cannot be generalized to all types of antioxidants, as will be shown later.

This determination was repeated, applying Marco's method<sup>14</sup> according to Miller's protocol,<sup>15</sup> with five BHA levels within the same domain [ $0-(1)-5 \mu\text{M}$ ] in tubes at  $50^\circ\text{C}$  and measurements at 10 times. The kinetics of the control was indistinguishable from that obtained in the microplate at the same temperature (Figure 1A1), and the linear correlation coefficient ( $r^2 = 0.992$ ) between the results calculated by using the respective controls proved the equivalence of both methods. As expected, absolute values of the response were higher at  $50^\circ\text{C}$  and reproduced the linear effect of BHA on  $\tau$  and the absence of an effect on  $a$ .

**pH Effect.** The criterion of omitting buffers<sup>14</sup> is not shared by other authors,<sup>21</sup> who found that the initial pH affects the progress of the oxidation. In our case, the effect of the pH was studied in buffered solutions (83 mM Briton in the reaction mixture; pH 3.5–(0.5)–11.0) at different BHA concentrations,  $0-(0.5)-5 \mu\text{M}$ , in the presence and in the absence of linoleic acid. Within this range, the pH did not determine hipso- or bathochromic shifts in the absorption spectrum of  $\beta C$ , and the maximum effect on the absorbance at 470 nm was less than 5%.

At  $45^\circ\text{C}$ , in the absence of BHA, the time required to achieve a given degree of bleaching was very high at low pH (3.0–4.0), falling sharply in the 4.0–5.5 pH range, remaining basically constant between 5.5 and 7.5, and increasing slowly within 7.5–11.0. Therefore, the 5.5–7.5 range seems the best option for providing a stable discriminating capacity at reasonable times. In the presence of BHA, the fitting of the bleaching kinetics to the model in eq 7 showed that the pH modifies the value of  $\tau$  ( $a$  remains constant) without altering its

linear dependence on the BHA concentration. These results (Figure 1B and Table 2B) confirmed the adequacy of the range 5.5–7.5 and led to selection of the value 6.5 as the pH of the routine assays.

As Table 2 shows, all the experimental data were satisfactorily modeled by either eq 3 or eq 7, with a good predictive capacity (adjusted coefficient of multiple determination), statistical consistence (Fisher's test), adequate parametric sensitivity, narrow parametric confidence intervals (Student's test), unbiased residuals, and accuracy and bias factors close to 1.

**Use of Initiators.** The suggestion to use free radicals as initiators is an unnecessary complication. Indeed, the reaction proceeds smoothly regardless of oxygen saturation, and moderate differences in the initial conditions (minimized by the described reagent preparation) are irrelevant when a calibration set is used and the proposed kinetic modeling is applied. Including a lipophilic initiator (such as  $\text{Fe}^{2+}$ ) would lead to a new problem, since the inclusion of a prooxidant in the system would require a more complex mathematical model.

**Size of the Micelles Formed.** It has been reported that the size of the micelles could be one of the factors responsible for differences in the initial conditions.<sup>4</sup> However, reagents recovered at the time of use by means of a vortex or ultrasonic bath for 30 s did not show statistically significant differences in the evaluation of the same series of antioxidants.

**Dissolved Oxygen.** Although the original method used oxygen-saturated solutions, other authors<sup>17</sup> quantified the proportion of radicals by measuring the absorbance at 270 nm and concluded that this condition was unnecessary. Our results confirmed this observation and showed that the initial oxygen saturation did not produce significant differences in the time course of the  $\beta C$  bleaching process. Consequently, the saturation step may be omitted.

**Synergistic or Antagonistic Interactions.** In no purified samples is the presence of interfering materials, able to produce synergistic or antagonistic effects,<sup>1</sup> a realistic assumption. However, this type of problem is not specific to the method because it would not be acceptable that the high specificity prevents the detection of the simultaneous effect of two antioxidants (or one antioxidant and one prooxidant) in a complex extract.

It is necessary in this context to distinguish between two different situations. When a crude extract of an antioxidant A contains an interfering material I (inhibitor, enhancer, prooxidant, another antioxidant) whose nature or concentration is not well-known, it is obvious that the evaluation, regardless of the method applied, translates the interaction without the possibility of making concrete attributions, since A and I are perfectly covariant in the dilutions to be tested. However, when we know the nature of I, and its concentration can be varied independently of A, either eq 3 or eq 7 provides useful tools to discriminate and quantify both effects, because the interference will modify the parameters of these models in a regular pattern (as suggested by preliminary results obtained in our laboratory about synergistic and antagonistic actions).

**Solubility and Polarity.** When the sample is slightly hydrosoluble, it is necessary to use other solvents, commonly ethanol, methanol, acetone, or 1,4-dioxane. Reported differences<sup>21</sup> of  $\sim 10\%$  in the response of  $\beta C$  due to the solvent effect require equalized proportions in samples, blanks, and calibration and control wells (as in any spectrophotometric assay).



**Table 3.** Parametric Estimates and Confidence Intervals ( $\alpha = 0.05$ ) of the  $\beta C$  Bleaching Kinetics As Affected by the Specified Antioxidant and Prooxidant Agents, According to the Bivariate Model in Eq 7<sup>a</sup>

agent	concn range ( $\mu M$ )	$K$	$\tau$	$a$	$u_r$	$v_r$	$u_a$	$v_a$	$R_{adj}^2$
<b>Antioxidants (See Figure 2)</b>									
BHT	0–(3)–30	$0.86 \pm 0.01$	$35.50 \pm 1.12$	$1.38 \pm 0.08$	$0.222 \pm 0.015$	$0.003 \pm 0.002$	$0.324 \pm 0.144$	$0.182 \pm 0.089$	0.9949
ETX	0–(0.0004)–0.004	$0.84 \pm 0.01$	$28.45 \pm 0.28$	$1.20 \pm 0.02$	$1112.7 \pm 36.60$		$1194.5 \pm 241.6$	$225.3 \pm 77.82$	0.9967
PG	0–(8)–80	$0.86 \pm 0.01$	$29.95 \pm 1.48$	$1.14 \pm 0.05$	$0.126 \pm 0.013$	$0.020 \pm 0.003$		$0.003 \pm 0.001$	0.9906
$\alpha$ -toc	0–(0.004)–0.04	$0.86 \pm 0.01$	$31.05 \pm 0.69$	$1.33 \pm 0.04$	$60.90 \pm 2.324$			$18.19 \pm 1.976$	0.9966
<b>Prooxidants (See Figure 3)</b>									
$Cu^{2+}$	0–(24)–240	$0.92 \pm 0.01$	$32.87 \pm 0.37$	$1.05 \pm 0.00$	$0.012 \pm 0.001$	$0.037 \pm 0.002$			0.9993
$Fe^{2+}$	0–(1.5)–15	$0.84 \pm 0.01$	$37.16 \pm 0.92$	$1.55 \pm 0.07$		$1.106 \pm 0.054$	$0.565 \pm 0.113$	$1.629 \pm 0.312$	0.9940
$Fe^{3+}$	0–(60)–600	$0.92 \pm 0.01$	$31.25 \pm 0.38$	$1.04 \pm 0.01$	$0.004 \pm 0.001$	$0.014 \pm 0.001$			0.9975
Hb	0–(2)–20	$0.87 \pm 0.01$	$30.14 \pm 0.63$	$0.96 \pm 0.02$		$1.099 \pm 0.036$			0.9960

<sup>a</sup>See also Figures 2 and 3.

A different problem concerns the aforementioned “polar paradox”:<sup>20</sup> since the hydrophobic repulsion tends to concentrate nonpolar antioxidants in the lipid environment, where the oxidation process takes place, the apparent activity is higher than that obtained with polar antioxidants. However, this effect cannot be considered artificial, since it explains an objective advantage of the lipophilic antioxidants when the oxidized substrate is lipidic. In fact, when  $\beta C$  (lipophilic substrate) was substituted by crocin (hydrophilic substrate) in a reagent prepared with increasing concentrations of linoleic acid ( $180\text{--}500 \mu g \cdot mL^{-1}$  in the final mixture), the complementary “nonpolar paradox” was produced: lipid radicals remained in the micellar zone, and crocin bleaching did not occur.

Therefore, selecting an antioxidant to apply to a specific material (e.g., food) should not be based on an abstract or universal antioxidant activity, but on the one resulting from all the effects that are combined in that material. From this perspective, the possibility of a universal method to evaluate OM activities seems unrealistic and even unhelpful, since real cases involve at least the combination of two binary criteria: the hydrophilic or lipophilic character of the oxidizable substrate and the involvement of radicals or hydrogen atoms in the transfer process.

**Application To Quantify and Characterize Antioxidant and Prooxidant Agents.** The time course of the reaction was studied at the condition previously discussed using five common antioxidants (BHA, BHT, ethoxyquin, propyl gallate, and  $\alpha$ -tocopherol), several metals ( $Cu^{2+}$ ,  $Fe^{2+}$ ,  $Fe^{3+}$ ,  $Cd^{2+}$ ,  $Ni^{2+}$ ,  $Sr^{2+}$ ,  $Mg^{2+}$ ,  $Zn^{2+}$ , and  $Mn^{2+}$ ), and hemoglobin. The last two were selected because they can be present, either as constituents or contaminants, in crude biological extracts and metals as traces in buffer salts.<sup>37,38</sup>

As a first general result, none of the tested compounds promoted, in the absence of linoleic acid, bleaching kinetics significantly different from that of the control. This suggests that in all the cases the activity (antioxidant or prooxidant) was not related to  $\beta C$  bleaching, but to the radical production by the fatty acid.

Beyond Quantitative differences (Figure 2 and Table 3), BHT and propyl gallate behaved like BHA (increase of  $\tau$  and not statistically significant or irrelevant variation of  $a$ ). The much stronger effects of ethoxyquin and  $\alpha$ -tocopherol were more complex as well, the first promoting an increase of  $a$  and the second a slight decrease. The increase of  $a$  can be explained, as was said earlier, in terms of a delay of the propagation phase due to a strong affinity of the antioxidant by oxygen or radicals, the opposite effect having a complementary explanation.

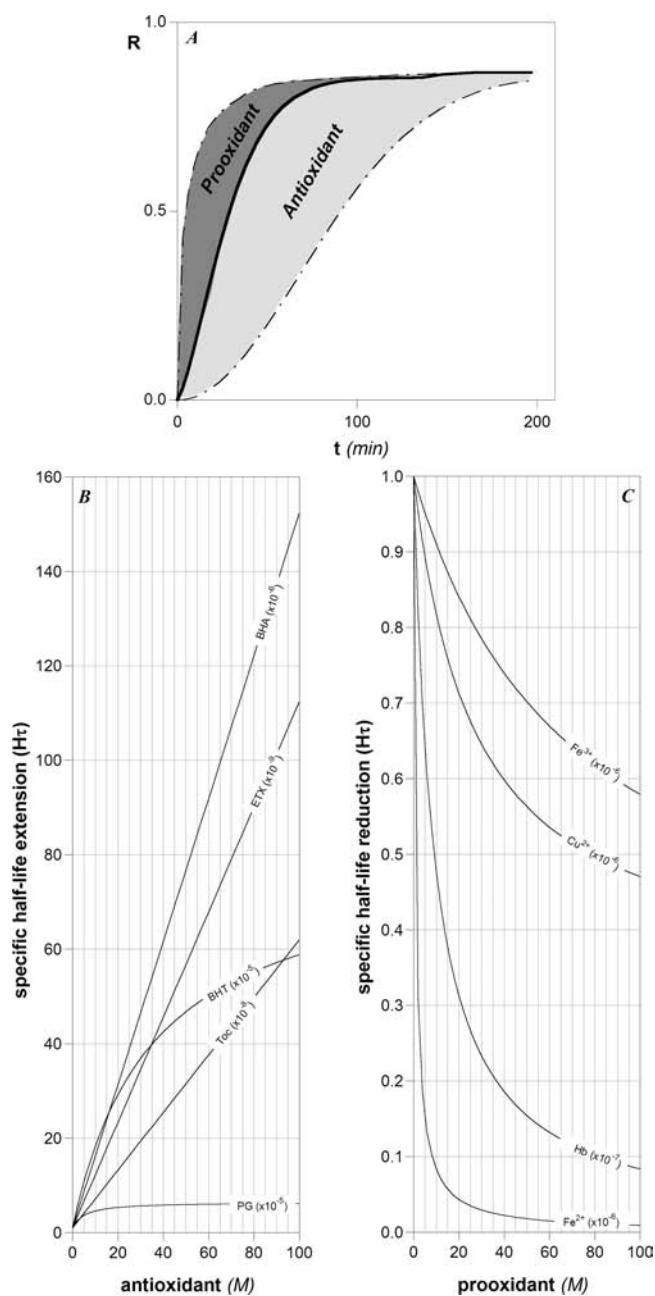
Equally consistent results (with the already specified statistical criteria) were obtained when eqs 3 and 7 were applied to the description of prooxidant activities (Figure 3 and Table 3). In all the cases the decrease of  $\tau$  was asymptotic, a behavior which probably can be generalized to any prooxidant. Indeed, while the theoretical limit of the  $\tau$  increase is  $+\infty$ , that of its decrease is zero (instead of  $-\infty$ ), which causes a progressive packing over the kinetic profile of the control. This is, otherwise, the reason by which a convenient methodological unity for antioxidant and prooxidants makes advisable temperatures avoiding excessively high profiles (high oxidation rates) in the control. The parameter  $a$  showed an asymptotic decrease in  $Fe^{2+}$  and  $Fe^{3+}$  (in this last case irrelevant, although statistically significant) and remained constant in  $Cu^{2+}$  and hemoglobin.

The high prooxidant activities of hemoglobin and  $Fe^{2+}$  confirmed the need of precautions to be taken with crude biological extracts and buffers and induced evaluation of other metals potentially present as contaminants in these cases. Activity was prooxidant in  $Co^{2+}$ , null in  $Cd^{2+}$ ,  $Ni^{2+}$ , and  $Sr^{2+}$ , slightly antioxidant in  $Mg^{2+}$ , and clearly antioxidant in  $Zn^{2+}$  and  $Mn^{2+}$ .

The fitting of the results was always satisfactory. The mathematical equations were robust and consistent ( $p$  values of  $<0.001$  from Fisher's  $F$  test), the residuals were randomly distributed, and autocorrelations were not observed by the 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 multiple determination between predicted and observed values were always  $R_{adj}^2 > 0.95$ , with a wide majority of the fittings superior at 0.99. Bias and accuracy factors ( $B_f$  and  $A_f$ ) also indicated the lack of bias and high accuracy of equations used to describe experimental effects of OM agents (data not shown).

A meaningful way to compare OM activities consists of plotting the specific variation of the half-life ( $H_r$ ), given by eq 6, as a function of the agent concentration. This can provide fixed values, as the concentration that doubles the half-life (antioxidants) or reduces it by half (prooxidants) and, more interestingly, when  $H_r$  is a nonlinear term, allows visualization of the agent-specific dynamics of these effects (Figure 4).

An oxidation modifier is a chemical entity that interferes in a process in which a substrate is oxidized through a specific mechanism in a particular environment. This has two implications of practical interest.



**Figure 4.** Top: Antioxidant and prooxidant responses. Bottom: specific half-life extensions (antioxidants) and reductions (prooxidants) of the agents considered in Figures 2 and 3. Note the concentration scales on the lines.

- (1) The natures of the OM, the substrate, and the chemical environment, as well as the mechanism involved, are factors that potentially modify the kinetic profile of the process. Since reality can provide many combinations of these factors in several quantitative relations, it should be expected that some methods will be appropriated for transferring their results to certain systems; however, it is problematic to assume the existence of a universal method able to predict the behavior of any antioxidant in any system.
- (2) In most real systems of practical interest, oxidation is an autocatalytic process with a sigmoidal profile. This means that, in principle, an OM can modify, simultaneously or not, any of the parameters defining that profile, which

prevents its activity from being quantified simply. In general, single value indexes can be defined for descriptive and comparative purposes, but no definition can disregard any previous formal model of the kinetic profile.

The first point has led to the admission that more than one method needs to be applied to characterize the OM activity in a useful way. However, the second point is less recognized, and the literature is plentiful in experimental resources which ignore kinetic profiles. This has increased the divergent results and promoted the idea that a rigorously standardized universal method is needed. The  $\beta C$  method operates on a system of lipid micelles in an aqueous environment, which could constitute an acceptable model for many foods and even some biological systems, but it is not a universal environment. Thus, some of the criticism that it has received could also be applied to many other resources used for assessing OM activities. Moreover, this method is also criticized for its sensitivity to variables such as temperature, pH, solvent effects, dissolved oxygen, and the possibility of interactions with other chemicals in complex samples. When kinetic models are disregarded and measures are performed at a single time, this sensitivity seems to require a complex and perhaps impractical standardization.

However, we hope to have shown that many of these problems disappear when, as in any analytical method, certain precautions are taken with the reagent and working pH and temperature, aiming to conserve the general autocatalytic profile of the process. In this case, the application of the proposed kinetic model produces stable and meaningful characterizations, and the microplate assay provides an appropriate tool for ensuring that sample series with a large number of items can be simultaneously assessed.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

Table showing the kinetic analysis of the spontaneous oxidation of  $\beta C$  by the effect produced at different temperatures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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