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Mathematical Model for the Characterization and Objective Comparison of Antioxidant Activities

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The available data about the interference of antioxidants in the kinetics of lipid oxidation are abundant, but often they allow only semiquantitative conclusions, not always with sufficient basis. One of the causes of this problem is the absence of formal models able to guide the experimental design and to calculate characterizing parameters. In this regard, the model which we propose allows us to obtain the simultaneous solution of a series of oxidation kinetics in the presence of any number of antioxidant concentrations. It describes satisfactorily simulations in which substrate and antioxidant compete for oxygen in a second order kinetic scheme, as well as experimental results from other authors, in different systems and under different conditions. Its application is simple, it provides parametric estimates which characterize both the oxidative process and the antioxidant activity, 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 parameters (*adj* r^2, adjusted coefficients of multiple determination) were up to 0.97.

KEYWORDS: Lipid oxidation; antioxidant effects; mathematical modeling; Weibull equation

INTRODUCTION

Almost two decades ago, Özilgen and Özilgen (*1*) pointed out that, in spite of the abundant bibliography about lipid oxidation in foods, mathematical models that allow formalization and prediction of the process have not been applied before. Recently, Frankel and Finley (*2*) showed their concern with regard to the lack of standardization in the multiplicity of methods applied to the evaluation of natural antioxidants and to the variable and confusing results that are produced in the context of working with complex substrates or, even more, with *in vivo* systems. Although this second group of problems has very diverse sides, the most elementary is still the absence of formal models which allow definition of at least what can be expected from the experimental designs, to establish the most appropriate domains for the independent variables which are included, and to quantify parameters which lead to objective comparisons among the effects of different antioxidants and the results of different approaches.

In their above-mentioned contribution, Özilgen and Özilgen (*1*) recognized the difficulty of verifying a detailed kinetic model in a process with the complexity of the lipid oxidation (*3*), and as a consequence, they proposed an empirical approach by means of a linear transformation of the logistic equation

$$C = \frac{C_0 \exp(kt)}{1 - \frac{C_0}{C_m}[1 - \exp(kt)]}$$

where C is the concentration of the total oxidation products (with C_0 and C_m as initial and maximum values, respectively), k is the maximum specific rate of the process, and t is the time. As was underlined by the authors, this equation—a model of autocatalytic reactions, and widely applied to the description of microbial growth in a limited medium—is appropriate for modeling a process as the lipid oxidation, with a slow initiation phase, a rapid propagation phase, and a termination phase which progresses asymptotically toward the final state. In fact, the equation is able to describe the habitual sigmoidal profiles of the lipid oxidation kinetics. However, the linearization can create a lack of precision due to the necessity of an informal estimation of the C_m parameter, the consideration of the intercept C_0 can be problematic (in general, it is preferable to consider that the oxidative response is null at zero time), and the model does not include the antioxidant effects, which should be evaluated by means of the independent fitting of the corresponding kinetics, with the inconveniences that we will see later on.

The empirical model that we propose is based on the cumulative function of Weibull's equation, and it represents a formal transfer from the field of the dose−response relationships. It allows inclusion of the effects of any number of antioxidant concentrations and evaluation of the system thus defined as a whole. By means of this approach, the simultaneous solution of all the kinetics is achieved, minimizing possible biases due to systematic and random experimental error, and different aspects of the antioxidant activity can be characterized through the corresponding parametric estimates. The model was assayed first using a simplistic kinetic simulation of the oxidative process

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(in terms of global second order reactions), and next it was applied to experimental data from other authors, with satisfactory results.

MATERIALS AND METHODS

The bibliographical abundance about the antioxidant activity of a great variety of natural and synthetic compounds, in raw and purified extracts, makes practically superfluous an 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. The preliminary test of the model was carried out on simulations which were developed as is described next.

1. Simulation of the Competition for Oxygen between a Substrate and an Antioxidant. A simplified way of considering the action of an antioxidant A, in a system with a substrate S which we want to preserve against oxidation, consists of supposing that A competes with S for the oxygen that enters in the system. With independence of the oxygen−acceptor chemical states and the steps of the process, it should be admitted that, if oxygen is not limiting, S and A can reach the maximum oxidation degree, and in exhaustive proportions. The preserving activity can derive from the superior affinity of the oxygen for A, the higher number of oxygen equivalents that A accepts, or both. Thus, disregarding for now the stoichiometric relationships, we can consider two global reactions:

$$S + O_2 \rightarrow SO_2 \quad (1)$$

$$A + O_2 \rightarrow AO_2 \quad (2)$$

If the nature of the system—together with initial conditions, stoichiometric relationships, and kinetic constants—allows a strong oxygen excess with regard to S or A, the corresponding process would be of pseudo-first-order, and the concentrations of S or A would drop according to a negative exponential model. In a more general way, and in better agreement with the available data, it should be supposed that both global reactions behave as if they were of second order (first in each reagent), and so the conversion rates of S and A should be formulated (where k_1 and k_2 are the corresponding kinetic constants) in the terms

$$v_1 = -\frac{d[S]}{dt} = k_1[S][O_2] \quad (3)$$

$$v_2 = -\frac{d[A]}{dt} = k_2[A][O_2] \quad (4)$$

With regard to the oxygen, two hypotheses are possible. The first one admits a semiopen system which receives oxygen from the atmosphere at the rate

$$v_{ox} = -\frac{d[O_2]}{dt} = k_L a([O_2] - [O_2]_E)$$

where k_L is the transfer coefficient, a the interface area, $[O_2]$ the oxygen concentration in the system, and $[O_2]_E$ the corresponding concentration in equilibrium. Working at constant area and volume, the product $k_L a$ can be substituted by a single coefficient k. Furthermore, if the transfer rate is higher than the consumption rate, the difference of concentrations, and so the oxygen intake rate, will remain essentially constant:

$$v_{ox} = k([O_2]_E - [O_2]) = k_{ox} \quad (5)$$

The second hypothesis implies a closed system, with a gas phase limited to a certain space, where the oxygen tends to exhaustion as the oxidation progresses.

In both hypotheses, the oxygen consumption rate is the sum of v_1 and v_2. The concrete stoichiometric relationships implied in the eqs **1** and **2** can now be included as factors (c_1 and c_2) of the corresponding rates, and so the net rate of variation in oxygen concentration is

$$v_3 = v_{ox} - (c_1 v_1 + c_2 v_2) \quad (6)$$

Although eqs **3** and **4** have explicit algebraic solutions, the group eq **3**, eq **4**, and eq **6** lacks it, since v_1 and v_2 depend on $[O_2]$, and this term depends in turn on those rates. The numeric solution is, however, simple, and even the Euler's method provides a good convergence when a sufficiently low time increment Δt is used for the iterative calculation of the concentrations of any chemical species X as a function of time:

$$[X]_{(t)} = [X]_{(t-\Delta t)} \pm v_X \Delta t \quad (7)$$

where v_X is the formation rate of the chemical species X.

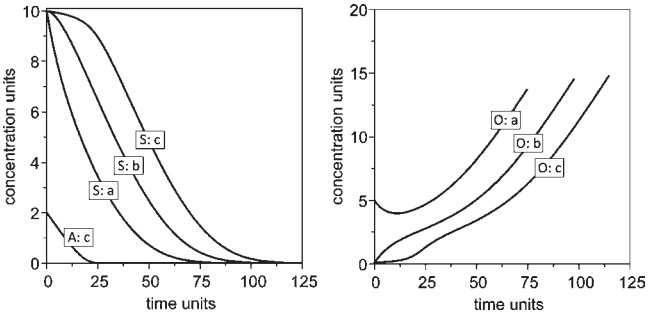

Figure 1. Simulations of the drops in substrate and antioxidant concentrations (left) and oxygen consumptions until exhaustive substrate oxidation (right), under the conditions specified in **Table 1**(a, b, c). S, substrate; A, antioxidant; O, oxygen.

Table 1. Arbitrary Parametric Values Used in the Simulations of **Figures 1 (a, b, c)** and **2** d, According to the Notations of Eqs 3−6

	a	b	c	d
S_0	10	10	10	10
A_0	0	0	2	0-(2)-18
O_0	5	0.1	0.1	1
k_1	0.01	0.01	0.01	0.01
k_2	0.50	0.50	0.50	0.50
k_{ox}	0.25	0.25	0.25	0.25
c_1	1	1	1	1
c_2	2	2	2	1

2. Other Numerical Methods. Fitting of the simulated and experimental results to the proposed model 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* spreadsheet. It allows an agile hypotheses assay with immediate visualization of their consequences. Subsequently, the estimates thus obtained were introduced as initial values in the nonlinear section of *DataFit 9* (Oakdale Engineering), to determine parametric confidence intervals and model consistency (Student's t and Fisher's F tests, respectively, in both cases with $\alpha = 0.05$).

RESULTS AND DISCUSSION

Using reasonable parametric values, eq **7** produces kinetic simulations such as those exemplified in **Figure 1** (data in **Table 1**) for a semiopen system. In this figure it should be noticed that (1) the profiles of the time-course concentrations of S and A change between negative exponential and sigmoidal models and (2) the initial conditions can impose initial irregularities to the time-course of the concentrations of oxygen and—although smoothed—oxidation products.

Figure 2 (data in **Table 1**) shows the kinetics of S resulting from this type of simulations for an increasing series of concentrations of A. The profiles are very similar to those of β-carotene oxidation in the presence of increasing concentrations of the antioxidant Santoquin (*4*), which we will consider later on. This suggests that, in spite of its simplistic character, the simulation is an acceptable model of our problem.

An advisable resource for the treatment of the data is to standardize the values S_t of the substrate (or the oxidation

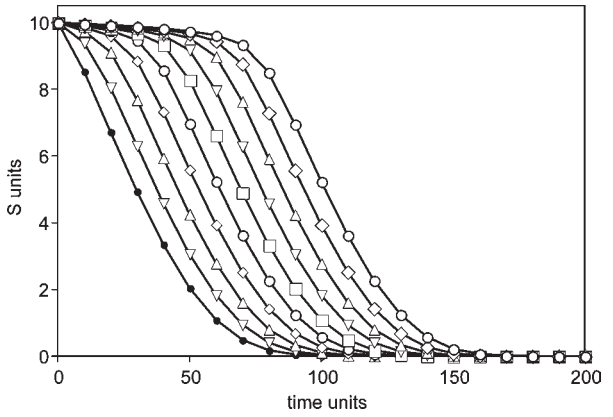

Figure 2. Simulation of the substrate kinetics at increasing antioxidant concentrations, under the conditions specified in **Table 1**d. Antioxidant concentration increases from left to right, with the first series at the left (●) corresponding to the absence of antioxidant.

products SO_t, as appropriate) at the different times and to transform them in time-increasing responses, R:

$$R_t = 1 - \left(\frac{S_t}{S_0}\right) \quad \text{or} \quad R_t = \left(\frac{SO_t}{SO_0}\right) - 1 \tag{8}$$

Since the definition of response implies that its value at zero time is null, it is also suitable to subtract possible non-null initial values. **Figure 3** (top) shows the data from **Figure 2** as responses.

1. Univariate Model of the Antioxidant Activity. The asymptotic curves of **Figure 3** are a logical consequence of the semiopen system which was postulated in the corresponding simulation. In such a system, S and A should be exhaustively oxidized at sufficiently long times. Nonetheless, the kinetics will be equally asymptotic, although with different final values, in a closed system, where the oxygen limitation can prevent the exhaustive oxidation. Thus, the dose of antioxidant, the exposure times, and the size of the experimental unit (as well as the volume of the gas phase in closed systems) should be carefully standardized in any test, so that the experimental results allow formal definition of the profiles of the studied process.

Although such a formal definition could be based on eqs **3**, **4**, and **6**, this procedure is not efficient. Such equations can be useful, as we saw, to justify on a simple kinetic basis the sigmoidal character of the process profiles, but the most efficient way for calculating the values of practical interest in the evaluation of an antioxidant is to model those profiles by means of a cumulative probability function.

In previous works (*5*−*8*) it was demonstrated that the most versatile resource to describe sigmoidal profiles such as those involved here is the cumulative function of the Weibull's distribution. In terms of the variables t (time) and R (response), the original function would be (where a and b are parameters of form and position, respectively)

$$R = 1 - \exp\left[-\left(\frac{t}{b}\right)^a\right]$$

But its use in our context requires two modifications: (1) to multiply the second member by the maximum response K, so that the asymptote can take values different from 1 and hence the model can represent oxygen-limited closed systems and (2) to reparametrize the equation, so that it includes explicitly the time for a semimaximum response or substrate half-life, an essential parameter for comparing antioxidant activities. It facilitates the

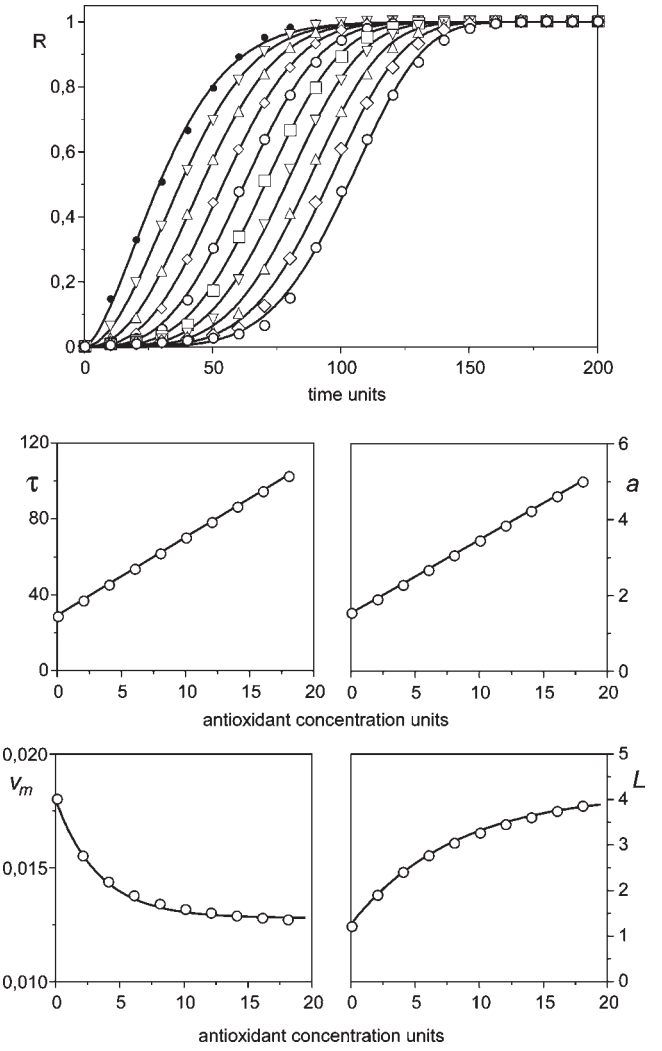

Figure 3. Kinetics of the substrate oxidation at different antioxidant concentrations, and relationships among the concentration of antioxidant and the parameters which characterize its activity. In the top graphic, points are the data from **Figure 2** formulated as responses, and continuous lines are the corresponding fittings to eq **9**. R, response; τ, substrate half-life; a, form parameter of eq **9**; v_m, maximum rate of substrate oxidation; L, logarithmic lag of substrate oxidation. Values of τ and a are fitted to linear equations; values of v_m and L are fitted to eqs **12** and **14**, respectively. Numerical data in **Table 2**.

test of initial values during the application of nonlinear fitting methods and allows the direct calculation of the corresponding confidence interval by means of the usual computer software. The final form, which we will denote ^{m}W, is

$$R = K\left\{1 - \exp\left[-\ln 2\left(\frac{t}{\tau}\right)^a\right]\right\} \tag{9}$$

where R is the response, t the time, τ the substrate half-life, and a a form parameter related with the maximum slope of the response. Especially interesting in the problem studied here is the fact that a particular case ($a = 1$) of eq **9** is the model of the first- and pseudo-first-order kinetics. Thus, all the expected profiles can be described with the same functional form.

Indeed, when eq **9** was used for describing the data—as responses—from **Figure 2**, the fittings were very precise in all the cases (**Figure 3** and **Table 2**), with determination coefficients always higher than 0.999, statistically significant parametric

Table 2. Kinetic Series from **Figure** 3, Fitted to the Specified Models ($\alpha = 0.05$):[a]

	univariate model (eq 9)				bivariate model (eq 16); *adj.* $r^2 = 0.9994$				
[*A*]	*K*	τ	*a*	r^2	*K*	τ	*a*	b_τ	b_a
0	1.00 ± 0.006	29.19 ± 0.63	1.55 ± 0.065	0.9991	**1.00 ± 0.002**	**29.25 ± 0.35**	**1.56 ± 0.04**	**0.140 ± 0.003**	**0.122 ± 0.007**
2	1.00 ± 0.003	37.40 ± 0.32	1.91 ± 0.039	0.9998		37.46 ± 0.59	1.95 ± 0.07		
4	1.00 ± 0.002	45.76 ± 0.24	2.30 ± 0.034	0.9999		45.66 ± 0.84	2.33 ± 0.11		
6	1.00 ± 0.004	54.03 ± 0.40	2.69 ± 0.066	0.9998		53.86 ± 1.09	2.71 ± 0.14		
8	1.00 ± 0.006	62.23 ± 0.55	3.09 ± 0.104	0.9996		62.07 ± 1.34	3.09 ± 0.17		
10	1.00 ± 0.007	70.38 ± 0.68	3.49 ± 0.143	0.9995		70.27 ± 1.59	3.48 ± 0.20		
12	1.00 ± 0.009	78.49 ± 0.78	3.88 ± 0.183	0.9994		78.48 ± 1.84	3.86 ± 0.23		
14	1.00 ± 0.010	86.56 ± 0.87	4.28 ± 0.22	0.9992		86.68 ± 2.09	4.24 ± 0.26		
16	0.99 ± 0.011	94.61 ± 0.94	4.68 ± 0.26	0.9991		94.88 ± 2.35	4.62 ± 0.29		
18	0.99 ± 0.012	102.6 ± 1.01	5.08 ± 0.30	0.9990		103.1 ± 2.59	5.01 ± 0.33		

[a] In the bivariate model (eq **16**), parametric estimates (highlighted) were subsequently used for calculating τ and *a* values for every antioxidant concentration by means of the expressions in eq **17**, accepting the limits of the involved confidence intervals. r^2 (and *adj.* r^2): coefficient (and adjusted coefficient) of simple (multiple) determination.

estimates (Student's *t*; $\alpha = 0.05$), and statistically consistent models (Fisher's *F*; $\alpha = 0.05$).

2. Characterizing Parameters of the Antioxidant Activity. According to that established in the precedent section, the parameter τ provides directly the substrate half-life at a given concentration of antioxidant. This is the most important parameter of the system, and the most robust, in the double sense of less dependent on the descriptive function used and less sensitive to the experimental error. Moreover, the model contains implicitly much more relevant information applicable to the evaluation and comparison of antioxidant properties. Thus:

2.1. Antioxidant Concentration Necessary for Duplicating Substrate Half-Life. The relationship between antioxidant concentration *A* and substrate half-life τ is linear (**Figure 3**):

$$\tau = h_0 + h_1 A$$

Thus, once the coefficients h_0 and h_1 are calculated, it is possible to estimate another interesting value: the antioxidant concentration required for duplicating (or multiplying by the factor *n*) the substrate half-life:

$$A_n = \frac{h_0}{h_1}(n-1) \tag{10}$$

2.2. Maximum Substrate Oxidation Rate at a Given Antioxidant Concentration. The relationship between the concentration of antioxidant and the parameter *a* is also linear (**Figure 3**). However, in practice, the maximum substrate conversion rate v_m at a given antioxidant concentration is more interesting. Such a rate is the slope of the tangent to the curve in the inflection point (*9*, *10*); it can be obtained with some algebraic manipulation starting from eq **9**, and it has the structure

$$v_m = \frac{Ka \ln 2}{\tau} G^G e^{-G};\ \text{where } G = \frac{a-1}{a} \tag{11}$$

This maximum rate drops asymptotically as the concentration of antioxidant increases (**Figure 3**), according to a relationship which can be described by means of a function of the type

$$v_m = v_{inf} + (v_{m0} - v_{inf}) \exp(-rA) \tag{12}$$

where v_{m0} is the maximum rate in the absence of antioxidant, *r* its drop rate with antioxidant concentration, and v_{inf} its inferior asymptote. This relationship does not have too much interest, but it can provide an approximate valuation of the limits of the system, in front of the apparent limitless capacity suggested by eq **10**.

2.3. Lag Time of the Substrate Oxidation Due to the Presence of Antioxidant. It is a parameter that is sometimes interesting, in spite of the fact that it is of doubtful estimation due to the above-mentioned possible irregularities in the initial border of the process. Analysis of variance could be applied for estimating when an experimental value of the substrate differs statistically from the initial one. But such a resource requires an onerous number of replicates and is inaccurate because it depends necessarily on the sampling intervals. It seems more objective to define the lag λ as the intersection of the tangent in the inflection point with the time axis, in the form

$$\lambda = \tau \ln 2 \left[G^{1/a} + \frac{e^{-G} - 1}{aG^G e^{-G}} \right] \tag{13}$$

It can be mentioned, although it is not very useful, that the natural logarithm *L* of the lag increases asymptotically as the concentration of antioxidant increases (**Figure 3**), according to a relationship which is the reverse of eq **12**:

$$L = L_0 + (L_m - L_0)[1 - \exp(-uA)] \tag{14}$$

where L_0 is the logarithmic lag in the absence of antioxidant, *u* the variation rate of *L* with antioxidant concentration, and L_m the superior asymptote.

The confidence intervals of v_m and λ could be estimated by means of the reparametrization of eq **9** to make explicit such values, but the result is too complex and not very operative. It is simpler to calculate the expressions in eqs **11** and **13** using the limits of the confidence intervals of τ and *a* obtained from the fitting of the experimental data with eq **9**.

2.4. Action Interval of an Antioxidant. A last quantitative feature is the action interval, or time that elapses, to a given concentration of antioxidant, until the conversion of 99% of the substrate, that is, until the response reaches the value 0.99*K*. This value can be obtained by isolating *t* in eq **9**, but if we need also the corresponding confidence interval, it is preferable to use eq **9** in a new reparametrized form, so that it includes explicitly the value 0.99*K* as a parameter, an operation which is not problematic in this case. In general, for the time τ_n corresponding to a response equivalent to *n*% of the maximum one (*nK*/100), the reparametrized equation is

$$R = K\left\{1 - \exp\left[\ln(1 - 0.01n)\left(\frac{t}{\tau_n}\right)^a\right]\right\} \tag{15}$$

Thus, when the experimental data are fitted to this form, any statistical software provides directly τ_n and its confidence interval for the established probability (α).

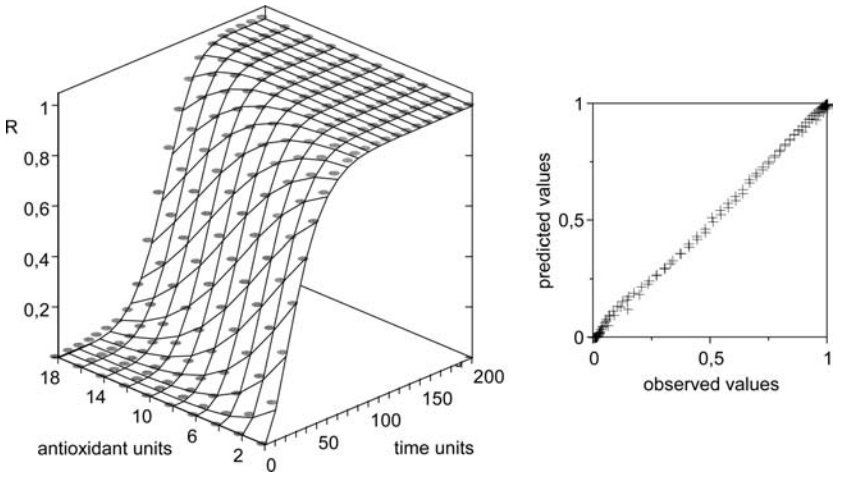

Figure 4. Left: kinetic series from **Figure 3** (points) jointly considered and fitted (surface) to the bivariate model in eq **16**. Right: correlation between observed and predicted values. Numerical data in **Table 2**.

3. Bivariate Model. A more efficient and statistically more consistent way to formulate the model discussed until now is to consider jointly the kinetic series in the presence of all the antioxidant concentrations assayed. It requires the use of a single equation with two independent variables (time and antioxidant concentration), which will be the simultaneous solution of the whole series of responses. This approach has, first, the advantage of minimizing the possible biases due to systematic and random experimental error. Additionally, if the increase in the number of parameters is smaller than the increase in the available number of observations—as in general should occur—the procedure will lead to a net gain in degrees of freedom. Finally, if after a preliminary—anyway necessary—assay to establish the domains of the variables, antioxidant concentrations and sampling times are adequately combined, the bivariate approach will economize experimental work.

In view of the modifications that the presence of antioxidant determines in the parameters τ and a of eq **9**, it can be accepted that the effect of a concentration A of antioxidant on the profile of the response will be proportional to A and able to be modeled by means of perturbing terms as

$$P_\tau = 1 + b_\tau A; P_a = 1 + b_a A$$

In a closed system, where the oxygen limitation can lead to nonexhaustive oxidations, the antioxidant level can also affect the asymptotic value K. Since in this case the effect will tend to reduce the parametric value, the perturbing term will be

$$P_K = \frac{1}{1 + b_K A}$$

Thus, eq **9** should be rewritten as

$$R = \frac{K}{1 + b_K A}\left\{1 - \exp\left[-\ln 2\left(\frac{t}{\tau(1 + b_\tau A)}\right)^{a(1 + b_a A)}\right]\right\} \quad (16)$$

With prooxidant compounds, which tend to reduce τ and a, the perturbing terms would be divisors of the corresponding parameters. In general, since the antioxidant action can proceed through different mechanisms of competition for oxygen between substrate and antioxidant, the form (factor or divisor) in which a perturbing term should be included in the equation, especially if it affects the parameter a, should be subjected to the usual statistical best-fit tests.

Using again the values from **Figure 3**, now joining all the series in a sole matrix, it could be verified that eq **16** describes the data with the same precision as that of eq **9** separately applied to the same series, as can be appreciated in **Table 2**. Therefore, the surface depicted in **Figure 4** represents the simultaneous solution of the kinetics corresponding to all the concentrations of antioxidant. The slight lack of fit which can be appreciated in the correlation between observed and predicted values is due to the above-mentioned effect of initial conditions, and it disappears if the initial level of oxygen is modified or suppressed. The parameters τ and a for a given concentration of antioxidant can be determined by means of

$$\tau_{i(A)} = \tau(1 + b_1 A_i); a_{i(A)} = a(1 + b_2 A_i) \quad (17)$$

4. Experimental Verifications. As it was said, the verification of the model with experimental data was performed by using bibliographical results, selected among those more appropriate to exemplify different systems, methods, and time domains. When it was necessary, original data were transformed in responses using the expressions in eq **8** defined previously.

4.1. Santoquin in β-Carotene Bleaching. The quantification through carotene loss of the coupled oxidation of β-carotene and linoleic acid in aqueous emulsion is the basis of one of the current methods for evaluating antioxidant activity. In the presence of increasing concentrations of the antioxidant Santoquin, Marco (*4*) obtained clearly sigmoid kinetics (**Figure 5**), and he defined the response, or extended induction time, as

$$E_t\ (\%) = \frac{t_n - t_0}{t_{ref} - t_0} \times 100 \quad (18)$$

where t_n is the time at which, at a given concentration of the antioxidant under consideration, the substrate concentration (with ordinate S_n) is n% of the initial one and t_0 ant t_{ref} are the times which correspond to the S_n ordinate on the curves obtained in the absence of antioxidant and in the presence of a reference antioxidant, respectively.

The author, who averaged out the results for $n = 50$ and $n = 70$, recommended the use of logit graph paper for graphically linearizing the curves in the ordinate interval 10−90%. He also observed that the relationship between the concentration of antioxidant and its effect (in terms of the response E_t) on the oxidation of the substrate is linear, thus providing an additional calibration resource. The calculation procedure was without a doubt useful at the date of its publication, and our univariate approach is in essence the same one, with the difference that it uses an explicit algebraic function. Given the current facilities for

nonlinear fitting methods, it is preferable that this second option, which allows a more precise calculation, provides important additional information and leads to the bivariate model in eq **16**.

In this regard, it is interesting to use the Marco's results (it is difficult to find in the bibliography so complete kinetic data) to compare the uni- and bivariate approaches which we propose. The independent application of the univariate model in eq **9** to the four kinetics from **Figure 5** produced individual satisfactory fittings, but the results showed two problems. First, the linear relationship between τ and A, which is an important condition of the system, was poor. In addition, the experimental system is closed, but its design prevents the oxygen deficiency and it allows the exhaustive oxidation. However, the two lower concentrations of Santoquin suggested lower asymptotes than the control, but the highest concentration (whose kinetics is incomplete) suggested a higher asymptote (in fact, the fitting provides a higher value than 1 if the corresponding restriction is not included), which seems contradictory.

Nevertheless, when the bivariate model in eq **16** was applied to the same four kinetics jointly considered, the only coefficient that lacked statistical significance ($\alpha = 0.05$) was b_k, which translates the effect of the antioxidant on the asymptote, whereas the recalculation after suppressing that coefficient produced a consistent model (**Table 3**). On the other hand, since eq **16** includes the linear relationship between A and τ, its result necessarily obeyed this condition, although at the expense of a higher discrepancy of the individual kinetics, especially at the lowest antioxidant level. Likely the best election in this type of alternatives is the bivariate model, which describes the system as a whole. To attribute the discrepancy to the experimental error is, in fact, plausible in this case, which demands individual preparation of emulsions with oxygen-saturated water, and so small differences in the diffusion of the gas can determine a cumulative effect with time.

4.2. Trolox and Ascorbic Acid in the Riboflavin Oxidation in Milk. Although in the study of Hall et al. (*11*) some kinetics—with time course of hours—were interrupted before the asymptotic level, the validity of the bivariate model **16** can be verified in cases like this through its ability to provide a reasonable approach to the global description of the system. Indeed, if at least the kinetics in the absence of antioxidant—necessarily the most complete one—allows estimation of an asymptote, it can be accepted that (1) any kinetics in the presence of antioxidant will have a lower or equal asymptote, which can be used as a restriction in the fitting process and (2) the linear relationship between τ and A included in eq **16** will act as an additional restriction that will give coherence to the solution of the system if the model is valid.

Equation **16** was applied in this case after smoothing the original results by means of the mobile average method (window = 3), for minimizing the effects of the random experimental error, and the antioxidant concentrations (in ppm) were coded dividing them by 100, so that the numerical domains of the independent variables were of the same order, thus avoiding biases in the parametric estimates. **Figure 6** shows two representative

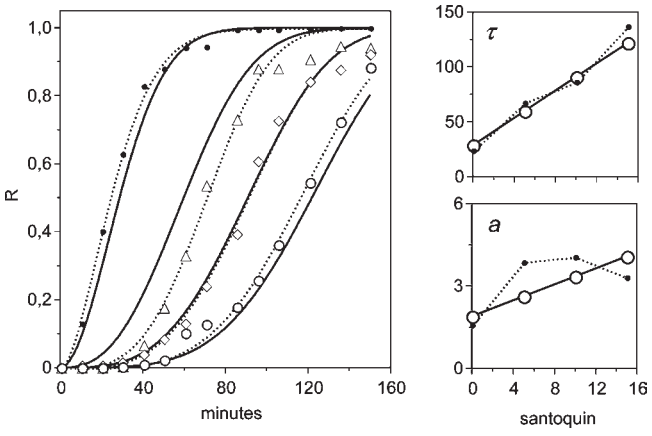

Figure 5. Experimental data from Marco (*4*) for the antioxidant Santoquin in the system β-carotene/linoleic acid, in terms of responses (points) and fitted to the univariate model in eq **9** (discontinuous lines) and the bivariate model in eq **16** (continuous lines). Concentrations of Santoquin: 0 (●), 5 (△), 10 (◇), and 15 (○) micrograms per experimental unit (52 mL). For more details, see text.

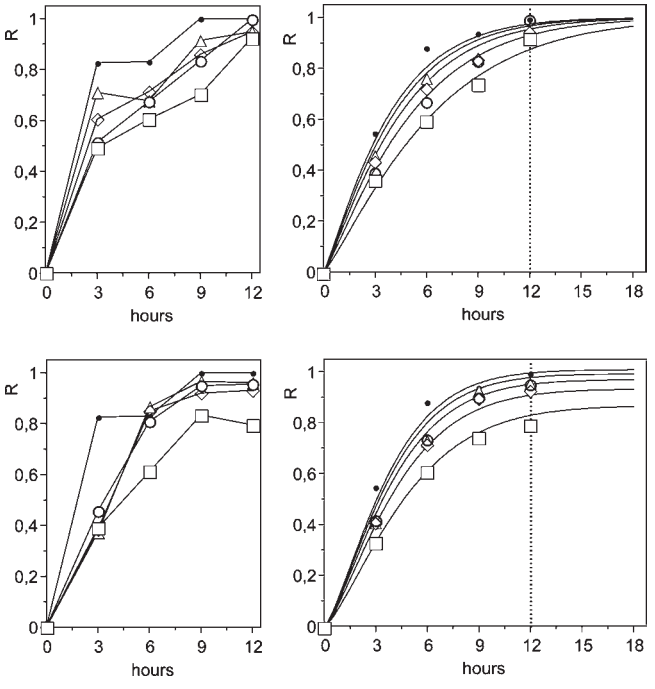

Figure 6. Riboflavin oxidation in milk (as response R) in the presence of increasing concentrations of Trolox (top) and ascorbic acid (bottom). In both cases: 0 (●), 100 (△), 250 (◇), 500 (○), and 1000 (□) ppm. Left: original data from Hall et al. (*11*); right: smoothed data (mobile average, window=3), jointly fitted (lines) to the bivariate model in eq **16**. Note the differences in final values that are suggested by the extrapolations.

Table 3. Results from Marco (*4*), Hall et al. (*11*), and Pazos et al. (*12*) Fitted to a Bivariate Model (Eq 16) ($\alpha = 0.05$)[b]

	K	τ	a	b_k	b_τ	b_a	adj r^{2a}
Marco (*4*)	1.00 ± 0.002	28.77 ± 3.01	1.90 ± 0.47		0.216 ± 0.034	0.076 ± 0.050	0.9746
Hall et al. (*11*) Trolox	1.00 ± 0.008	2.88 ± 0.282	1.172 ± 0.126		0.065 ± 0.023		0.9902
Hall et al. (*11*) ascorbic acid	1.03 ± 0.067	3.34 ± 0.309	1.345 ± 0.264	0.026 ± 0.008			0.9884
Pazos et al. (*12*) emulsion	74.7[c]	3.11 ± 0.096	3.27 ± 0.41	0.078 ± 0.065	0.079 ± 0.032		0.9931
Pazos et al. (*12*) fish oil	104.0[c]	6.10 ± 0.082	3.01 ± 0.17		0.036 ± 0.004		0.9939

[a] Adjusted coefficient of multiple determination. [b] See also **Figures 5**–**7**. [c] Estimated asymptotic value in the absence of antioxidant, used as a restriction (maximum acceptable value) for global fitting.

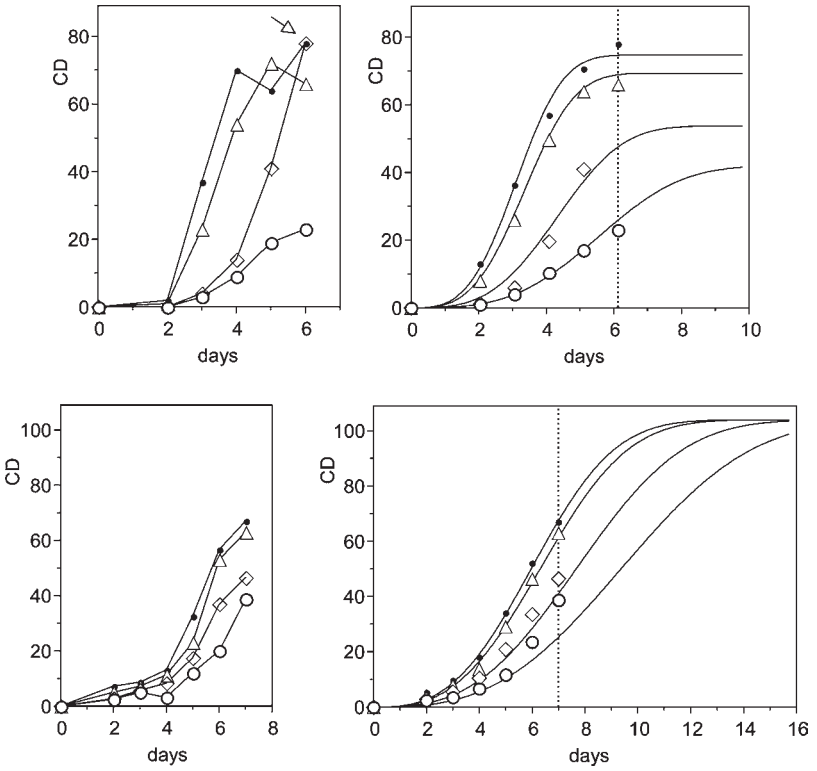

Figure 7. Formation of conjugated diene hydroperoxides (CD) in water-emulsified (top) and pure (bottom) fish oil in the presence of three concentrations of hydroxytyrosol: 0 (●), 10 (△), 50 (◇), and 100 (○) ppm. Left: original data of Pazos et al. (*12*); right: smoothed data (mobile average, window=3), jointly fitted (lines) to the bivariate model in eq **16**. Note the differences in final values that are suggested by the extrapolations. In the case of the emulsion, the experimental value at 10 ppm (◇), marked with an arrow and only explainable as an outlier, was suppressed.

experimental series, and in both of them eq **16** provided a statistically significant and consistent (Student's *t* test and Fisher's F-test, respectively, for $\alpha = 0.05$) description.

It can be pointed out that in the case of Trolox the results required acceptance of the same asymptotic value for all the kinetic series (**Table 3**), in which the extension of the riboflavin half-life was due to the antioxidant effect. However, in the case of ascorbic acid, the equation was significant and consistent, simply accepting different asymptotes, whereas the coefficient b_τ—which translates the extension of the riboflavin half-life—was not statistically significant. The result could be explained supposing that the antioxidant drains all the oxygen from the initial gas phase, and since τ corresponds to the semiasymptotic response, the asymptotic drop is enough to justify the effect.

4.3. Hydroxytyrosol in the Oxidative Deterioration of Fish Lipids. Pazos et al. (*12*) studied the effects of several antioxidants in different modalities of this system, with results which allowed us to establish interesting gradations in their effectiveness. As is habitual, again several kinetic series were interrupted at subasymptotic levels, hindering their individual fitting to any sigmoid model and thus requiring the same criteria applied in the previous case for validating eq **16**. Fittings were carried out also, with original results smoothed by the mobile average method (window = 3) and antioxidant concentrations (in ppm) coded dividing them by 10, to balance the domains of the independent variables. As an illustration, responses were considered in natural values instead.

Figure 7 shows two representative experimental series, both described in a statistically significant way (**Table 3**) by means of eq **16**. Although with the available data the asymptotic values are relatively chancy extrapolations, it has interest to point out that the model produces clearly different values in emulsions (that is the expected result in a closed system with oxygen limitation) and the same values in pure oils (that is the expected result if the limiting reagent is the substrate).

As in the previous case, this type of question is beyond the perspective of this work. Here we mention them to illustrate that the model which we propose is useful to detect problems of practical interest, although, as it happens in any kinetic model, it cannot explain the underlying mechanism, as well as to establish quantitative comparisons on an accurate formal basis.

In conclusion, the lack of standardization (*2*) in methods for evaluating antioxidant activity begins with the lack of formal models applicable to the lipid oxidation, already pointed out by Özilgen and Özilgen (*1*). Under these conditions, the experimental designs are often merely intuitive, and many of the abundant bibliographical data in this regard are incomplete from a kinetic point of view, which prevents suitable characterization of the relevant properties of the tested compounds. The model which we propose provides a simultaneous description of the kinetics of the oxidation of a substrate in the presence of any number of concentrations of an antioxidant. Although it does not have a mechanistic form, it coincides in one of its particular cases with the first order kinetic model, and it fits accurately the profiles of simulations which postulate the competition of substrate and antioxidant for oxygen, in a second order kinetic scheme. The model also fits in a statistically significant way the results obtained by other authors, in processes involving different substrates, antioxidants, conditions, and time domains. The application of the model is simple: in its bivariate option, it reduces the sensitivity of the univariate option to possible biases due to different types of experimental error, and its mathematical form constitutes a useful orientation to prepare more economic and efficient experimental designs than the conventional ones in this field. Finally, its results allow the calculation of several parameters which characterize both the oxidative process and

antioxidant action, and they facilitate objective comparisons among different compounds and methods.

LITERATURE CITED

(1) Özilgen, S.; Özilgen, M. Kinetic model of lipid oxidation in foods. *J. Food Sci.* **1990**, *55*, 498–501.

(2) Frankel, E. N.; Finley, J. W. How to standardize the multiplicity of methods to evaluate natural antioxidants. *J. Agric. Food Chem.* **2008**, *56*, 4901–4908.

(3) Melton, S. L. Methodology for following lipid oxidation in muscle foods. *Food Technol.* **1983**, *37*, 105–111.

(4) Marco, G. J. A rapid method for evaluation of antioxidants. *J. Am. Oil Chem. Soc.* **1968**, *45*, 594–598.

(5) Murado, M. A.; González, M. P.; Vázquez, J. A. Dose-response relationships. An overview, a generative model and its application to the verification of descriptive models. *Enzyme Microb. Technol.* **2002**, *31*, 439–55.

(6) Murado, M. A.; Vázquez, J. A. The notion of hormesis and the dose−response theory: A unified approach. *J. Theor. Biol.* **2007**, *244*, 489–499.

(7) Riobó, P.; Paz, B.; Franco, J. M.; Vázquez, J. A.; Murado, M. A.; Cacho, E. Mouse bioassay for palytoxin. Specific symptoms and dose-response against dose-death time relationships. *Food Chem. Toxicol.* **2008**, *46*, 2639–2647.

(8) Riobó, P.; Paz, B.; Franco, J. M.; Vázquez, J. A.; Murado, M. A. Proposal for a simple and sensitive haemolytic assay for palytoxin. Toxicological dynamics, kinetics, ouabain inhibition and thermal stability. *Harmful Algae* **2008**, *7*, 415–429.

(9) Vázquez, J. A.; Murado, M. A. Mathematical tools for objective comparison of microbial cultures. Application to evaluation of 15 peptones for lactic acid bacteria productions. *Biochem. Eng. J.* **2008**, *39*, 276–287.

(10) Zwietering, M. H.; Jongenburger, I.; Rombouts, F. M.; Van't Riet, K. Modeling of the bacterial growth curve. *Appl. Environ. Microbiol.* **1990**, *56*, 1875–1881.

(11) Hall, N. K.; Chapman, T. M.; Kim, H. J.; Min, D. B. Antioxidant mechanisms of Trolox and ascorbic acid on the oxidation of riboflavin in milk under light. *Food Chem.* **2010**, *118*, 534–539.

(12) Pazos, M.; Alonso, A.; Sánchez, I.; Medina, I. Hydroxytyrosol prevents oxidative deterioration in foodstuffs rich in fish lipids. *J. Agric. Food Chem.* **2008**, *56*, 3334–3340.

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