β-carotene assay revisited. Application to characterize and quantify antioxidant and pro-oxidant activities in microplate.

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ABSTRACT

The β-carotene (βC) 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 pro-oxidant 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 pro-oxidants, and can be easily applied to natural extracts, food samples and many other type of compounds. As example, we have tested a set of commercial antioxidants and some typical lipophilic pro-oxidants. The activity of the tested antioxidants decreased in the following order: ethoxyquin >> α-tocopherol > butyl-hydroxyanisole (BHA) > butyl-hydroxytoluene (BHT) >> propyl gallate (PG). On the other hand, haemoglobin and Fe^{2+}, Fe^{3+}, Co^{2+} and Cu^{2+} showed a strong pro-oxidant effect, the activity was null in Cd^{2+}, Ni^{2+} and Sr^{2+}, slightly antioxidant in Mg^{2+}, and strongly antioxidant in Zn^{2+} and Mn^{2+}.

Keywords: antioxidant activity; β-carotene method; mathematical modeling; Weibull equation.

INTRODUCTION

Methods to quantify the anti- or pro-oxidant 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, multiphasic). Under these conditions, the effort has been focused on multiplying assessment methods (it is common to apply 3 to 6 different procedures in each evaluation), and less attention has been done 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 last decade many authors have claimed to unify the approaches *(1-10)*, and have proposed standardized criteria to assess the real effectiveness of an antioxidant, both *in vitro* and *in vivo (11-13)*.

The main antioxidants used in the food industry inhibit lipid oxidation by acting as metal chelator (such as ethylenediaminetetraacetic acid) or hydrogen donors (such as BHA). In the latter case, one of the most common methods for assessing antioxidant activity is the β-carotene

(βC) bleaching assay *(14, 15)*. The method works in an aqueous emulsion of linoleic acid and βC, which is discolored by the radicals generated by the spontaneous oxidation of the fatty acid *(7)*, promoted by thermal induction, typically at 50°C. Quantification is based on varying the rate at which βC absorbance decays (~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 βC reaction is not representative of the lipid oxidation in foods, where the fatty acids are mainly present as triglycerides *(1, 7)*, and whose crude extracts can contain interfering materials *(1)*, and even other OM agents, potentially causing synergistic or antagonistic interactions, which have not been studied yet in depth *(16, 17)*.

Second, βC is sensitive to oxygen and temperature even in the absence of linoleic acid *(18, 19)*. Moreover, in a lipid emulsion, the hydrophobic repulsion phenomena favour the activity of apolar OM against the polar ones (polar paradox: *(20)*).

Finally, the assay reproducibility can be low due to: a) the reagent complexity *(4)*; b) the non-specific conditions of heat induction *(5, 8)*, which has led to suggest the use of free radicals (*e.g.* AAPH or Fe^{2+}) as initiators; c) the frequent use of a single reaction time, which does not guarantee the reliability of the results *(5, 9)*; d) the effects on the reaction of factors such as pH, solvent ratios and the presence of metals *(21)*, 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. Other 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 *(22-25)*. MO agents interfere with a process which is necessarily asymptotic and generally sigmoidal. In this context, one can look for conditions that hide this fact and make acceptable linear approximations based in 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 *(1, 4, 5, 7, 8, 16, 26)* between the samples under evaluation, but also it makes the identification of the key critical points and the standardization of the assessment conditions *(21)* 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 to study the response in detail, providing parameters whose factual meanings are able to characterize anti- and pro-oxidant activities in a consistent, practical, useful and reproducible way.

MATERIALS AND METHODS

Equipment and reagents

Equipment: Multiskan Spectrum microplate photometers from Thermo Fisher Scientific; 96-well polypropylene microwell plate with flat bottom.

Main reagents: linoleic acid, βC and Tween 40.

Antioxidant agents: butyl-hydroxyanisole (BHA); butyl-hydroxytoluene (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 (α-tocopherol).

Predictably pro-oxidant agents: iron (II) and (III) sulfides, copper (II) sulfate, magnesium sulfate, manganese sulfate, cadmium nitrate, nickel nitrate, zinc chloride and hemoglobin.

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

The β-carotene bleaching method.

Reagent and procedure conditions

The method described by Marco *(14)* is the reference for many subsequent modifications that simplified the operative *(15)* or transferred the procedure to microplate *(4)*. 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 *(14)*, 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:

$$E_t(\%) = \frac{t_n - t_0}{t_{ref} - t_0} \times 100 \qquad [1]$$

where t_0, t_n and t_{ref} are the times at which the βC 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 βC extended induction time was close to linearity.

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* de *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 α=0.05) were calculated using the '*SolverAid*' macro previously used *(27)*, freely available from *de Levie's Excellaneous* website: http://www. bowdoin.edu/~rdelevie/excellaneous/. The *'SolverStat'* macro *(28)* 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 *(29, 30)*:

$$Af = 10^{\frac{\sum\left|\log\left(\frac{pred}{obs}\right)\right|}{n}} \quad \text{and} \quad Bf = 10^{\frac{\sum\log\left(\frac{pred}{obs}\right)}{n}} \qquad [2]$$

where *pred* and *obs* are the predicted and experimental values, respectively, and *n* the number of observations. The nearer the values of B_f and A_f are to 1, the better is 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 modifies the response (linoleic acid, antioxidant concentration, pH and temperature, among others). These effects have induced in some cases to over-standardize the protocol, and in some others to overlook 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 anti- and pro-oxidant 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 other. Next, the assay performances used in this work are described and its differences from other alternatives will be discussed and justified.

Assay performances: reagent, procedure, analytical time and quantification criterion

Reagent

A common criticism of the βC method is the lack of reproducibility due to the complexity and instability of the reagent. Some authors *(31)* store a stock solution of βC and Tween in chloroform at –16ºC, and add linoleic acid at the time of use. Under these conditions the stock solution becomes concentrated by evaporation and significantly bleached in ~40 h. The daily preparation of the reagent corrects this problem, but causes variations in the initial conditions *(4)*. The procedure described next will provide enough reagent for testing 2,400 samples and should be stable at least for one week, without significant changes in the initial conditions.

β-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 50ºC 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 –18ºC under nitrogen. A single tube provides sufficient reagent to fill a microplate (120 wells), which is obtained by adding 30 ml of 100 mM Briton buffer, pH=6.5 in pre-heated mili-Q water at the assay temperature (45ºC). The absorbance at 470 nm of the reagent thus prepared is ~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 µl) by combining 50 µl of the sample to be tested and 250 µl of reagent. The reader device was programmed to 45°C with agitation (660 sections per min and 2 mm amplitude). In addition to the sample under evaluation, the microplate must contain:

a. A series (calibration) in which the sample is replaced by a reference OM in the selected solvent (water:ethanol 9:1) at increasing concentrations.
b. Two or three wells (control) in which the sample is replaced by solvent.
c. If the effect of βC 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.
d. If sample or calibration antioxidant absorb at 470 nm, an additional series (correction) in which the reagent is replaced by solvent should be included.

The analytical time

The analytical time is a non-trivial aspect. Short analysis times (~50 min) prevent the kinetic definition of the process, whereas longer times (~500 min) enhance the effect of factors as solvent evaporation and thermal discoloration of βC. 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 an reproducible data sets were systematically obtained.

On the other hand, as we will see later on, the theoretical limit of the half-life extension promoted by an antioxidant is +∞, but the limit of half-life contraction promoted by a pro-oxidant is zero. Therefore, a progressive packing of the kinetic profiles takes place in this last case, what can difficult accurate evaluations. Conditions that allow to a control half-life of ~35 min were found as optimal for analyzing both anti- and pro-oxidants. If a especially efficient pro-oxidant determination is necessary, a simple way to achieve it consist of increasing the half-life of the control by reducing the temperature of the assay (at 35ºC, the half-life is ~115 min).

Quantification

Quantification was carried out using as a basis the Murado and Vázquez model *(25)*, which describes the whole kinetic profile by applying the Weibull mass function *(32)*. When S_0 and S_t are the substrate (βC) 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:

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

where *K* is the asymptote, τ 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}):

$$v_{max} = \frac{Ka}{\tau}\left(\ln 2\right)^{1/a} G^{G}\exp\left(-G\right) \;\; ; \;\; \text{where:} \;\; G = \frac{a-1}{a} \qquad [4]$$

given the form of *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_{med} (corresponding with the median abscissa τ) may be used instead v_{max}:

$$v_{med} = \frac{Ka \ln 2}{2\tau} \qquad [5]$$

The equation [3] is very versatile: if $a<1$ it can describe the profiles generated by the model developed by Terpinc and Abramovič *(24)*; 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, what 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 (τ) is always increased by the presence of an antioxidant and because the relation [4], the maximum slope (v_{max}) decreases if a remains constant. Additionally, a could vary as well, modifying the relationship between τ 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 pro-oxidant shall promote the opposite effects: half-life decrease (with corresponding v_{max} increase) and a decrease if the effect on τ is not enough to explain the earlier beginning of the propagation phase.

Therefore, the model [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 τ 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 θ, due to an OM agent, can be described by means of a hyperbolic factor H_θ like:

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

where *OM* is the agent concentration, and u_θ, v_θ fitting coefficients (when v_θ=0 the dependence is linear). This leads to formulate a bivariate equation, as a function of time and the agent concentration, in the terms:

$$R(t, OM) = K\left\{1 - \exp\left[-\ln 2\left(t/H_\tau \cdot \tau\right)^{H_a \cdot a}\right]\right\} \qquad [7]$$

Thus, the entire set of kinetic profiles can be simultaneously described, in the most complex case, by the seven parameters of the model [7]. A useful characterization of *OM* activity is now provided by the term H_τ, which represents the increase (antioxidants) or decrease (pro-oxidants) 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 *(33, 34)*, the simultaneous description of all curves is 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 βC bleaching rate (both in the presence and absence of linoleic acid), the solvent evaporation and the microplate thermal gradient, this last a usual problem is such devices *(35)*. 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 (pro-oxidant) values in the same assay; b) βC bleaching in the absence of linoleic acid, evaporation and thermal gradient are 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 50ºC) and wavelengths (470, 900 and 975 nm), with the 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 2, part A). Thus, the βC bleaching includes the effect of temperature and 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 higher than 12 hours, can be neglected by using transparent, oxygen-permeable films *(36)*. 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 between the absorbances at 975 and 900 nm (figure 2A4). Although over 35ºC 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 (~120 µl) at which the meniscus corrupts the measure.

Thermal gradient is a more problematic issue, as it is illustrated in figure 1A5. 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 τ parameter from equation [3] with

temperature, within the range 30-50ºC (see table A in the supplemental material for the parametric stimations), both in the presence and absence of linoleic acid. Second, using this linear relationship, the values of τ provided in the absence of linoleic acid, in each of 96 wells, enabled to calculate the corresponding temperatures. The thermal gradient thus revealed allows to conclude that: 1) a temperature of 45ºC, close to values used by other authors, was a less risky condition (see figure 1A.5); 2) the gradient cannot be detected at ~32 ºC, and over 55ºC is very strong, disabling the possibility of an accurate analysis; 3) at 45ºC, the first 3 columns on the left and the 2 bottom rows of the microplate should be excluded.

Both the original method *(14)* and its adaptation to microplate *(4)* operate between 55-50ºC. Our results, however, showed that, although equations [3] or [7] provided always 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 45ºC because: a) it significantly reduces the spontaneous oxidation of βC; b) it leads to better discrimination between different levels of antioxidant; c) it is significantly more sensitive to pro-oxidant effects. In fact, these effects cannot be accurately detected at 50ºC (see figure 1) and their determination would improve even at 40ºC; d) it significantly reduces the temperature gradients; e) although it extends the analytical time, it is no problematic from the point of view of any collateral effect.

Lower temperatures produce even statistically better results, but extend unnecessarily the analysis time. Higher temperatures reduce this time, but tend to infringe the conditions a and b above established 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 τ 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 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 non-linear 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 anti- and pro-oxidants.

The time-dependent response in the βC method is inherently sigmoidal, and it represents its most important factor. The reduction of the study at one single time and expect 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 βC bleaching reaction, by combining the use of the equation [7] with a high amount od data with low experimental error. As a reference, the case odf BHA will be discussed at first.

The individual fitting to the equation [3] (figure 1 C1) of the kinetic profiles obtained in the presence of BHA showed that the antioxidant determines: a) an increase of the half-life (τ) following a linear relation ($\tau=54.58\cdot BHA+39.73$; $r^2=0.993$); b) a slight variation of *a* that reaches a stable minimum from 2,0 μM BHA, in anyway scarcely relevant. The simultaneous fitting (figure 1 C2) of all profiles to the bivariate equation [7] confirmed the linear effect on τ,

and the lack of statistical significance for *a* variation. Although this last model showed deviations in some profiles (compare figure 1 C1 with C2), it is preferable because it treats the system as a whole, enabling to verify the statistical consistence of all the sides involved in the resulting characterization. The asymptotic drop of v_{med} was consistently described by both approaches (dots and lines for uni- and bi-variate models, respectively, in figures 1 C, 4, 5 and 6).

Moreover, the isoboles (projections on the plane *A*-*t* of the lines of equal response) of the surface from equation [7] formed a straight beam converging at one point (figure 1 C3). In other words: the relationship between 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, what facilitates predicting its effects. Since *a* does not vary with BHT concentration, and τ varies linearly, the mean value of the half-life concentration ratio (55.81 ± 1.25 min·μM^{-1} in this case) can provide a specific half-life extension as characterizing index. Unfortunately, this behavior cannot be generalized to all types of antioxidants, as it will be shown later.

This determination was repeated applying the Marco's method *(14)* according to Miller's protocol *(15)*, with five BHA levels within the same domain [0-(1)-5 μM], in tubes at 50ºC and measures at ten times. The kinetic of the control was indistinguishable from that obtained in microplate at the same temperature (figure 1 A1), 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 50ºC, and reproduced the linear effect of BHA on τ and the absence of effect on *a*.

pH effect

The criterion of omitting buffers *(14)* is not shared by other authors *(21)*, who found that initial pH affects the progress of the oxidation. In our case, the effect of 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 μM, in the presence and absence of linoleic acid. Within this range, pH did not determine hipso or bathochromic shifts in the absorption spectrum of βC, and the maximum effect on the absorbance at 470 nm was less than 5%.

At 45ºC, 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 [7] showed that the pH modifies the value of τ (*a* remains constant) without altering its linear dependence on BHA concentration. These results (figures 1 B and table 2 part B) confirmed the adequacy of the range 5.5-7.5 and led to select the value 6.5 as pH of the routine assays.

As table 2 shows, all the experimental data were satisfactorily modeled either by equations [3] or [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.

The 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 (like Fe^{2+}) would lead to a new problem, since the inclusion of a pro-oxidant in the system would require a more complex mathematical model.

The 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 *(4)*. 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 *(17)* 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 βC bleaching process. Consequently, the saturation step may be omitted.

Synergistic or antagonistic interactions

In no purified samples, the presence of interfering materials, able to produce synergistic or antagonistic effects *(1)* is 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 pro-oxidant) 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, pro-oxidant, another antioxidant) whose nature or concentration are 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 equation [3] or [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 *(21)* of ~10% in the response of βC due to the solvent effect requires equalized proportions in samples, blanks, calibration and control wells (as in any spectrophotometric assay).

A different problem concerns the aforementioned "polar paradox" *(20)*: since the hydrophobic repulsion tends to concentrate non-polar 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 artifactual, since it explains an objective advantage of the lipophilic antioxidants when the oxidized substrate is lipidic. In fact, when βC (lipophilic substrate) was substituted by crocin (hydrophilic substrate) in a reagent prepared with

increasing concentrations of linoleic acid (180-500 μg ml^{-1} in the final mixture), the complementary "non-polar 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 anti- and pro-oxidant agents

The time-course of the reaction was studied at the condition previously discussed, using five common antioxidants (BHA, BHT, ethoxyquin, propylgallate and α-tocopherol), several metals (Cu^{2+}, Fe^{2+}, Fe^{3+}, Cd^{2+}, Ni^{2+}, Sr^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}) and hemoglobin. These last were selected because they can be present, either as constituents or contaminants, in crude biological extracts, and metals as traces in buffer salts *(37, 38)*.

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

Beyond quantitative differences (figure 2 and table 3), BHT and propylgallate behaved like BHA (increase of τ and not statistically significant or irrelevant variation of *a*). The much stronger

effects of ethoxyquin and α-tocopherol were more complex as well, the first promoting an increase of *a*, and the second a slight decrease. Increase of *a* can be explained, as it was said, 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 the equations [3] and [7] were applied to the description of pro-oxidant activities (figure 3 and table 3). In all the cases the decrease of τ was asymptotic, a behavior which probably can be generalized to any pro-oxidant. Indeed, while the theoretical limit of the τ increase is $+\infty$, that of its decrease is zero (instead $-\infty$), what causes a progressive packing over the kinetic profile of the control. This is, otherwise, the reason by which a convenient methodological unity for anti- and pro-oxidants 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 pro-oxidant activities of hemoglobin and Fe^{2+} confirmed the need of the precautions to be taken with crude biological extracts and buffers, and induced to evaluate other metals potentially present as contaminants in these cases. Activity was pro-oxidant 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 results was always satisfactory. The mathematical equations were robust and consistent (p-values < 0.001 from Fisher's F test), the residuals were randomly distributed and autocorrelations were not observed by Durbin-Watson test (data not shown). The statistical analysis, parameter assessment tools and model prediction uncertainties provided by the *'SolverStat'* macro agreed accordingly. Furthermore, all the adjusted coefficients of multiple

determination between predicted and observed values were always $R^2_{adj} > 0.95$, 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 consist of plotting the specific variation of the half-life (H_τ), given by the expression [6], as a function of the agent concentration. This can provide fixed values, as concentration that doubles the half-life (antioxidants) or reduces it by half (pro-oxidants), and, more interestingly when H_τ is a non-linear term, allows to visualize 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:

1) The nature 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 admit 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 βC 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.

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FIGURE CAPTIONS

Figure 1: Factors affecting βC bleaching in microplate assay. A: temperature effects (30: ○, 35: ■, 40: □, 45: ▲ and 50ºC: △) on the bleaching kinetics in the presence (A1) and absence (A2) of linoleic acid, the evaporation (A3) and the thermal gradient in microplate (A4). B: pH effects on the parameters of the equation [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 of BHA (0: △, 0.5: ▲, 1.0: □, 1.5: ■, 2.0: ○, 2.5: ●, 3.0: ◇, 3.5: ◆, 4.0: ▽, 4.5: ▼ and 5.0 μM: **+**). C: kinetics of βC bleaching, at 45ºC and pH=6.5, in the presence of the same concentrations of BHA, according to the univariate (C1) and bivariate (C2) models [3] and [7]. Isoboles of the response surface from model [7] are also represented (C3), as well as the effects of BHA concentration on τ (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.

Figure 2: Effects of the specified antioxidants (ranges in table 3) on βC bleaching reaction. A: experimental results (dots) and fittings (lines) to the model [3]; B: *ibid*. to the bivariate model [7]; C: Parametric variations as a function of the antioxidant concentration, obtained from the models [3] (dots) and [7] (lines); D: correlations between observations and predictions, and isobolograms. In all cases a control series (△) and ten dilutions of the maximum concentration were tested (▲: 1/10, □: 2/10, ■: 3/10, ○: 4/10, ●: 5/10, ◇: 6/10, ◆: 7/10, ▽: 8/10, ▼: 9/10, **+**: 10/10). See also table 3.

Figure 3: Effects of the specified pro-oxidants (ranges in table 3) on βC bleaching reaction. Graphic criteria and keys as in figure 2. See also table 3.

Figure 4: Anti- and pro-oxidant responses. Bottom: specific half-life extensions (antioxidants) and reductions (pro-oxidants) of the agents considered in figures 2 and 3. Note concentration scales on lines.

TABLES

Table 1: Main conditions commonly used in the βC method. The ratios between reagent components (βC: β-carotene, LA: linoleic acid, Tw: Tween-40, R: reagent and S: sample) are established on the βC basis, whose concentration is specified in the first column.

βC	component relationships			T (ºC)	R:S (ml:ml)	source
μg.ml^{-1}	βC	LA	Tw			
12.00	1	150.00	333.3	50	50:2	Marco (1968)
40.00	1	0.09	1000	50	5:0.2	Miller (1971) [a]
4.00	1	0.11	1000	55	0.25:0.03	Dapkevicius et al., (1998)
5.00	1	36.00	200	50	0.25:0.03	Mikami et al., (2009)
6.67	1	0.11	1000	45	0.25:0.05	Present work

([a]) we have assumed that the original work contains a typo in the units which requires dividing by 1000 the concentrations of βC and LA specified in that report.

Table 2: Parametric estimates and confidence intervals (α=0.05) of the βC bleaching kinetics as affected by: A) temperature, according to the model [3]; B) pH, according to the model [7] applied, at each pH value, to the series of BHA concentrations tested in C; C) BHA concentration, at T=45ºC and pH=6.5, according to the models [3] and [7]. Not statistically significant parameters of the model [7] were omitted. See also figure 1.

	K	τ	a	u_τ	R^2_{adj}
A: temperature (ºC)					
30	0.940±0.01	181.61±0.91	0.86±0.03	--	0.9983
35	0.961±0.02	116.04±2.05	0.86±0.02	--	0.9993
40	0.945±0.01	73.66±6.83	2.03±0.04	--	0.9992
45	0.932±0.03	38.61±17.6	1.15±0.04	--	0.9987
50	0.930±0.02	12.05±5.01	1.22±0.06	--	0.9977
B: pH					
3.5	1.000±0.23	120.46±51.0	0.78±0.06	1.43±0.10	0.9682
4.0	1.000±0.10	120.59±17.6	1.04±0.04	1.43±0.05	0.9885
4.5	1.000±0.02	84.08±6.83	1.10±0.03	1.56±0.06	0.9918
5.0	1.000±0.02	55.45±2.0	1.09±0.02	1.77±0.05	0.9958
5.5	0.962±0.01	25.95±0.91	1.03±0.03	2.67±0.10	0.9942
6.0	0.963±0.02	18.14±1.05	1.00±0.04	3.27±0.20	0.9863
6.5	0.954±0.02	16.67±1.20	1.06±0.05	3.66±0.29	0.9779
7.0	0.956±0.02	17.89±1.27	1.09±0.06	3.28±0.26	0.9778

7.5	0.932±0.02	20.46±1.61	1.03±0.06	3.18±0.27	0.9714
8.0	0.929±0.02	24.73±1.51	1.12±0.05	2.77±0.19	0.9765
8.5	0.945±0.02	30.16±1.57	1.09±0.04	2.41±0.13	0.9819
9.0	0.998±0.03	38.14±2.66	1.02±0.05	1.96±0.12	0.9784
9.5	0.957±0.03	33.22±2.26	1.07±0.05	2.06±0.14	0.9749
10.0	1.000±0.04	35.94±3.02	1.02±0.06	1.68±0.13	0.9711
10.5	1.000±0.03	35.37±2.64	1.03±0.06	1.47±0.10	0.9771
11.0	0.999±0.02	31.09±1.99	1.11±0.06	1.19±0.07	0.9747
C: BHA (μM)					
univariate model [3]					
0.0	0.869±0.01	36.83±0.70	1.29±0.04	--	0.9993
0.5	0.908±0.01	63.56±0.71	1.37±0.03	--	0.9993
1.0	0.898±0.01	92.89±0.91	1.28±0.03	--	0.9991
1.5	0.923±0.02	122.27±1.67	1.15±0.03	--	0.9981
2.0	0.895±0.01	148.31±2.09	1.08±0.03	--	0.9982
2.5	0.885±0.03	180.38±3.03	1.04±0.03	--	0.9981
3.0	0.884±0.02	206.49±4.86	1.02±0.03	--	0.9973
3.5	0.880±0.03	233.90±5.13	1.04±0.03	--	0.9979
4.0	0.881±0.05	273.46±8.09	1.02±0.03	--	0.9979
4.5	0.879±0.09	283.22±5.77	1.02±0.02	--	0.9986
5.0	0.878±0.11	296.95±7.71	1.05±0.03	--	0.9981
bivariate model [7]					
BHA	0.878±0.01	35.88±0.57	1.14±0.01	1.49±0.02	0.9952

Table 3: Parametric estimates and confidence intervals (α=0.05) of the βC bleaching kinetics as affected by the specified anti- and pro-oxidant agents, according to the bivariate model [7]. See also figures 2 and 3.

agent	range (μM)	K	τ	a	u_τ	v_τ	u_a	v_a	R^2_{adj}
antioxidants (see figure 2)									
BHT	0-(3)-30	0.86 ±0.01	35.50 ±1.12	1.38 ±0.08	0.222 ±0.015	0.003 ±0.002	0.324 ±0.144	0.182 ±0.089	0.9949
ETX	0-(0.0004)-0.004	0.84 ±0.01	28.45 ±0.28	1.20 ±0.02	1112.7 ±36.60	--	1194.5 ±241.6	225.3 ±77.82	0.9967
PG	0-(8)-80	0.86 ±0.01	29.95 ±1.48	1.14 ±0.05	0.126 ±0.013	0.020 ±0.003	--	0.003 ±0.001	0.9906
α-toc	0-(0.004)-0.04	0.86 ±0.01	31.05 ±0.69	1.33 ±0.04	60.90 ±2.324	--	--	18.19 ±1.976	0.9966
pro-oxidants (see figure 3)									
Cu^{2+}	0-(24)-240	0.92 ±0.01	32.87 ±0.37	1.05 ±0.00	0.012 ±0.001	0.037 ±0.002	--	--	0.9993
Fe^{2+}	0-(1.5)-15	0.84 ±0.01	37.16 ±0.92	1.55 ±0.07	--	1.106 ±0.054	0.565 ±0.113	1.629 ±0.312	0.9940
Fe^{3+}	0-(60)-600	0.92 ±0.01	31.25 ±0.38	1.04 ±0.01	0.004 ±0.001	0.014 ±0.001	--	--	0.9975
Hb	0-(2)-20	0.87 ±0.01	30.14 ±0.63	0.96 ±0.02	--	1.099 ±0.036	--	--	0.9960

FIGURES

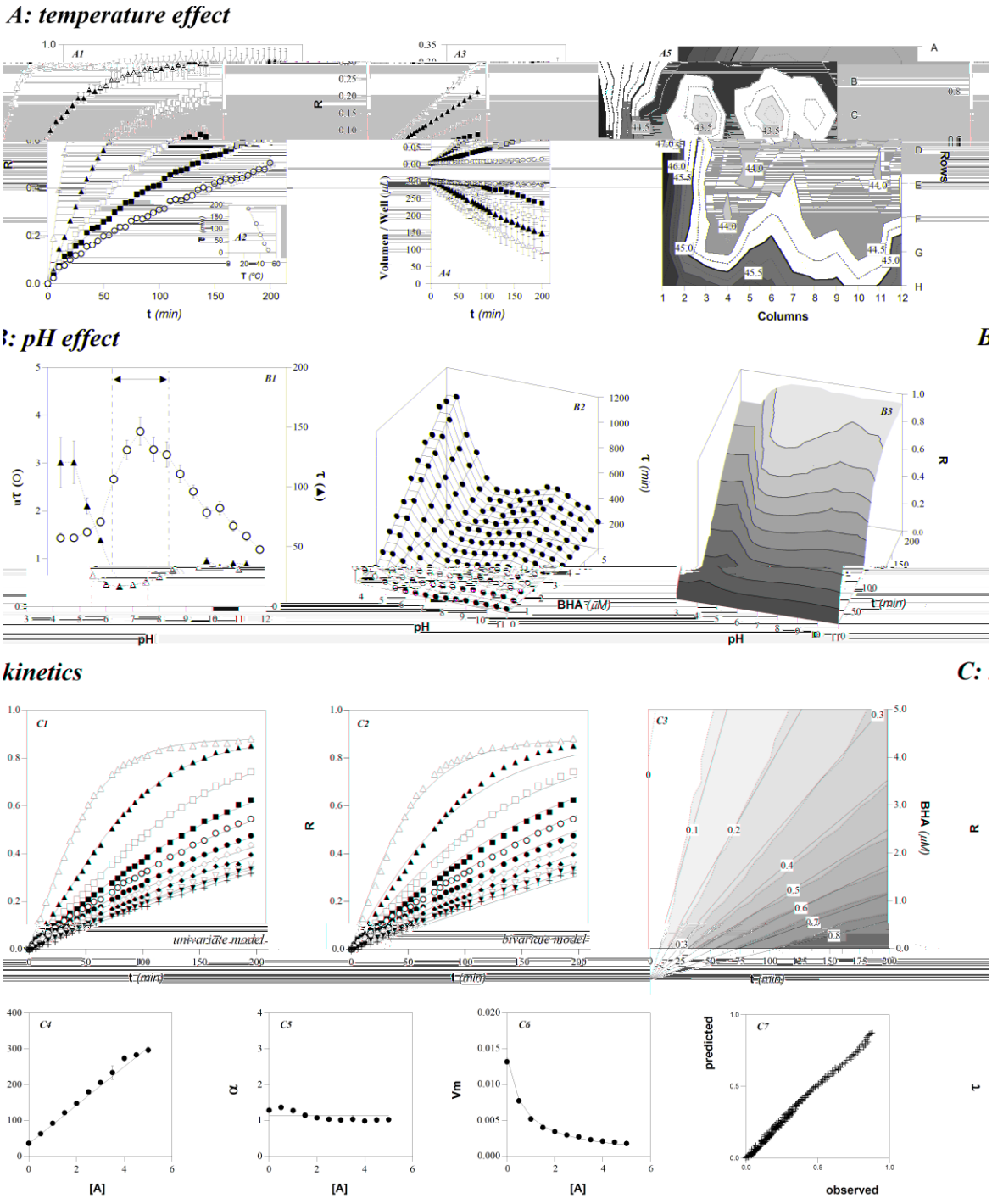

Figure 1: Factors affecting βC bleaching in microplate assay. A: temperature effects (30: ○, 35: ■, 40: □, 45: ▲ and 50ºC: △) on the bleaching kinetics in the presence (A1) and absence (A2) of linoleic acid, the evaporation (A3) and the thermal gradient in microplate (A4). B: pH effects on the parameters of the equation [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 of BHA (0: △, 0.5: ▲, 1.0: □, 1.5: ■, 2.0: ○, 2.5: ●, 3.0: ◇, 3.5: ◆, 4.0: ▽, 4.5: ▼ and 5.0 µM: +). C: kinetics of βC bleaching, at 45ºC and pH=6.5, in the presence of the same concentrations of BHA, according to the univariate (C1) and bivariate (C2) models [3] and [7]. Isoboles of the response surface from model [7] are also represented (C3), as well as the effects of BHA concentration on τ (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.

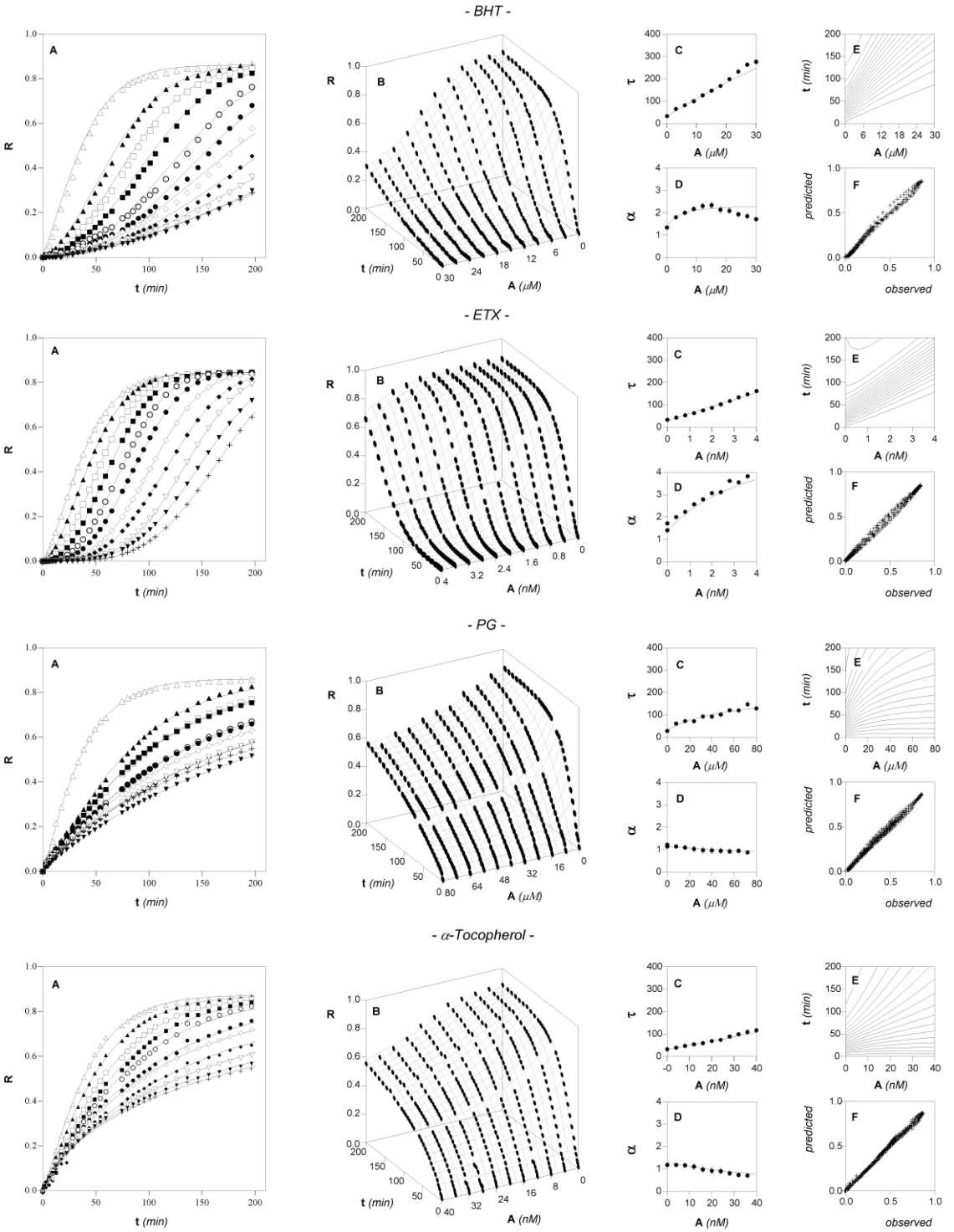

Figure 2: Effects of the specified antioxidants (ranges in table 3) on βC bleaching reaction. A: experimental results (dots) and fittings (lines) to the univariate model [3]; B: experimental results (dots) and fittings (grid line) to the dose-time-dependent model [7]; C: Parametric variations as a function of the antioxidant concentration, obtained from the models [3] (dots) and [7] (lines); D: correlations between observations and predictions, and isobolograms. In all cases a control series (△) and ten dilutions of the maximum concentration were tested (▲: 1/10, □: 2/10, ■: 3/10, ○: 4/10, ●: 5/10, ◇: 6/10, ◆: 7/10, ▽: 8/10, ▼: 9/10, ✚: 10/10). See also table 3.

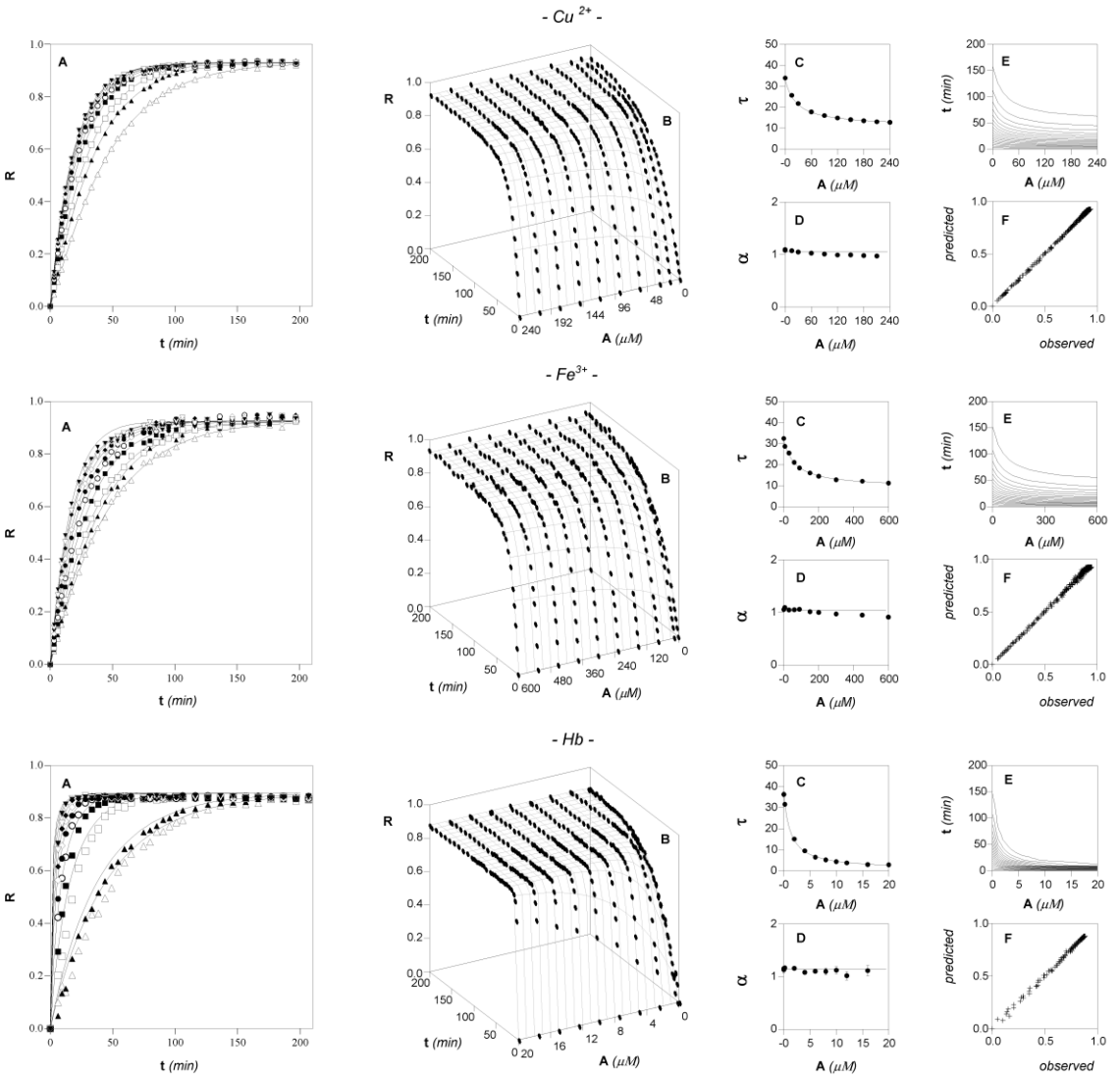

Figure 3: Effects of the specified pro-oxidants (ranges in table 3) on βC bleaching reaction. Graphic criteria and keys as in figure 2. See also table 3.

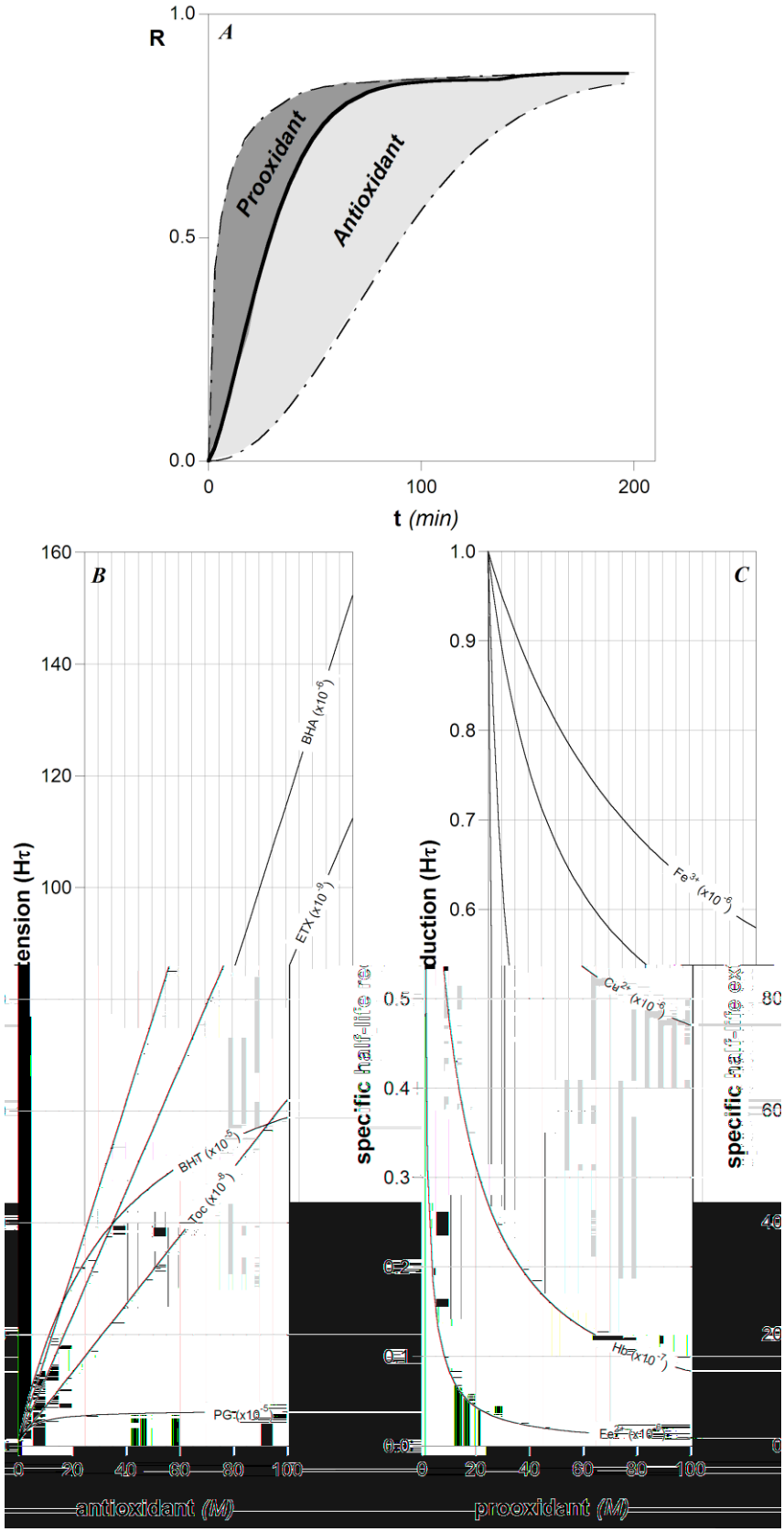

Figure 4: Anti- and pro-oxidant responses. Bottom: specific half-life extensions (antioxidants) and reductions (pro-oxidants) of the agents considered in figures 2 and 3. Note concentration scales on lines.