Crocin bleaching antioxidant assay revisited: Application to microplate to analyze antioxidant and pro-oxidant activities.

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ABSTRACT

The crocin bleaching assay (CBA) is a common method for evaluating the antioxidant activity of hydrosoluble samples. It is criticized due to its low reproducibility, problematic quantification of results, differences in reagent preparation, doubtful need for a preheating phase and sensitivity to factors such as temperature, pH, solvents and metals. Here, the critical points of the method were extensively revised, and a highly reproducible procedure for microplate readers redeveloped. The problems of using quantification procedures, disregarding kinetic considerations, are discussed in detail and a model is proposed for quantifying simultaneously anti- and pro-oxidant activities as function of concentration and time. Thus, the combined use of a reproducible procedure and robust mathematical modeling produced consistent and meaningful criteria for comparative characterization of any oxidation modifier, taking into account the dose-time-dependent behavior. The method was verified by characterizing several commercial antioxidants and some

metal compounds using the parametric values of the proposed models. The activity of the tested antioxidants decreased in the order ETX > TR > PG > AA> TBHQ > BHA. Others, such as the lipophilic antioxidants of BHT and α-Tocopherol did not show any activity. Interference from metals were null for Fe^{2+}, Fe^{3+}, Cd^{2+}, Ni^{2+}, Mg^{2+}, Zn^{2+} and Sr^{2+}, slightly antioxidant for Cu^{1+} and Cu^{2+}, and strongly antioxidant for Mn^{2+}. None of the tested metals showed a pro-oxidant activity.

Keywords: antioxidant activity; crocin bleaching assay; non-linear responses; mathematical modeling.

1. INTRODUCTION

During oxidation reactions in organic systems - not only respiration - active forms of oxygen are produced including free radicals or other reaction oxygen species (ROS) that initiate free radical reactions. The activity of ROS facilitates an indiscriminate oxidation of many biological structures, which is associated with pathological processes such as chronic inflammation, atherosclerosis and cancer as well as natural aging (Laguerre, Lecomte, & Villeneuve, 2007; Schaich, 2004). Although several endogenous mechanisms can terminate ROS free radical reactions, exogenous antioxidants from the diet may also counteract their effects, which explains more than 20 years of antioxidant research globally characterizing a range of mechanisms (McCall & Frei, 1999; Zock & Katan, 1998).

Differences in these mechanisms make a universal assay impractical (Apak et al., 2013). The practice of selecting several assays to analyze compounds of interest, does not follow any mechanistic consideration, but rather attempts to minimize problems regarding variability of results arising from differences in the matrix, substrate and oxidizing agent, control, characteristics of the system (e.g. aqueous, lipid or multiphasic) and variables such as

54 temperature and pH. In addition, the acquisition of large datasets has promoted the use of a
55 simplified formula, which can encourage dubious conclusions. Comparisons are difficult leading
56 to demands for unified analytical criteria (Frankel & Finley, 2008; Frankel & Meyer, 2000;
57 Hamilton, 1997; Huang, Ou, Prior, & Ronald, 2005; Niki, 2010; Prieto, Rodríguez-Amado,
58 Vázquez, & Murado, 2012; Roginsky & Lissi, 2005) as well as standardization of methods and
59 well defined protocols (Dawidowicz & Olszowy, 2010; Frankel, 1993, 1994; Jiménez-Escrig,
60 Jiménez-Jiménez, Sánchez-Moreno, & Saura-Calixto, 2000; Ordoudi & Tsimidou, 2006; Prior,
61 Wu, & Schaich, 2005; Sharma & Bhat, 2009). A common method proposed by Bors et al. (1984)
62 uses crocin as the substrate and AAPH (2.2 '-azobis-2-amidinopropane: R-N=N-R) as a source of
63 free radicals. The antioxidant to be tested competes with crocin, and the bleaching rate of crocin
64 is measured at 450 nm. Without discussing the implications of AAPH radicals on the oxidation
65 process in food or biological systems, this assay can be classified among those that interfere with
66 the transfer of one hydrogen atom. It is suitable for aqueous systems, producing very consistent
67 results. The original method has been modified several times to simplify the protocol (Tubaro,
68 Micossi, & Ursini, 1996), transferring it to microplate assay (Lussignoli, Fraccaroli, Andrioli,
69 Brocco, & Bellavite, 1999), applied for lipophilic environments using AMVN (2,2'-azobis-2,4-
70 dimthylvaleronitrile) as a source of radicals, and adapted to the measure of pro-oxidant activities
71 (Manzocco, Calligaris, & Nicoli, 2002).
72
73 Although such revisions have extended the scope of this assay, several problems remain. The
74 comparison of results is hindered by differences in the preparation, proportions and conservation
75 of reagents, the need or not to incorporate a preheating phase and potential interference caused
76 by metals in the samples as well as pH and temperature effects. In addition, results are generally
77 assessed at a single time point, and often, reactions are assumed to be linear, resulting potentially
78 in loss of information and increasing the risk of erroneous conclusions (Prieto, Vázquez &
79 Murado, 2014).

In this work, we revise unresolved problems and propose criteria to quantify antioxidant (*A*) and pro-oxidant (*P*) activities using a formal model with parameters that enable detailed characterization of *A* and *P* oxidation modifiers (*M*). When applied in crocin bleaching assay, these criteria allowed: 1) clarification of the method critical points, providing a revised protocol, which is more reproducible and discriminative than previously whilst avoiding prescriptive standardization; 2) determination of the effects of temperature and pH; 3) description of problems associated with over-simplified analyses such as those based on measurement at a single time point; and 4) identification of complex trends that emerge when equivalent dose systems must be used.

2. MATERIALS AND METHODS

2.1. Equipment and reagents

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

Main reagents: crocin and 2,2'-azobis-(2-amidinopropane)dihydrochloride (AAPH or ABAP).

Antioxidants: butyl-hydroxyanisole (BHA); butyl-hydroxytoluene (BHT); 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline (Ethoxyquin or ETX); propyl 3,4,5-trihydroxybenzoate (Propyl Gallate or PG); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox or TR); L(+)-ascorbic acid (AA); tert-butil hidroquinone (TBHQ) and (2R)-2,5,7,8-tetramethyl-2-[(4R,8R)-(4,8,12-trimethyltridecyl)]-6-chromanol (α-Tocopherol).

Metallic salts: zinc chloride (Zn^{2+}); magnesium sulfate (Mg^{2+}); manganese sulfate (Mn^{2+}); copper (II) sulfate (Cu^{2+}); copper (I) chloride (Cu^{1+}); cadmium (II) nitrate (Cd^{2+}); nickel (II) chloride (Ni^{2+}); strontium chloride (Sr^{2+}) and iron (II) and (III) sulfides (Fe^{2+}, Fe^{3+}).

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

2.2. Crocin bleaching method

The subsequent sections describe the final revised procedure defined by this work. Its differences, from alternatives, will be discussed and justified.

2.2.1. Reagent

Crocin (4 mg; 100 μmol.L^{-1} in the reaction mixture) and AAPH (75 mg; 7.68 mmol.L^{-1}) were dissolved in 30 mL of Mili-Q water 100 mmol.L^{-1} Briton buffer (pH 5.5, 40 °C). To avoid any degradation of both reagents, the solution was prepared and mixed just before use. The absorbance at 450 nm (~1.400) is dependent on the origin and conservation state of crocin. The molar extinction coefficient for crocin (ε_{450}=15,117 L.mol^{-1}.cm^{-1}, Sigma-17304) was less than previously reported (ε_{443}=133,000 L.mol^{-1}.cm^{-1}) where the product was purified from natural sources (Bors, Michel, & Saran, 1984; Ordoudi & Tsimidou, 2006; Tubaro et al., 1996). This made it necessary to use a more concentrated reagent of crocin, but did not affect the results.

2.2.2. Procedure to assess the action of oxidation modifiers

Where the modifier was an antioxidant, the procedure was:

In each well of a preheated (37°C) microplate (96 wells, 350 μL) were added 250 μL of reagent and 50 μl of sample in water:ethanol (9:1) (in triplicate). The apparatus was programmed for 200 min at 37°C, with agitation at 660 cycles/min (1 mm amplitude) and interruptions for readings at

intervals of three, five and 10 min (initiation, propagation and asymptotic phase). In addition to the sample set under study, the microplates contained:

a) A series (calibration) in which the sample was replaced by a standard antioxidant, in water:ethanol (9:1), at concentrations necessary to obtain a bleaching of 50% at least.
b) Three wells (blank) in which the sample was replaced by solvent.
c) Three wells (control) with a reagent without AAPH and the sample was replaced by solvent. Thus, spontaneous bleaching of crocin is quantified for correction purposes.
d) If the sample or the standard antioxidant absorb at 450 nm, the corresponding series (correction), in which the reagent was replaced by solvent, must be included.

If the modifier was a pro-oxidant, the procedure is the same, but AAPH is omitted.

2.2.3. Quantification

Procedures differed essentially with regard to kinetics, which are inherently sigmoidal. Although it has been recognized that measures for short reaction times (<10 min) do not always lead to appropriate characterizations (Apak et al., 2013; Prior et al., 2005). Next, we have summarized the usual non-kinetic approaches, as well as the kinetic alternative proposed in this work.

Non-kinetic approaches

A1. It is accepted that the CBA acts in the form of reaction 1 to 5 in the following sequence (Bors et al., 1984):

1. $R\text{-}N{=}N\text{-}R \rightarrow 2R^{\bullet} + N_2$
2. $R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$

3. $CH + ROO^{\bullet} \rightarrow C^{\bullet} + ROOH$
4. $AH + ROO^{\bullet} \rightarrow A^{\bullet} + ROOH$
5. $CH + A^{\bullet} \rightarrow C^{\bullet} + AH$

156

157 in which antioxidant (AH) competes with crocin (CH) for the peroxyls ($ROO^{\bullet}$) formed in the
158 reaction with oxygen of the decomposition products of AAPH. As a result of this analysis
159 (Tubaro, Ghiselli, Rapuzzi, Maiorino, & Ursini, 1998), the usual procedure (Chatterjee, Poduval,
160 Tilak, & Devasagayam, 2005; Lussignoli et al., 1999; Tubaro et al., 1996) is formulated as:

161

$$v = v_0 \frac{k_C C}{k_C C + k_A A}; \text{ or: } \frac{v_0}{v} = 1 + \frac{k_A A}{k_C C} \quad [1]$$

162

163 where v and v_0 are the bleaching rates of crocin (C) in the presence and absence of antioxidant
164 (A), and k_C and k_A the rate constants of the reactions of the radicals with C and A. As the crocin
165 concentration remains constant, and the rate constants ratio can be simplified to a new constant
166 (k), the rate v can be written as a linear function of A:

167

$$v = \frac{1}{v_0} + \frac{k}{v_0 C} A \quad [2]$$

168

169 in which k is the characterizing parameter and v the bleaching rate of crocin, calculated from the
170 difference between initial (a_0) and final (a_t) absorbency at a time (t) within 1-10 min:

171

$$v = (a_0 - a_t)/t \quad [3]$$

172

173 The restriction of the analysis to the initial rate interval neglects the time-course of the oxidation
174 which however, is important for the characterization of the process. Therefore, this method,
175 despite its kinetic analysis, is considered as a non-kinetic approach.

176

A2. Another common method is based on the inhibition of oxidation as a percentage or relative antioxidant activity (*I*), defined as (Ordoudi & Tsimidou, 2006):

$$I = (a_s - a_o)100/a_o \quad [4]$$

where a_s and a_0 are the absorbance of sample and reaction mixture when the sample is replaced by solvent, respectively, both at a fixed reaction time. Additionally, some authors (Chatterjee et al., 2005) determine *I* in the presence of increasing concentrations of sample and estimate the concentration required for *I*=50% (IC_{50}). Usually, the calculation is applied within the interval of 5-20 min, and IC_{50} is computed by linear interpolation, without any model describing *I* as a function of *A*.

Often, due to the uncertainty of results, the methods A1 and A2 are simultaneously used (Bountagkidou, Ordoudi, & Tsimidou, 2010).

A kinetic approach

Bors et al. (1984) started from mechanistic considerations, but restricted the analysis to the initial stages allowing the application of linear approaches, while (Murado & Vázquez, 2010) used the mass function of the Weibull distribution (Weibull & Sweden, 1951) as an empiric model but, described satisfactorily the entire kinetic profile. This improvement was transferred to the crocin CBA by considering C_0 and C_t as the crocin concentrations (oxidizable substrate) at times 0 and *t*, and defining the oxidative response as $R=1-(C_t/C_0)$. Therefore, its time-course can be fitted to following equation:

$$R = K\left\{1 - \exp\left[-\ln 2\left(t/\tau\right)^{\alpha}\right]\right\} \quad [5]$$

where K is the asymptote, τ the time required for 50% oxidation (substrate half-life) and α a shape parameter related to the maximum slope of the response. Equation [5] is very versatile: when $\alpha<1$, it can adjust the profiles of the model of (Terpinc, Bezjak, & Abramovič, 2009); when $\alpha=1$, a first-order kinetic is described; when $\alpha>1$, a variety of sigmoidal profiles is produced.

When a modifier M alters the oxidation kinetics, the authors suppose that any parameter θ from equation [5] is modified according to a perturbation term P_θ, which is defined as:

$$P_\theta = 1 + a_\theta M \qquad [6]$$

where the subscript θ indicates the modified parameter, M is the modifier concentration, a_θ is a proportionality coefficient and the term P_θ multiplies or divides the parameter, depending on whether the modifier increases or decreases its value.

Therefore, an oxidation modifier can be characterized in detail, through the variations that cause the perturbations of the parameters of equation [5], all of them with precise meanings, as well as theoretical and practical interest with respect to the oxidation kinetics. In open systems K=1, and in most common cases (as suggested by the authors), τ and α change linearly. Therefore, by inserting [6] into [5], a bivariate model was obtained, as a simultaneous function of time and the modifier concentration. As we shall see later, the original bivariate approach (Murado & Vázquez, 2010) could be modified to a more complete equation.

2.3. Numerical and statistical methods

Fitting the experimental results to the proposed equations was carried out in two phases. First, parametric estimates were obtained by minimization of the sum of quadratic differences between observed and model-predicted values, using the nonlinear least-square (quasi-Newton) method provided by the macro *Solver* in *Microsoft Excel* 2003, which allows quick testing of a hypotheses and its consequences (Prieto, Vázquez, & Murado, 2012). Next, the determination of the parametric confidence intervals and model consistency (Student´s *t* and Fisher´s *F* tests, respectively, in both cases with α=0.05) were calculated using the '*SolverAid*' (Prikler, 2009).

Other statistical assessment criteria, which were applied to re-check the consistency of model, are:

- The 'SolverStat' macro (Comuzzi, Polese, Melchior, Portanova, & Tolazzi, 2003), which is used for the assessment of parameter and model prediction uncertainties allowing the analysis of different solutions in the parameter space.
- Distribution of residuals, which always were randomly scattered around zero and grouped data and autocorrelations were not observed.
- Adjusted coefficients of multiple determination (R^2_{adj}), which indicates the goodness of fit.
- Bias and accuracy factors of all equations were calculated to evaluate the fittings to experimental data.

3. RESULTS AND DISCUSSION

3.1. An extension of the kinetic model

In an open system, it can be accepted that substrate oxidation is exhaustive at a sufficient time, implying a constant asymptote (K=1) in model [5]. Half-life (τ) is always increased by the presence of an antioxidant, causing a decrease in the slope of the function, even when α remains

constant. In some cases, α varies, which modifies the slope of the curve independent of the modification induced by the antioxidant in τ. Thus, when the affinity of the antioxidant for the oxygen or radicals is much higher than for the substrate, the propagation phase is delayed, which translates to an increase in α. Pro-oxidants promote the opposite effects.

As stated by Murado & Vázquez (2010), any alteration of the oxidative kinetics modifies at least one of the two parameters τ and α, promoting their variation as a linear function of the concentration of the modifying agent. Although, the model was successfully applied to the data published by different authors, the linear variations of τ and α are restrictions that not always are satisfied. However, when the perturbation term is re-written as a hyperbolic function:

$$\pi_\theta = \frac{1 + a_\theta M}{1 + b_\theta M} \quad ; \quad (\theta = \tau, \alpha) \qquad [7]$$

where M is the modifier concentration, and a_θ, b_θ are merely fitting coefficients (when b_θ=0, the parameter depends linearly on M), a bivariate equation is formulated, as a function of the time and the modifier concentration, increasing significantly the descriptive capabilities of the model for real cases:

$$R(t, M) = K\left\{1 - \exp\left[-\ln 2\left(t/\pi_\tau \cdot \tau\right)^{\pi_{\alpha \cdot \alpha}}\right]\right\} \qquad [8]$$

Thus, when an entire set of kinetic profiles was simultaneously described by [8], the term π_τ typifies the specific substrate half-life extension (antioxidants) or contraction (pro-oxidants), and can be used to compare values of different agents. The term π_α describes the relative affinity of the modifier for oxygen or the substrate. The dose-time-dependent characterization was especially robust, minimizing the effects of random and systematic errors. As stated by many

authors before (De Lean, Munson, & Rodbard, 1978; Prieto, Vázquez, & Murado, 2011) optimally, efficient data analysis should involve simultaneous description of all curves, rather than fitting each one individually. The simultaneous curve-fitting reduces the number of parameters needed to analyze the response, is a more informative approach and provides better estimations of parameters and reduces intervals of confidence. In addition, if the experimental curves obtained do not span the full range and some of them fail to provide information about one or more of the parameters of the equation, the bivariate application describes simply and accurately the responses.

Later on, we will propose some reparametrizations for equation [5], which would make it useful for modeling the effects of temperature and pH on the rate of the crocin reaction.

3.2. Kinetic behavior of the crocin reaction

The oxidant action implies interfering in an autocatalytic process in which no less than four chemical species are present (oxygen, oxidizable substrate, antioxidants and oxidation products); reactions of first and second order can take place and interactions can occur at several levels in the sequence.

The time-dependent response of the CBA is inherently sigmoidal. Dose-response at one time point, with the expectation to find linear form (as described by the non-kinetic approaches A1 and A2) often leads to unreliable results and misinterpretation of the effects of response modifying factors (AAPH and antioxidant concentrations, pH, temperature). Today, the preference for apparently, simple and routinely applicable assays with minimal calculation requirements, is justifiable, given the availability of computational applications and microplate

readers. Their combination provides adequate tools to work with data sets, which allow accurate evaluations, enabled by non-linear modeling.

If we assume that equation [5] describes appropriately the oxidation process –in accordance with experimental results– equation [8] can be used to simulate time-concentration-dependent responses, and test the suitability of the single-time methods (non-kinetic approaches A1 and A2) to quantify the responses of the CBA. In Figure 1, an illustrative set of simulations (A, B, C and D) is presented, being in all cases the asymptote K=1 and all others parameters varying as described below:

	τ	a_τ	b_τ	α	a_α	b_α
A	20	0.1	0	1.0	0	0
B	20	0.1	0	1.4	0	0
C	20	0.1	0.01	1.0	0	0
D	20	0.1	0	1.4	0.01	0

In general, for both non-kinetic approaches (A1 and A2), unsatisfactory solutions were found andare described next:

a) For the quantification approach A1, any single-time procedure at any time is only linear in case A where the time-dependent response is a first order reaction (π_α has to be constant and equal to 1) and the dose-dependent response variation of the specific half-life extension coefficient (π_τ) has to be linear (b_τ=0). In any other case, the dose-response will be a non-linear relationship. In Figure 1, case A shows the specific circumstance where the dose-response is linear. For cases B, C and D, the responses will always be non-linear, to different degrees, independent of the time selected. In some cases, a concentration-range exists at one given time, in which the result may appear linear, but it is not. Focusing on the response produced at the earliest stage (as it is indicated for CBA, 1-10 minutes) hides statistically

more common non-linear relationship, which assisted by the experimental error, leads to less reproducible results.

b) On the other hand, for the quantification solution A2, the IC_{50} value, computed as described by procedure A2, exhibited an asymptotic variation, typically sigmoidal, as function of the analytical time. A2 assumes that the IC_{50} value calculated is time-independent, as it can be seen in all four cases presented in Figure 1. When computing the activity of an antioxidant using criterion A2, the results will be highly dependent on the time of application.

From the point of practical application – for example, in the food industry- the exclusive focus on finding the most linear solution or simple responses is not helpful for improving the translation of the results found in laboratory assays. Perhaps by using non-linear solutions to describe the oxidation process, we are not helping to translate the results, because they may be related to the response, but at least we are able to: 1) describe precisely the kinetics detected in the many different reactions with antioxidants of very different nature; 2) obtain reproducible values of practical interest, 3) incorporate, if necessary, environmental variables that modify the process, 4) infer mechanistic details that can be verified by other methods.

In the CBA, as in many other methods to quantify the antioxidant activity, authors have selected the conditions that hide the sigmoidal character of the oxidation kinetics (**¡Error! No se encuentra el origen de la referencia.**) and selected commercial antioxidants that generate similar results to the linear specific response (π_α=cte=1 and π_τ=linear; using commercial antioxidants of TR or PG as we will see later on). Furthermore, instead of comparing dose-responses between each other, the common practice is to use the dose-response of one commercial antioxidant as a calibration curve to compute the equivalent antioxidant activity of a sample that is only tested at one single-time-dose, assuming too many aspects as true.

In our opinion, any criterion that avoids a kinetic focus is a misleading simplification. We are aware that equation [8] is slightly more complex than a linear one, but it is also much less deceiving, because it produces characterizing values of practical interest with high reproducibility, and enables the inclusion of environmental variables that modify the process as well as the mechanistic details that can be verified by other methods. In the following, we will focus on the standardization of the assay, before applying the kinetic approach to the behavior of the crocin reaction, when affected by temperature, pH and a set of oxidation modifiers.

3.3. Reagent preparation

3.3.1. Crocin solution

The problems associated with the reagent preparation have been extensively described by (Ordoudi & Tsimidou, 2006). Although the purification of crocin is no longer a difficulty, because the product is commercially available, its conservation state must be checked by verifying that the absorbance at 450 nm of the final reagent with 100 $\mu mol.L^{-1}$ crocin is ~1.40. Minor inaccuracies are not important when the results are analyzed by using kinetic models.

3.3.2. AAPH solution

The issues concerning AAPH are less assessed. Its role is to provide radicals at a constant specific rate but, is highly dependent on the amount used and the assay conditions such as pH and T, which will be analyzed and discussed later on. Regarding the reagent preparation, the main aspect is related to preheating or not, and with storing (as stock solution) or not the AAPH solution:

- *The preheating treatment of AAPH solution*: is a controversial matter that, according to some authors, reduces variability and economizes the reagent (Chatterjee et al., 2005; Lussignoli et al., 1999), while others described it as an inappropriate measure (Ordoudi & Tsimidou, 2006). Our results suggest that preheating is always inadvisable because: 1) it generates an initial high radical concentration that finishes the reaction in a few minutes and only contributes to the appearance of linear kinetics, hiding the part of the profile that provides the characterizing information; 2) the obtained results are redundant with those provided by other methods such as ABTS or DPPH; 3) incomplete thermal degradations can produce biphasic curves of problematic interpretation; 4) by avoiding the preheating treatment, the radical breaks down at a constant rate, which reproduces more appropriately the real conditions.

- *Stock solution*: some authors (Ordoudi & Tsimidou, 2006) propose to store AAPH solutions at 4ºC. However, the error associated with the fresh preparation is lower than that produced due to the degradation of AAPH in a stock solution, even in short time responses. When AAPH is stored, biphasic curves are frequently observed, with a fast initial bleaching phase as a consequence of the high level of radicals accumulated during the storage.

Thus, our recommendation is –as described in the methodological section– to prepare AAPH and crocin solutions freshly each time, and combine them just before use, avoiding preheating and any other oxidation instances before starting the assay.

3.4. Critical factors that affect the quantification of the response

In this work, we have carefully selected those conditions that do not interfere with the response, and that do not vary excessively with those previously reported by other authors (see **¡Error! No**

400 **se encuentra el origen de la referencia.**) thus, enabling a more complete and realistic analysis.
401 Therefore, the method itself is revisited and some of the critical factors are analyzed.
402
403 The apparently simple assays, routinely applied, with minimal calculation requirements can
404 misunderstand the effect of some factors that modify the response (such as AAPH and
405 antioxidant concentration, pH, T, among others), leading to over-standardization of the protocol
406 in some cases, or to overlooking those aspects that need to be standardized in other situations
407 (Apak et al., 2013; Frankel, 1993; Murado & Prieto, 2013). Next, we will revise some of these
408 factors independently, taking into account the kinetic behavior. The proposed model will be used
409 and applied as: univariate (the time as the only dependent variable, parametric results in **¡Error!**
410 **No se encuentra el origen de la referencia.**) and bivariate (the effect of time and the factor
411 combined as dependent variables, parametric and graphic results in **¡Error! No se encuentra el**
412 **origen de la referencia.** and Figure 2).
413
414 *3.4.1. AAPH effect as an example of a pro-oxidant action*
415
416 Crocin and AAPH concentrations are the most controversial aspects of the CBA procedures
417 summarized in **¡Error! No se encuentra el origen de la referencia.**. The first aspect can be
418 attributed to the differences in purity of the reagent (note the respective molar extinction
419 coefficients). The second one seems to be connected to the need of adapting the kinetic profile to
420 the quantification method applied. With the aim of determining the effect of AAPH
421 concentration, the assay (100 $\mu mol.L^{-1}$ crocin; 37ºC; pH=5.5) was performed in the presence of
422 12 concentrations of this reagent within a 0-20 $mmol.L^{-1}$ interval.
423
424 The results represent an example which can be related to the case of a pro-oxidant (Figure 2, plot
425 A), in which crocin oxidation was described with notable precision by the model proposed, in

both the univariate and bivariate form. The individual fitting to model [5] of the values obtained at each AAPH concentration (**¡Error! No se encuentra el origen de la referencia.**, part A), showed that AAPH causes a non-linear decrease of the half-life (τ) and a linear increase of α. The application of the bivariate model [8] to the simultaneous fitting of all the kinetic profiles (**¡Error! No se encuentra el origen de la referencia.**, part A and Figure 2, plot A) confirmed the conclusions drawn from model [5], with equally high statistical significance.

Since the AAPH concentration affects strongly the crocin oxidation, it is important to decide its adequate value, taking into account two basic considerations, regarding the time of analysis and the nature of the modifier assayed:

a) *The analytical time:* Short analysis times (~50 min) favor the effect of the experimental error, while longer times (~500 min) favor solvent evaporation and thermal discoloration of crocin. In our experience, a middle point of 150-200 min continuously provided highly reproducible results, without undesirable consequences on evaporation and bleaching processes.
b) *The type of modifier agent:* If the testing agent is an antioxidant, half-life extensions of ~25 min in an assay of ~200 min have to be considered, as they are time ranges that enable very accurate evaluations. To accomplish such time conditions, the concentration of 7.68 $mmol.L^{-1}$ of AAPH is needed, as computed by equation [8]. If we want to test pro-oxidants, AAPH must be omitted, because the crocin reaction in the absence of AAPH is itself an appropriate method for assessing pro-oxidant activities.

3.4.2. Temperature effect

Since temperature accelerates the AAPH degradation and the spontaneous bleaching of crocin (Prior et al., 2005), a strong effect of this variable on the response can be expected. Although the

usual working range is 37-40ºC, the temperature effect was assessed at four temperatures (32, 37, 40 and 45°C) in the absence and presence of AAPH at the established concentration (7.68 mmol.L^{-1} in a 100 mmol.L^{-1} Briton-buffered reaction mixture, pH=5.5) following the protocol described in the methodological section.

The individual fitting to equation [5] of the profiles corresponding to each temperature (**¡Error! No se encuentra el origen de la referencia.**, part B) showed a statistically significant linear decrease of τ without variation of α, indicating that the oxidation process is more sensitive than the antioxidant action to the temperature enhancement.

Interestingly, equation [5] also was able to incorporate formally the temperature effect. Such incorporation can be done in two ways. One way can include a hyperbolic term like [7] affecting the parameter τ but temperature-dependent (**¡Error! No se encuentra el origen de la referencia.**, part B). Although this option was acceptable, the statistical significance was higher by using the second one, an expression less empirical, involving the Arrhenius equation. As it is detailed in the Appendix, the second option requires performing a reparametrization of model [5] to make explicitly a rate parameter (v_τ) that represents the reaction rate at time τ. This leads to the following final expression:

$$R(t,T) = K\left\{1-\exp\left[-(\ln 2)^{1-\alpha}\left(\frac{2t}{K\alpha}\left[\exp\left(-E/R_gT\right)\right]\right)^{\alpha}\right]\right\} \qquad [9]$$

in which the exponential term from the Arrhenius equation (see Appendix) acts as a temperature-dependent factor replacing v_τ from equation [A4] (we denote the constant of gases R with a g subscript for avoiding homonymy with response). This bivariate description of the temperature

475 effect on the rate v_τ was statistically satisfactory and fully consistent with the results of the
476 univariate approach.
477
478 The graphical results of the response are presented in Figure 2 (plot B1), displaying the different
479 T in the crocin reaction (dots) and the fittings, obtained by applying equation [9] (lines). Figure 2
480 (plot B2) shows the behavior of v_τ as a result of the parametric estimations, obtained by the
481 individual fittings to model [5] (dots), which indicates agreement with those fittings produced by
482 equation [9] (lines). **¡Error! No se encuentra el origen de la referencia.** (part B) shows the
483 fitting parameters for bivariate model [9] in the presence and absence of AAPH.
484
485 Even in the absence of AAPH, temperature increased the bleaching rate of crocin in agreement
486 with the Arrhenius equation, while the presence of AAPH reduced the coefficient *E* from 65.5 to
487 35.8, proportional to the activation energy required for the crocin oxidation.
488
489 Besides these effects, temperature increased –as mentioned already– by both evaporation and
490 thermal gradient in the microplate. We decided to confine to the most standard temperature
491 condition, the 37ºC value. Nonetheless, the most stable results were obtained at 32ºC, and even
492 further reductions would be advisable, whenever they were accompanied by a correlative
493 increase in the AAPH level, to maintain similar kinetic responses (~30°C and ~15 $mmol.L^{-1}$
494 would maximize the accuracy). Although it is not common practice, the spontaneous bleaching
495 in the absence of AAPH must be excluded from the analysis, using the control as described in
496 the methods section.
497
498 *3.4.3. pH effect*
499

500 The use of buffers is an extended practice, however, there is no consensus on the appropriate
501 initial pH (**¡Error! No se encuentra el origen de la referencia.**) or the effect of this variable on
502 the response. Some authors (Bors et al., 1984; Ordoudi & Tsimidou, 2006; Tubaro et al., 1996)
503 applied approach A1 to analyze crocin bleaching in the presence of three antioxidants (caffeic
504 acid, catechol and trolox) at two pH values (5.5 and 7.4), concluding that the pH causes
505 significant differences in the first two cases, but not for the case of trolox. Our preliminary
506 assays, in the crocin-AAPH system, showed the difficulty in distinguishing the effect of pH from
507 that produced by an antioxidant, especially when the antioxidant activity is measured at a single
508 time. Thus, we decided to revise the effect of this variable on the crocin reaction by using 100
509 mmol.L^{-1} Briton buffer at 16 pH values: 3.5-(0.5)-11.0 (no hypso- or bathochromic shifts in the
510 absorption spectrum of crocin were detected in this range).
511
512 Results presented in Figure 2 (plot C) show a progressive reduction of the oxidation rate as the
513 pH increases. Because the variable of pH does not affect the spontaneous discoloration rate of
514 crocin, the effect must be attributed either to the inhibition of the AAPH degradation or to the
515 capture of radicals from such a degradation. In any case, the increase of pH had an antioxidant-
516 like effect.
517
518 The individual description of the kinetic profiles was satisfactory with model [5] (parametric
519 results in **¡Error! No se encuentra el origen de la referencia.**, part C). Again, the possibility to
520 incorporate the pH variable into model [5], to describe its effect, requires making explicitly a rate
521 parameter, as in the reparametrized expression [9] with temperature. However, to describe the
522 effect of pH, there is no general formulation, compared to the effect of T applying the Arrhenius
523 equation. First, in order to identify the effect of pH on the crocin reaction, the individual fittings
524 of the responses to expression [A2] (from Appendix) were calculated. Then, the behavior of v_τ
525 against pH was determined. The pH modifies the rate value of v_τ, exponentially decreasing,

while all the other parameters remain constant. When, in equation [A4] (Appendix), v_τ was replaced by an exponential decreasing expression, the following pH-time dependent analysis can be formulated:

$$R(t, pH) = K\left\{1-\exp\left[-(\ln 2)^{1-\alpha}\left(\frac{2t}{K\alpha}\left[v_\tau \exp(-b\cdot pH)\right]\right)^{\alpha}\right]\right\} \qquad [10]$$

in which v_τ is the rate at the minimum pH and b is a fitting coefficient. This model offers a simultaneous description, highly predictive and statistically significant in all the parameters, of the results obtained at the entire set of pH values (Figure 2, plot C and **¡Error! No se encuentra el origen de la referencia.**).

The results obtained are presented in Figure 2 (plot C1), adjusted to the bivariate model [10], displaying the pH effect response of the crocin reaction (dots) and the fittings obtained by applying model [10] (lines). Figure 2 (plot C2) shows the behavior of v_τ for the parametric estimations, obtained by the individual fittings (dots) to model expression [A2] (from Appendix) in agreement with those fittings produced by model [10] (lines). The parametric estimations to model [10] are presented in **¡Error! No se encuentra el origen de la referencia.** (part C).

In practice, the working pH in CBA is commonly around 7.0 (**¡Error! No se encuentra el origen de la referencia.**). At this value, the oxidation rate of crocin by AAPH is significantly reduced, which forces an increase of temperature for avoiding an excessive increase in the analytical time. However, this solution increases the effect of the experimental error and produces a high base-line, due to the spontaneous oxidation of crocin, complicating the data analysis. The alternative of increasing the concentration of AAPH causes an equally increase of the experimental error, especially at brief reaction times. Additionally, pH=7 is located within a

550 domain, in which the effect of the variable pH on the response is sharp, and small variations
551 could cause noticeably changes on the discoloration rates. To overcome these problems, we have
552 selected a pH of 5.5, at which possible small variations (0.5-1.0 units), during the kinetic
553 process, do not prevent an accurate evaluation.
554
555 *3.4.4. Antioxidant concentration. Trolox as a case of study.*
556
557 Trolox is commonly used as a standard antioxidant in hydrophilic systems because of its
558 potency, but it should be kept in mind that it represents a quite particular case. When the effect
559 of eight different concentrations of trolox (0-(0.5)-4 $\mu mol.L^{-1}$) on the crocin reaction was studied
560 under selected conditions (7.68 $mmol.L^{-1}$ AAPH, 37°C, pH=5.5), both equations [5] and [8]
561 provided statistically significant descriptions of the results (Tables 2 and 3). Such descriptions
562 showed an paraticular simple outlook (Figure 2, plot D), with an increase of the half-life τ as a
563 linear function of the antioxidant concentration. This response corresponds to hypothesis A from
564 Figure 1 and is the only case in which the non-kinetic approaches produce acceptable results, in
565 particular when temperature or AAPH concentrations are relatively high. Nonetheless, this
566 behavior is far from being able to be generalized to any antioxidant and consequently, those
567 approaches can still lead to erroneous equivalences, when unknown samples are tested against
568 trolox.
569
570 *3.4.5. Result's reproducibility*
571
572 The CBA revisited assay, presented here, is a powerful tool to simplify hydrophilic responses
573 found in other assays. Although we have revised the effects of several factors which some
574 authors have found occasionally problematic (probably due to the absence of a proper kinetic
575 model), our conclusions do not over-standardize, in fact reduce the variability of the assay. When

576 we establish certain precautions with the reagents used, the adequate working range of pH, the
577 usual working temperature and applying appropriate criteria to quantify the responses, the assay
578 becomes highly reproducible. As demonstrated by the standard deviation error bars of each of
579 the spectrophotometric kinetic responses obtained from four genuine replicates presented in
580 Figure 2 (plots A1, B1 and C1). As Figure 2 and **¡Error! No se encuentra el origen de la**
581 **referencia.** show all the experimental data were satisfactorily modeled, with a good predictive
582 capacity (adjusted coefficient of multiple determination), statistical consistence (Fisher's test),
583 adequate parametric sensitivity, narrow parametric confidence intervals (Student's test),
584 unbiased residuals and accuracy and bias factors. In addition, the statistical analysis of the
585 parameters calculated for the univariate fittings (also presented in **¡Error! No se encuentra el**
586 **origen de la referencia.**) are represented in their respective Figure 2 plots (A2, B2, C2 and D2).
587
588 **3.5. Application: assessment of several commercial antioxidants and metal cations**
589
590 The revised protocol (with 7.68 mmol.L^{-1} AAPH, 37ºC, pH=5.5) and the proposed models [5]
591 and [8] were finally applied to a comparative study of several commercial antioxidants, as well
592 as the possible interfering effects of metal salts that can be present –as part of complex natural
593 extracts or as trace impurities of buffers– in the solutions to be tested. The results (**¡Error! No se**
594 **encuentra el origen de la referencia.** and Figure 3) allowed us to conclude:
595
596 a) Antioxidant activity inhibiting the spontaneous bleaching of crocin (in the absence of AAPH)
597 was not detected in any case. This indicates that the detected activities are only related to the
598 trapping of the radicals released in the APPH degradation.
599
600 b) BHA, TBHQ, ETX, PG, TR, TBHQ, Mn^{+2}, Cu^{+2} and Cu^{+1} showed antioxidant activity.
601 BHT, α-tocopherol, Zn^{+2}, Mg^{+2}, Sr^{+2}, Fe^{+2} and Fe^{+3} did not show such an activity (neither pro-

oxidant). Others, such as Mn^{+2}, Cu^{+2} and Cu^{+1} acted as strong antioxidants. The behavior of BHT and α-tocopherol (well-known antioxidant in lipidic systems) can be explained as examples of "polar paradox". The high antioxidant activity of metals (Mn^{+2}, Cu^{+2} and Cu^{+1}), even at very low concentrations, emphasizes the precautions that need to be taken when using complex extracts or buffer solutions with possible salt traces. Copper ions are known to participate in the formation of reactive oxygen species (ROS). Both cupric and cuprous ions can participate in oxidation and reduction reactions. In the presence of reducing agents, Cu^{2+} can be reduced to Cu^{1+}, which is capable of catalyzing the formation of hydroxyl radicals from hydrogen peroxide via the Haber–Weiss reaction (Gaetke & Chow, 2003). The oxidation inhibition by copper could be attributed to its interaction with the peroxyl radicals produced by AAPH, breaking the propagation of the radical chain (Costanzo, Guidi, & Giuffrida, 1995). The inhibiting effect of Mn^{2+} has been previously reported by several authors (Coassin, Ursini, & Bindoli, 1992), arguing that this could be ascribed to the lack of a facile way for the electron transfer from the metal to the hydroperoxyl radical.

c) Equations [5] and [8] described accurately all the kinetics studied (**¡Error! No se encuentra el origen de la referencia.**). Using the specific half-life extension (term π_τ in [8]) for an antioxidant concentration of 300 $\mu mol.L^{-1}$in an assay under the specified conditions, the following order of activities can be established:

τ: $Mn^{+2} > TR > ETX > PG > AA > Cu^{+1} > Cu^{+2} > TBHQ > BHA$

d) A way to compare modifying-oxidation activities in a meaningful and visual form (Figure 3, plot B) could consist of plotting the specific variation of the half-life from equation [7] as a function of the agent concentration.

For all cases, the fitting of results was always satisfactory. The mathematical equations were robust and consistent (p-values < 0.001 from Fisher's F test), the residuals were randomly distributed and autocorrelations were not observed by Durbin-Watson test (data not shown). The statistical analysis, parameter assessment tools and model prediction uncertainties provided by the 'SolverStat' macro agreed accordingly. Furthermore, the adjusted coefficient of multiple determination (R^2_{adj}) between predicted and observed values were always > 0.98, with a wide majority of the fittings superior of 0.99.

3.6. A possible mechanistic inference from the kinetic approach

When the increase of τ –and the concomitant drop of the slope– is not sufficient to explain the effect of a concrete antioxidant, and an increase of α is needed, the kinetic profile begins its exponential rise (propagation phase) with a delay that increases with the antioxidant concentration. In this respect, ethoxyquin (Figure 3 and **¡Error! No se encuentra el origen de la referencia.**) is the clearest example. This delay, or lag phase, is difficult to explain within the framework of the reaction sequence admitted for the crocin bleaching (see section 2.2.3), and suggests that the antioxidant not only captures peroxyls (reaction 4), but also acts at the level of the source of radicals (reaction 1), preventing the formation of $R^{\bullet}$ or capturing the $R^{\bullet}$ formed but, in any case, reducing the contribution of $R^{\bullet}$ to the peroxyl formation. Although we do not have any mechanistic proof, a tentative application of the Runge-Kutta method to the rate equations, and expectable mass balances, formulated from that sequence, seem to confirm our hypothesis. If our hypothesis is correct, models [5] and [8] would have additional capability, to a certain extent, to detect some evidences of some modes of action involved in the antioxidant activities.

4. CONCLUSIONS

Repeatedly identified problems in connection to the assessment of the antioxidant activity are the low reproducibility, the inability to establish useful comparisons and the need for knowing the effects of the state variables, with the aim of achieving standardized methods which can be generalized to any oxidation-modifying agent (Prieto, Vázquez & Murado, 2014). These problems, multiplied by the diversity of methods arising from the interest in this field, make the current situation chaotic (Frankel, 1993, 1994; Huang et al., 2005; Koleva, Beek, Linssen, Groot, & Evstatieva, 2002; Laguerre et al., 2007; Naguib, 2000; Roginsky & Lissi, 2005). The formal treatment of the experimental data is, without doubt, the most basic issue, since it is the only way to quantify the response and to control any affecting variable that should be considered. In this regard, linear models are very simple, but very unsatisfactory. In fact, from the work of few authors that reported the progress of the response, even at short times, sigmoidal and potential profiles clearly are observed (Lussignoli et al., 1999; Manzocco et al., 2002), which forces authors to neglect part of the experimental data to maintain the non-kinetic approaches (Dimajo, Laguardia, Giammanco, Laneve, & Giammanco, 2008). In contrast, the use of rate equations and mass balances provides unquestionable kinetic descriptions, however, it does not solve the problem, because it does not directly provide characterizing values of practical interest. Furthermore, the absence of explicit analytical solutions makes the calculation prolix.

The approach proposed here represents an intermediate option whose reliability and versatility, we believe, was demonstrated. Indeed, equations [5] and [8] enabled: 1) to describe, with precision, the different modalities of the sigmoidal kinetics detected in the crocin reaction as affected by oxidation modifiers of different nature and behavior; 2) to obtain, in a reproducible and statistically significant way, parametric structures that characterized these modifying activities in a more practical, accurate and detailed mode than the usual ones; 3) to incorporate consistently, if necessary, the effects of the state and composition variables that act on and or

alter the process and; 4) to infer some mechanistic details with a concrete hypothesis which can be verified by complementary methods.

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692

693 **APPENDIX SECTION**

694

695 **Inclusion of the Arrhenius model in the kinetic description of CBA**

696

697 The Arrhenius equation establishes that the rate constant (k) of a chemical reaction is a function
698 of the absolute temperature (T) according to:

699

$$k = B\exp\left(-E/RT\right) \qquad \text{[A1]}$$

700

701 where B represents the frequency of collisions among reacting molecules, E is the activation
702 energy and R the constant of gases. In our context, B and E can be considered as fitting
703 parameters.

704

705 The equation [5] used to describe the bleaching kinetics has no parameters that can be
706 dimensionally assimilated to the constant k. However, such assimilation is possible if the
707 equation is reparametrized in such a way that it includes explicitly a rate parameter, that is, a
708 slope. Two meaningful slopes can be considered in [5]: that corresponding to the inflection point
709 (v_τ), and the maximum slope (v_m), whose relations with the shape parameter α are:

710

$$v_\tau = \frac{K\alpha\ln 2}{2\tau} \qquad \text{[A2]}$$

711

$$v_m = \frac{K\alpha}{\tau}\left(\ln 2\right)^{1/\alpha} G^G \exp\left(-G\right);\ \text{where}\ G = \frac{\alpha-1}{\alpha} \qquad \text{[A3]}$$

712

713 When the function is symmetrical (K=1, α=3.259), v_τ=v_m. If α<3.259, the abscissa of v_m is less
714 than τ, and it becomes zero when α=1 (note that the form of the term G implies that v_τ exists for

715 $\alpha>0$, while v_m only for $\alpha>1$). As long as these meanings are not forgotten, any of the two slope
716 expressions can be used for reparametrizing purposes. Thus, by substituting τ in [5] for its value
717 isolated in [A2] or [A3], we obtain:
718

$$R = K\left\{1-\exp\left[-(\ln 2)^{1-\alpha}\left(\frac{2v_\tau}{K\alpha}t\right)^{\alpha}\right]\right\} \qquad [A4]$$

719

$$R = K\left\{1-\exp\left[-\left(\frac{v_m}{K\alpha G^G \exp(-G)}t\right)^{\alpha}\right]\right\};\ \text{with:}\quad G=\frac{\alpha-1}{\alpha} \qquad [A5]$$

720

721 Since v_τ or v_m, as true rate parameters, can be replaced by the second member of [A1], we can
722 use the the exponential term of the Arrhenius equation as a temperature-dependent perturbation
723 factor of the considered rate. This leads to the model [9], where the option including v_τ –the
724 simplest one– was used.
725

FIGURE CAPTIONS

Figure 1: Simulation of the crocin bleaching kinetics (compare with real cases in fig. 3), using the eq. [8] with the four parametric combinations specified in section 3.2. For each case, five sub-figures are presented in which the following items are shown: **1)** the simulated dose-time dependent response of crocin oxidation using equation [8]; **2)** effect of antioxidant concentration on the parameters τ and α of eq. [8]; **3)** reaction rate (v) as a function of time for each antioxidant level; **4)** relationship between V_0/V and [A]/[C] ratios (supposed linear by A1 criterion) at different times along the range 1-200 min.; and **5)** time dependency of the $IC_{50\%}$ value (supposed constant by criterion A2). Note the problems associated with the acceptation of a linear hypothesis and the use of a single time for quantification purposes.

Figure 2: Evaluation of the different critical points on the CBA. Numerical results in **¡Error! No se encuentra el origen de la referencia.** and 3. For all cases Experimental results are points and fittings to the corresponding models are lines: **A:** Kinetic effect of AAPH. ***A1:*** Simultaneous fittings of equation [8] (lines) to Increasing concentrations of (0-20 mmol.L^{-1}) at 37ºC. ***B2:*** variation of parameter τ according to the individual fittings [5] (points) and simultaneous fittings [8] (lines). **B:** Kinetic effect of temperature. ***B1:*** Fittings of model [9] (lines) in absence and presence of AAPH (7.6 mmol.L^{-1}) at five different temperatures (●: 32, ○: 37, ▲: 40, △: 45 and 50ºC). ***B2:*** estimates of V□ obtained from individual fittings to the model [A2] (points) and its simultaneous description by model [9] (lines). **C:** Kinetic effect of pH on the bleaching rate in the crocin-AAPH system (37ºC, 7.68 mmol.L^{-1} AAPH). ***C1:*** kinetic results (points) fitted to the equation [10] (lines). ***C2*****:** $V\tau$ obtained from individual fittings to the model [A2] (points) and its simultaneous description by model [10] (lines).**D:** Effect of antioxidant concentration on the bleaching of crocin-AAPH system (37ºC, 7.68 mmol.L^{-1}

AAPH, pH=5.5) using trolox (0-(0.5)-4 μM) in the reaction mixture as example. ***D1:*** kinetic results (points) jointly fitted to the equation [8] (surface). ***D2:*** variation of parameter τ according to the individual fittings [5] (points) and simultaneous fittings to the model [8] (lines).

Figure 3: ***A:*** Effects of several antioxidants on crocin-AAPH system (7.68 $mmol.L^{-1}$ AAPH, 37ºC, pH=5.5). Experimental results (points) and fittings to the eq. [8] (lines; see also **¡Error! No se encuentra el origen de la referencia.**). In all cases the kinetic profiles drop orderly with the increase of the agent concentrations, which are ($\mu mol.L^{-1}$): BHA: 0-(37)-370, AA: 0-(30.3)-303, ETX: 0-(3.0)-30, TBHQ: 0-(80.0)-800, PG: 0-(30.0)-300, Cu^{+2}: 0-(20)-200, Cu^{+1}: 0-(20)-200, Mn^{+2}: 0-(12.5)-125. ***B:*** Characterization of all the agents studied through the specific half-life extension coefficient (π_τ from eq. [8]).

766

767

768 **TABLE CAPTIONS**

769

770 Table 1: Work conditions and usual quantification approaches in the crocin bleaching assay.

771

772 Table 2: Individual fittings to the eq. [5] of the kinetic data corresponding to the crocin reaction
773 in the specified cases. Parametric estimates and confidence intervals (n=3; α=0.05). v_τ: reaction
774 rate at the half-life time; r^2: correlation coefficient between observed and predicted values.

775

776 Table 3: Simultaneous fittings of the kinetic data from crocin reaction in the specified cases to
777 the bivariate models [8] (and [9] in temperature effect or [11] in pH effect). Parametric estimates
778 and confidence intervals (n=3; α=0.05). v_τ: reaction rate at the half-life time in [9] and [11]; E:
779 fitting coefficient proportional to the activations energy in [9]; r^2: correlation coefficient between
780 observed and predicted values. Note that A1, A2, A 3 and A4 are the cases also analysed with
781 the model [5] in the **¡Error! No se encuentra el origen de la referencia.**.

782

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TABLES

Table 1: Work conditions and usual quantification approaches in the crocin bleaching assay.

crocin			AAPH	T (ºC)	buffer	pH	formal analysis	reference
µmmol.L^{-1}	ε: M^{-1}.cm^{-1}	λ (nm)	(mmol.L^{-1})					
12	133,000	443	5	40	10 mmol.L^{-1} P [c]	7	A1	(Tubaro, Micossi & Ursini, 1996)
10	133,000	443	12.5	40	100 mmol.L^{-1} P [c]	7	A1	(Tubaro, Micossi & Ursini, 1996)
-	89,900	450	1	37 [b]	10 mmol.L^{-1} P	7.4	A2	(Lussignoli et. al., 1999)
25	89,900	450	5	37 [b]	10 mmol.L^{-1} PBS	7.4	A2	(Chatterjee et. al., 2005)
10	133,000	440	25	40	10 mmol.L^{-1} PBS	7	A1	(Ordoudi & Tsimidou, 2006)
100	15,117 [a]	450	7.68	37	100 mmol.L^{-1} Briton	5.5	eqs. [5] and [8]	This work

([a]) Sigma-17304; ([b]) preheated at 37 ºC, 5-20 minutes; ([c]) in water:ethanol (9:1); the rest in water.

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Table 2: Individual fittings to the eq. [5] of the kinetic data corresponding to the crocin reaction in the specified cases. Parametric estimates and confidence intervals (n=3; α=0.05). v_τ: reaction rate at the half-life time; r^2: correlation coefficient between observed and predicted values.

	K	***τ***	***α***	v_τ	r^2
***A:** Effect of AAPH concentration (AAPH as a pro-oxidant agent) at 37ºC*					
mmol.L^{-1} AAPH					
0.0	0.96±3.43	577.3±16.5	1.37±0.20	0.0003	0.9954
1.0	0.96±3.43	107.9±1.16	1.54±0.04	0.0053	0.9987
2.0	0.96±3.43	69.1±0.81	1.59±0.03	0.0084	0.9992
4.0	0.96±3.43	40.3±0.35	1.62±0.02	0.0146	0.9997
6.0	0.96±3.43	30.3±0.19	1.69±0.02	0.0200	0.9998
8.0	0.96±3.43	24.6±0.13	1.75±0.02	0.0252	0.9999
10.0	0.96±3.43	21.2±0.18	1.78±0.03	0.0296	0.9997
12.0	0.96±3.43	19.8±0.13	1.79±0.03	0.0318	0.9998
14.0	0.96±3.43	17.6±0.15	1.78±0.03	0.0357	0.9997
16.0	0.96±3.43	16.7±0.10	1.84±0.02	0.0386	0.9999
18.0	0.96±3.43	15.1±0.12	1.83±0.03	0.0424	0.9997
20.0	0.96±3.43	12.8±0.12	1.90±0.03	0.0515	0.9999
***B:** Effect of temperature*					
TºC (without AAPH)					
32	0.95±0.02	350.0±28.5	1.27±0.02	0.0009	0.9959
37	0.95±0.02	255.8±61.3	1.33±0.03	0.0012	0.9981
40	0.95±0.02	209.1±47.6	1.38±0.04	0.0015	0.9978
45	0.95±0.02	176.8±51.1	1.43±0.06	0.0017	0.9970
TºC (with 7.68 mmol.L^{-1} AAPH)					
32	0.97±0.01	38.61±0.22	1.17±0.01	0.0320	0.9987
37	0.94±0.01	28.58±0.26	1.81±0.01	0.0439	0.9992
40	0.95±0.01	19.49±0.52	1.38±0.01	0.0631	0.9993
45	0.94±0.01	12.29±0.11	1.42±0.01	0.1159	0.9983
***C:** Effect of pH*					
pH					
3.5	0.96±0.08	18.3±0.55	1.33±0.02	0.029	0.9993
4.0	0.97±0.08	21.8±0.63	1.34±0.02	0.025	0.9992
4.5	0.97±0.01	22.3±0.76	1.27±0.03	0.024	0.9991
5.0	0.98±0.01	23.8±0.92	1.21±0.04	0.022	0.9995
5.5	0.97±0.06	27.2±0.44	1.32±0.01	0.020	0.9993
6.0	0.97±0.06	26.6±0.49	1.29±0.01	0.020	0.9982
6.5	1.00±0.01	50.6±1.22	1.35±0.02	0.011	0.9991
7.0	1.00±0.03	71.4±3.89	1.41±0.04	0.008	0.9992
7.5	1.00±0.05	90.1±5.41	1.57±0.05	0.007	0.9993
8.0	1.00±0.09	124.5±11.2	1.77±0.07	0.006	0.9989
8.5	1.00±0.17	147.5±21.2	1.79±0.08	0.005	0.9979
9.0	1.00±0.03	167.7±3.89	1.64±0.04	0.004	0.9963
9.5	1.00±0.51	184.5±71.1	1.77±0.14	0.003	0.9994
10.0	1.00±0.79	234.9±60.1	1.89±0.13	0.002	0.9991
10.5	0.97±0.03	420.5±3.89	1.98±0.04	0.001	0.9963
11.0	0.97±0.03	675.6±3.89	2.05±0.04	0.001	0.9983
***D:** Effect of antioxidant (Trolox) concentration (7.68 mmol.L^{-1} AAPH, 37ºC, pH=5.5)*					
µmol.L^{-1} Trolox					
0.0	0.95 ±0.03	27.2±0.18	1.40±0.01	0.0199	0.9999
20.0	0.95 ±0.03	48.5±0.98	1.32±0.04	0.0110	0.9984
40.0	0.95 ±0.03	68.1±0.91	1.34±0.03	0.0078	0.9989
60.0	0.95 ±0.03	91.9±0.81	1.33±0.02	0.0058	0.9993
80.0	0.95 ±0.03	117.3±0.89	1.30±0.02	0.0045	0.9993
100.0	0.95 ±0.03	145.3±1.19	1.30±0.02	0.0037	0.9992
120.0	0.95 ±0.03	162.9±1.14	1.32±0.01	0.0033	0.9995
140.0	0.95 ±0.03	187.5±1.72	1.27±0.02	0.0028	0.9993
160.0	0.95 ±0.03	218.3±2.62	1.26±0.02	0.0024	0.9992

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Table 3: Simultaneous fittings of the kinetic data from crocin reaction in the specified cases to the bivariate models [8] (and [9] in temperature effect or [11] in pH effect). Parametric estimates and confidence intervals (n=3; α=0.05). v_τ: reaction rate at the half-life time in [9] and [11]; E: fitting coefficient proportional to the activations energy in [9]; r^2: correlation coefficient between observed and predicted values. Note that A1, A2, A 3 and A4 are the cases also analysed with the model [5] in the table 2.

	K	τ (v_τ in A2 and A3)	α (E in A2)	a_τ (b in A3)	b_τ	a_α	b_α	r^2
A: ANALYSIS OF THE FACTORS THAT AFFECT THE OXIDATION PROCESS IN THE CBA								
A1: AAPH	0.96±0.02	576.7±43.7	1.49±0.03	$0.001\pm1\times10^{-4}$	$0.004\pm1\times10^{-4}$	$1.5\times10^{-5}\pm3\times10^{-6}$	-	0.9984
A2: TEMPERATURE								
without AAPH	0.95±0.29	$1.4\times10^{3}\pm1\times10^{2}$	1.24±0.06	35.81±1.241	-	-	-	0.9993
with AAPH	0.95±0.02	$5.5\times10^{9}\pm1\times10^{7}$	1.58±0.06	65.59±3.272	-	-	-	0.9971
A3: pH	0.96±0.01	0.922±0.021	1.52±0.13	0.383±0.035	-	-	-	0.9986
A4: Trolox	0.96±0.01	27.21±0.441	1.33±0.01	0.045±0.001	-	-	-	0.9987
B: ANALYSIS OF SEVERAL COMPOUNDS								
B1: ANTIOXIDANT								
ascorbic acid	0.93±0.07	23.95±0.79	1.30±0.07	0.055±0.004	0.009±0.001	0.061±0.015	0.0217±0.006	0.9971
BHA	0.95±0.04	23.06±0.42	1.21±0.01	0.009±0.001	0.002±0.001	-	-	0.9986
TBHQ	0.96±0.08	32.22±0.75	1.24±0.02	0.007±0.001	0.002±0.001	-	-	0.9976
ethoxyquin	0.93±0.09	23.95±1.07	1.30±0.10	0.315±0.025	0.019±0.002	0.307±0.083	0.0622±0.022	0.9940
propyl gallate	0.96±0.09	27.41±0.65	1.19±0.04	0.049±0.002	0.003±0.001	-	-	0.9962
B2: METAL IONS								
Cu^{+2}	0.93±0.09	23.95±0.94	1.30±0.06	0.036±0.004	0.007±0.001	0.002±0.001	-	0.9943
Mn^{+2}	0.97±0.08	31.29±0.67	1.16±0.02	0.234±0.007	0.011±0.001	-	-	0.9979
Cu^{+1}	0.95±0.04	30.82±0.42	1.32±0.01	0.012±0.001	0.001±0.001	0.008±0.012	0.0022±0.014	0.9985

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FIGURES

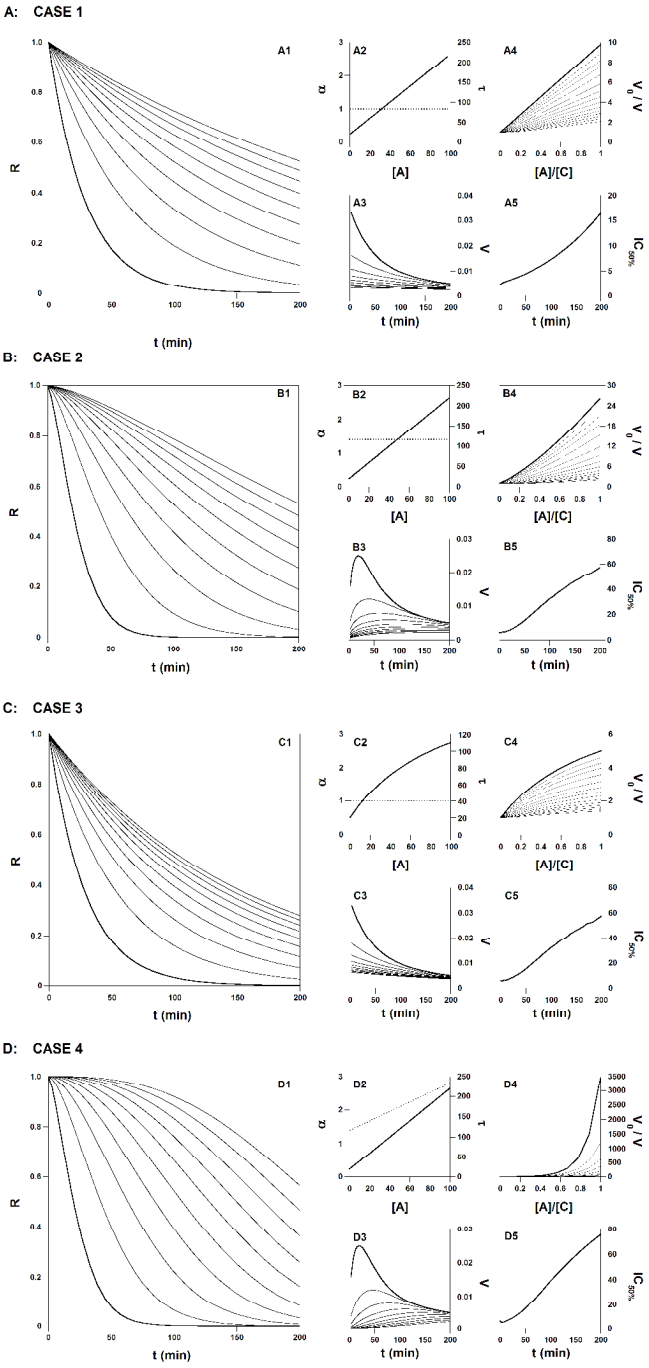

Figure 1: Simulation of the crocin bleaching kinetics (compare with real cases in fig. 3), using the eq. [8] with the four parametric combinations specified in section 3.2. For each case, five sub-figures are presented in which the following items are shown: **1)** the simulated dose-time dependent response of crocin oxidation using equation [8]; **2)** effect of antioxidant concentration on the parameters τ and α of eq. [8]; **3)** reaction rate (v) as a function of time for each antioxidant level; **4)** relationship between V_0/V and [A]/[C] ratios (supposed linear by A1 criterion) at different times along the range 1-200 min.; and **5)** time dependency of the $IC_{50\%}$ value (supposed constant by criterion A2). Note the problems associated with the acceptation of a linear hypothesis and the use of a single time for quantification purposes.

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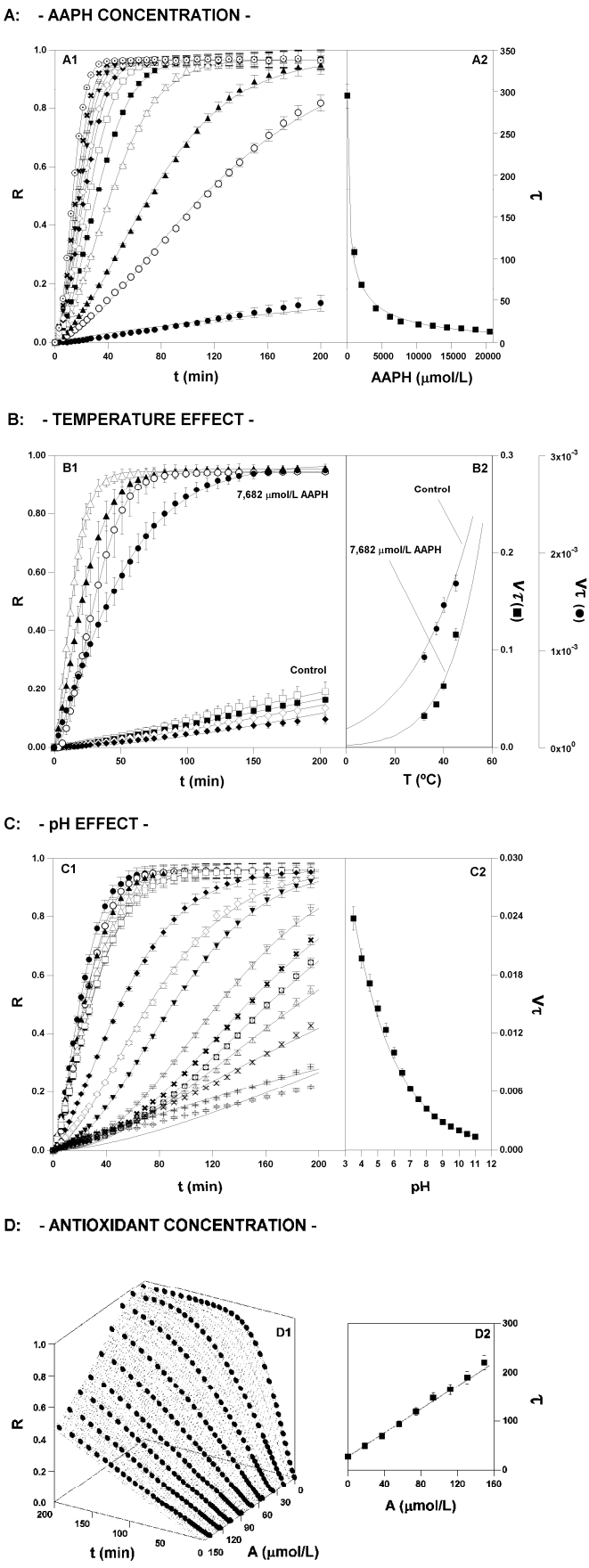

Figure 2: Evaluation of the different critical points on the CBA. Numerical results in Table 2 and 3. For all cases Experimental results are points and fittings to the corresponding models are lines:

A: Kinetic effect of AAPH. **A*1:*** Simultaneous fittings of equation [8] (lines) to Increasing concentrations of (0-20 mM) at 37ºC. ***B2:*** variation of parameter τ according to the individual fittings [5] (points) and simultaneous fittings [8] (lines).

B: Kinetic effect of temperature. ***B1:*** Fittings of model [9] (lines) in absence and presence of AAPH (7.6 mmol.L^{-1}) at five different temperatures (●: 32, ○: 37, ▲: 40, △: 45 and 50ºC). ***B2:*** estimates of $V\tau$ obtained from individual fittings to the model [A2] (points) and its simultaneous description by model [9] (lines).

C: Kinetic effect of pH on the bleaching rate in the crocin-AAPH system (37ºC, 7.68 mmol.L^{-1} AAPH). ***C1:*** kinetic results (points) fitted to the equation [10] (lines). ***C2*****:** $V\tau$ obtained from individual fittings to the model [A2] (points) and its simultaneous description by model [10] (lines).

D: Effect of antioxidant concentration on the bleaching of crocin-AAPH system (37ºC, 7.68 mmol.L^{-1} AAPH, pH=5.5) using trolox (0-(0.5)-4 μM) in the reaction mixture as example. ***D1:*** kinetic results (points) jointly fitted to the equation [8] (surface). ***D2:*** variation of parameter τ according to the individual fittings [5] (points) and simultaneous fittings to the model [8] (lines).

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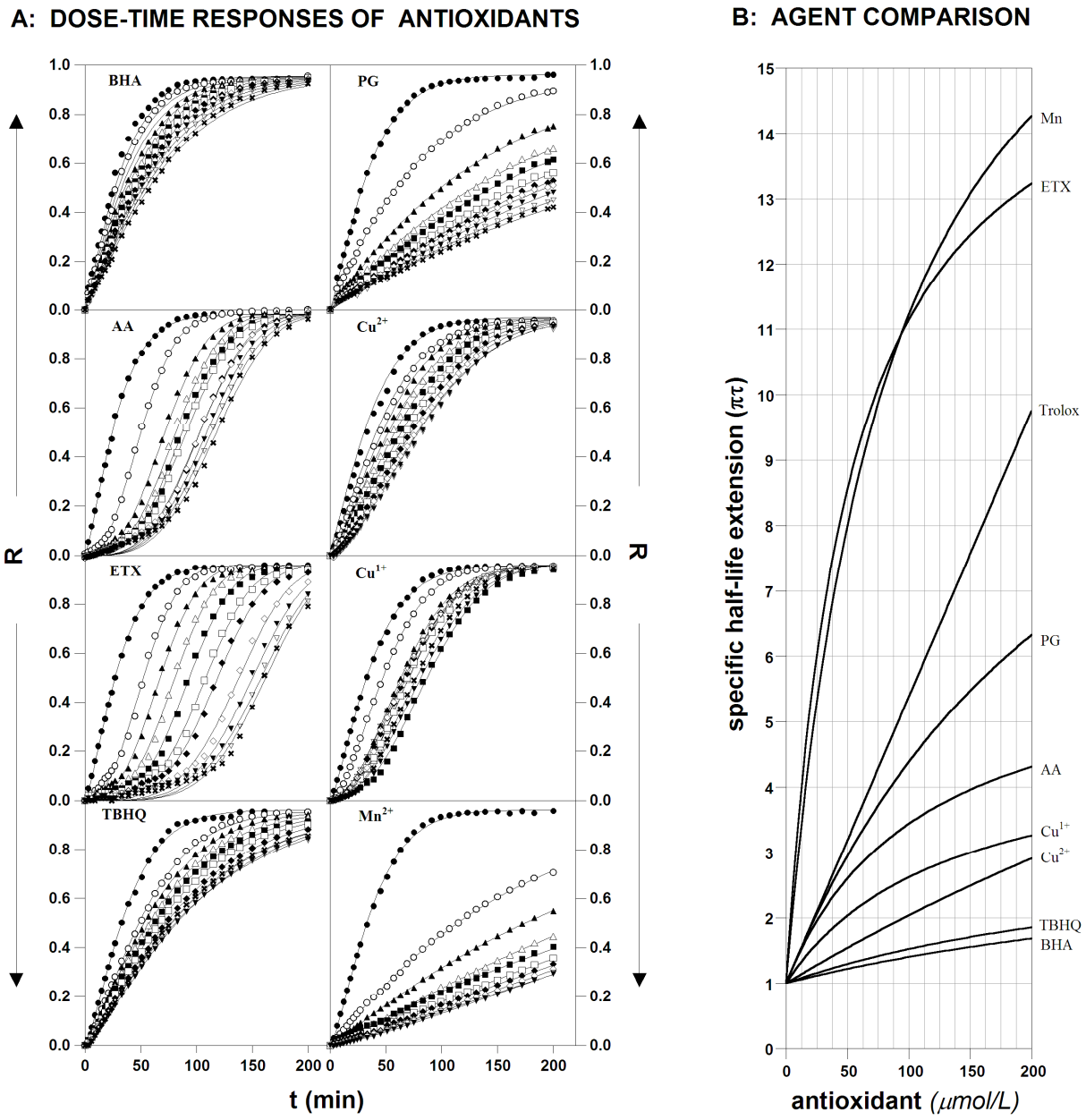

Figure 3: ***A:*** Effects of several antioxidants on crocin-AAPH system (7.68 mmol.L^{-1} AAPH, 37ºC, pH=5.5). Experimental results (points) and fittings to the eq. [8] (lines; see also table 3). In all cases the kinetic profiles drop orderly with the increase of the agent concentrations, which are (μmol.L^{-1}): BHA: 0-(37)-370, AA: 0-(30.3)-303, ETX: 0-(3.0)-30, TBHQ: 0-(80.0)-800, PG: 0-(30.0)-300, Cu^{+2}: 0-(20)-200, Cu^{+1}: 0-(20)-200, Mn^{+2}: 0-(12.5)-125. ***B:*** Characterization of all the agents studied through the specific half-life extension coefficient (π_τ from eq. [8]).