A new model to quantify and characterize the response to pro- and anti-oxidants of the copper-induced oxidation of LDL assay. A redefinition of a possible tool for oxidative stress diseases risk prediction and for rapid examination of potential preventive compounds.

M.A. Prieto, J.A. Vázquez & M.A. Murado

Grupo de Reciclado y Valorización de Materiales Residuales

Instituto de Investigaciones Mariñas (CSIC). r/ Eduardo Cabello, 6. Vigo-36208. Galicia, Spain.

ABSTRACT

The copper-induced oxidation of low density lipoprotein (LDL) particles represents a particularly suitable method for studying, both *in vitro* and *in vivo*, the effects of a number of alimentary, environmental and lifestyle factors on the mechanism of a biochemical process involved in many oxidative stress diseases, such as atherosclerosis, which affects around 50% of the occidental people. The available data about the role of pro- and anti-oxidants in LDL dynamics are abundant, but often, only semi-quantitative conclusions can be achieved. The lack of detailed algebraic models, the concomitant difficulties for assessing the statistical consistence of the results and designing definite experiments, and a certain shortage in characterizing criteria are some of the causes hindering the advances in a field which is *per se* very complex. The approach we propose describes, in an algebraically explicit way, the complete and generally biphasic time-course of the diene formation –as well as the peroxide profile– in LDL particles, including the separate or joint effects of any series of pro- and anti-oxidant concentrations. The model was tested using experimental results from other authors under different conditions, and very accurate and statistically consistent fittings were obtained in all the cases. Results, which enabled rigorous and detailed comparisons between LDL responses to oxidation modifiers, suggest an alternative criteria for the prediction of risks of occurrence of stress diseases. Thus, the basis of more complex multivariable models is provided. In all experimental data used, the calculated parameters were always statistically significant (Student's t-test, $\alpha = 0.05$), the equations were consistent (Fisher's F-test) and the goodness of fit coefficient of determination was higher than 0.99.

Keywords: copper-induced LDL oxidation assay; new robust mathematical model; pro- and anti-oxidant effects; coefficients to measure compound activity; oxidative stress disease risk prediction.

HIGHLIGHTS

A robust quantification alternative to investigate *in vitro* the oxidation of LDL.
Dose-time criteria to test the anti- and pro-oxidant activities.
Possible solution to help to define the risk of oxidative stress diseases.
Further capabilities of the criteria are illustrated to more complex responses.

1. INTRODUCTION

LDL particles can be a risk for cardiovascular disease when they invade the endothelium and become oxidized (Princen, Van Poppel, Vogelesang, Buytenhek, & Kok, 1992). The oxidized forms are more easily retained by arterial proteoglycans and they attract macrophages that engulf them, starting the formation of plaques. A high plasmatic level LDL promotes an excessive cholesterol deposit on the arterial walls and a proliferation of fibrous tissue producing the plaques causing atherosclerosis. Over time vulnerable plaques rupture, activate blood clotting and produce arterial stenosis, which if severe enough, results in heart attack, stroke, and peripheral vascular disease symptoms and major debilitating events.

The LDL are spherical particles (mean 2.5×10^6 MW) containing about 1600 molecules of cholesterylester and 170 of triglycerides, which form a central lipophilic core. This core is surrounded by a monolayer of about 700 phospholipid and 600 free cholesterol molecules. The polar heads of the phospholipids are located at the surface of the LDL particle and contribute to the solubility of LDL in an aqueous phase. A large protein termed apolipoprotein B-100 (apo B-100) is embedded in the outer layer. Half of the total fatty acid molecules bound into different lipid classes in an average LDL molecule are polyunsaturated fatty acids (PUFAs), mainly linoleic acid with minor amounts of arachidonic acid and docosahexaenoic acid (Esterbauer, Gebicki, Puhl, & Jürgens, 1992; Mertens & Holvoet, 2001). The fatty acid content of LDL and their distribution pattern can vary considerably from donor to donor. The PUFAs in LDL are protected against free radical attack and oxidation by a number of endogenous lipophilic antioxidants (i.e., α - and γ -tocopherol, carotenoids, oxycarotenoids, ubiquinol-10), of which α -tocopherol is by far the major antioxidant. Additionally, the plasma contains a great variety of water-soluble and lipid-soluble antioxidants. Similarly to the PUFAs, the endogenous and exogenous antioxidant present in or around the LDL molecules varies significantly between individuals. These individual variations from PUFAs and antioxidants are likely to have a significant effect on the oxidation behavior of different LDL samples (Kleinveld, Hak-Lemmers, Stalenhoef, & Demacker, 1992) and also are very likely to be influenced by different dietary habits (Gutteridge & Halliwell, 2010; Stocker & Keaney, 2004). Epidemiological studies have established an inverse correlation between the intake of natural exogenous antioxidants and the occurrence of oxidative stress diseases such as atherosclerosis, inflammation, cardiovascular problems, cancer, and aging-related disorders. Those facts have been demonstrated in clinical trials (Esterbauer, Puhl, Dieber-Rotheneder, Waeg, & Rabl, 1991; Li, Devaraj, Fuller, & Bucala, 1996; Parks et al., 1998; Princen et al., 1992; Ujhelyi et al., 1998) by inducing diets of higher antioxidant content, founding reductions of the LDL oxidation. Therefore, researchers have suggested to use the LDL oxidation as regular laboratory test to evaluate the early risks of oxidative stress diseases and to assess the clinical state of some patients with specific medical profiles. In addition, researchers have also used the oxidation analysis of LDL for testing the benefits of food and beverages as antioxidants for disease prevention (Chatterjee, Poduval, Tilak, & Devasagayam, 2005) and the identification of possible pro-oxidant substances (Esterbauer et al., 1992, 1991; Pinchuk, Schnitzer, & Lichtenberg, 1998).

In general, the LDL oxidation processes (El-Saadani et al., 1989; Lezerovich, 1986; Pinchuk et al., 1998) includes the four following stages: 1) *lag-initiation*: fatty acid (LH) oxidation is inhibited by the antioxidant content of the particle, until the exhaustion of its capability and the consequent beginning of the diene formation, mainly as peroxy radicals ($\text{LOO}^\bullet$); 2) *propagation*: in an autocatalytic process, LH are exponentially oxidized by peroxy radicals, meanwhile a simultaneous production of hydroperoxides (LOOH) takes place; 3) *decomposition*: alkoxy and peroxy radicals ($\text{LO}^\bullet$, $\text{LOO}^\bullet$) are derived from hydroperoxides; 4) *termination*: degradation products are formed from alkoxy and hydroperoxy radicals (aldehydes, ketones and epoxides). However, one should

keep in mind that the phases temporarily overlap and the activity of endogenous antioxidants plays an important role in the reaction rate at different stages, and not only on the initial lag-phase.

The oxidation state of LDL can be assessed through the time-related analyses of several oxidation products, such as, tiobarbiturate-reacting compounds (TBARS), conjugated dienes, hydroperoxides, aldehydes, disappearance of antioxidants or polyunsaturated fatty acids (PUFAs), the fragmentation of apo B-100, etc. Alone, none of them can tell us perfectly the oxidation state of LDL and the possible causal interrelationships between the different events. However, in general, it is accepted that LOOH concentration until the middle of the decomposition phase is directly reflected by the diene formation (Lezerovich, 1986), thus a common approach is based on the kinetics measurements of the formation and decomposition of conjugated dienes after inducing the oxidation of LDL by copper ions (El-Saadani et al., 1989; Esterbauer et al., 1991; Esterbauer & Striegl, 1989). The copper-induced oxidative LDL method facilitates to consider the kinetic perspective -an essential aspect that is not always taken into account- and since the superficial electronic charge of LDL particles gives them a hydrosoluble character in any oxidation state, the reaction can be effortlessly followed in an aqua solution measuring directly the diene formation. Its main weakness is an insufficient formalism, which prevents the quantification of its statistical reliability and loses a part of the relevant information that can be drawn from the experimental results (Devasagayam, Boloor, & Ramasarma, 2003).

In the current study, we propose a non-linear mathematical application to describe formally the diene time course throughout the entirety of the mentioned reaction sequence by means of a bivariate model, as a simultaneous function of the time and the concentration of any agent able to modify the oxidative process. This description proved to be statistically consistent and robust against error. At the same time, it produced a more global and detailed characterization than the usual one, and it revealed regularities suggesting some interesting general mechanisms underlying the behavior of the system studied.

2. MATERIAL AND METHODS

2.1. The copper-induced oxidative LDL method

Esterbauer and co-workers (El-Saadani et al., 1989; Esterbauer et al., 1991; Esterbauer & Striegl, 1989) established the method for continuous monitoring of in vitro oxidation of human LDL. Briefly, after dialysis against phosphate buffer saline to remove EDTA at 4 °C, LDL (50–100 µg/mL) is oxidized with 1–10 µmol of copper at 37 °C. The common evaluation methods of LDL susceptibility to oxidation in vitro are based on LDL oxidation, induced either by transition metals (usually copper) or by organic free radical generators (Lee, Chiang, Chen, & Hsu, 2000; Osakabe, Yasuda, & Natsume, 2002; Seccia, Albano, & Bellomo, 1997). Conjugated diene formation during LDL oxidation is monitored by changes in wavelength absorbance at 234 nm spectrophotometrically usually for periods of 200–400 minutes. Although the relevance of oxidation induced by either of these “pro-oxidants” to in vivo oxidation is questionable, the simplicity and reproducibility of the copper induced oxidation assay have made it attractive for routine and research assays.

2.2. Analytical procedures for the quantification of the results

The available data regarding the involvement of pro- and anti-oxidants in the kinetics of LDL oxidation are abundant, but often only semi-quantitative conclusions can be achieved, due to the difficulty of verifying a detailed kinetic model considering the complexity of the LDL oxidation. Besides, under these conditions, the experimental designs are frequently intuitive, and many of the respective abundant bibliographical data are incomplete from a dose- and time-dependent point of

view, preventing the full characterization of the relevant regularities of the response. In general, authors analyze the kinetic diene formation after the LDL oxidation started by a graphical method or by very simplistic mathematical analysis. The lack of detailed algebraic models, causes difficulties for assessing the statistical consistence of the results and expansion of the outcomes obtained by experiments. Additionally, when assessing the capability of compounds to delay the oxidation of LDL, it is important to note that each subject's LDL exhibits its own characteristic kinetics and that sample-to-sample variation can therefore be a serious problem if kinetic data from different experiments with different LDL preparation need to be compared (Esterbauer et al., 1992; Mosinger, 1997; Yoshida & Kisugi, 2010). Although those weakness points are not trivial, the method has become a particularly suitable tool for studying the LDL oxidation process and the relationships between the oxidizability of the PUFAs content and the endogenous/exogenous antioxidant capabilities (Hur & Reicks, 2012; Patterson, Horsley, & Leake, 2003; Staprans, Pan, Rapp, & Feingold, 2003; Wang et al., 2004).

2.3. Data used

The bibliographical abundance in clinical and food and technology fields of study, about the oxidation kinetics of LDL molecule makes it practically superfluous to extend the experimental work specifically devoted to validate the model proposed here. In this respect, its descriptive accuracy was verified using results from other authors (taken from the published figures by means of GetData Graph Digitizer 2.24), selected in such a way that they implied different methods, substrates and time domains. A summary of the anti- and pro-oxidants (oxidation modifiers, *OM*) collected is presented in Table 1. As it will be noted, the studies from which the data was collected are those that had been involved in developing, reviewing, extending or applying the methodology responses here evaluated. We hope that the reader would find them as a key data, rather than a lack of experimental effort.

2.4. Numerical methods

Fitting of the experimental results to the proposed equations was carried out in two phases. First, parametric estimates were obtained by minimization of the sum of quadratic differences between observed and model-predicted values, using the nonlinear least-squares (quasi-Newton) method provided by the macro *Solver* of *Microsoft Excel* 2003 spreadsheet (Kemmer & Keller, 2010). It allows a quick hypotheses testing and results displaying. Subsequently, parametric confidence intervals and model consistency (Student's *t* and Fisher's *F* tests, respectively, in both cases with $\alpha=0.05$) were calculated with *SolverAid* macro (Prikler, 2009). *SolverStat* macro (Comuzzi, Polese, Melchior, Portanova, & Tolazzi, 2003) was used for detecting possible anomalies in the distribution of parametric estimates and residuals.

3. RESULTS AND DISCUSSION

At first, the problems of the conventional analytical procedures, based on graphical analysis, to describe the pro- and anti-oxidants in the kinetics of LDL oxidation, are discussed. Then, by bringing across well establish mathematical models from other similar analytical methods, a more robust quantification tool, statistically consistent and free from the controversial aspects of the conventional procedures, were successfully applied to different combinations of *OM* agents. However, both solutions ignored the different phases of diene formation and focus only on the first phase, which is believed to be the relevant one. We have considered such a believe as a mere simplification and therefore, a new, complete and meaningful time and dose dependent model is developed to completely characterize the different diene formation phases produced during the oxidation of LDL. Then, the model was validated and tested with pro- and anti-oxidant data from

bibliographic references. Finally, to illustrate its capabilities, the new outcomes of the developed solution will be confronted against these established analytical methods.

3.1. The conventional analytical procedure

It is applied combining a kinetic perspective with the consideration of the variations in the kinetic parameters determined by the *OM* under study.

3.1.1. Graphical analysis of the time response of each dose to extract the relevant kinetic parameter

The kinetic part is solved in general by the graphical method illustrated in Figure 1, in which the simultaneous consideration of levels (Figure 1A) and rates (the corresponding numerical derivative: Figure 1B) makes easy to visualize the resource (Diekman, 1998; Ghaffari & Ghiasvand, 2010, 2011; Mosinger, 1997; Rikitake, Hirata, Kawashima, Akita, & Yokoyama, 1998). Although the use of all of them is not frequent, the essential parameters for characterizing the process are:

- K : maximum diene level, at the time in which its formation rate becomes null (intersection of the derivative with the abscissa axis). It would be an asymptotic value if dienes were stable, but in fact it is a maximum followed by a drop and a subsequent increase. The latest two phases are excluded from analysis, despite they are not irrelevant.
- t_K : time at which the maximum diene of the first formation phase is achieved.
- τ or t_{vm} : time corresponding to half-maximum ($K/2$) diene level, which also corresponds to the time at which the maximum rate (v_m) is achieved.
- v_m : maximum rate (maximum of the numerical derivative).
- λ or t_λ : lag time, in which the rate of the process can be considered negligible. It corresponds to the intersection of the tangent to the curve in the inflection point with the abscissa axis.

3.1.2. Mathematical dose-response analysis of the *OM*

After the graphical analysis, the treatment is completed by quantifying the variation of one or more of the specified parameters as a function of the concentration of the *OM* agents under study. The more frequently selected parameter is λ , which in general increases linearly with the antioxidant concentration (Esterbauer et al., 1991). When the λ results behave linearly as a function of the *OM*, the slope is used as the comparison term and when the response behaves non-linear typically, the dose at which the inhibition reaches the 50% (IC_{50}) is used as the parameter for comparative analyses. Also, the v_m has been suggested, because of it increases with the Cu^{2+} concentration according to a hyperbolic equation like the Michaelis-Menten one (Gieseg & Esterbauer, 1994).

3.1.3. Problems with the traditional step by step analyses

Although such a double approach is correct, it seems contradictory that the algebraic tools are reserved only to the second part of the procedure, which prevents the statistical validation of the results as a whole. The statistical information of the parameters found in the first graphical step is lost, and even if we compute the parametric estimations of the second step, it does not take into account the variable involved in the first step. Thus, among other problems, the two-step procedure lacks in a proper statistical estimation.

In addition, the graphical definition of λ –or its algebraic translation– does not imply a null response during the lag time, but only a low response, which varies depending on the rest of the kinetic parameters. In this sense, many authors emphasized the particular difficulties in estimating the lag phase parameter (Baty & Delignette-Muller, 2004). The value of λ is very sensitive to the experimental error and the inaccuracies of the graphical method. Lastly, λ values are inadequate for

assessing pro-oxidants, which promote their decrease and pile up their variations into a small interval. Even accepting to limit the analysis to the stretch up to the first maximum, we believe that a preferable general solution is the half-life parameter (τ) obtained when this maximum is considered as the asymptotic value.

When the kinetic responses were produced in a test-tube format, simple approaches seem to be acceptable. However, since the development of microplate routines, effortless time-dose data can be obtained and more complex and robust approaches to describe the responses are recommended. Next, general alternatives, from similar fields of studies, including those to describe the responses to build simultaneous solutions for most typical modifications, are discussed and tested.

3.2. Application of current mathematical quantification alternatives to partially describe the LDL oxidation.

Although the previous graphical analysis was and is widely used, with the expansion of computer programs, authors have chosen simpler applications and less susceptible to human error to obtain the previous parameters to characterize the response. In fact, to avoid this lack of statistical meaning of the results, the kinetic perspective is solved sometimes –rarely– using the logistic S-shaped equation (López-Alarcón, Speisky, & Lissi, 2007; Mosinger, 1997).

As discussed in the introduction, the profiles of formation of conjugated diene during the lag and propagation phase are believed to reflect the concentration of LOOH derived from the oxidation of the lipidic fraction present in the LDL. The traditional option is the analysis with S-shaped equations to characterize the response. Several S-shaped equations (Weibull, Hill, Gompertz or Richards-Chapman) could be very suitable options. Authors (Huang, Ou, Prior, & Ronald, 2005; Laguerre, Lecomte, & Villeneuve, 2007; Murado & Vázquez, 2010; Özilgen & Özilgen, 1990; Prieto, Rodríguez-Amado, Vázquez, & Murado, 2012) have identified, directly or indirectly, the ability of S-shaped functions to describe the corresponding kinetic profile. However, these type of functions can only be used to partially adjust the response of the first part of the formation of dienes to the cutting point with the abscissa of the first derivative (or partial asymptote as described in Figure 1), irrespective of the subsequent phases of diene formation, which are assumed negligible value in their characterization. Adequate mathematical models are available for the analysis of such phenomena. In this regard, recently, the Weibull mass function has been proposed and applied to describe the kinetic profile of the oxidation of diverse systems (Murado & Vázquez, 2010; Prieto, Murado, Vazquez, Anders, & Curran, 2013; Prieto, Murado, & Vázquez, 2013; Prieto et al., 2012). These approaches are next transferred to describe partially the kinetic first phase of diene formation, individually to each dose (univariate) or globally to all doses (bivariate).

3.2.1. Kinetic individual description of each dose: univariate approach

The time dependent Weibull (Weibull & Sweden, 1951) model can be presented in the following reparametrized form:

$$R(t) = K \left\{ 1 - \exp \left[-\ln(2) \left(t/\tau \right)^a \right] \right\} ; \text{ or, briefly: } R(t) = W(K, \tau, a) \quad [1]$$

where R is the oxidative response (with asymptotic maximum $K \leq 1$), τ the half-life of the oxidizable substrate, or time corresponding to 50% oxidation, and a a shape parameter. If the corresponding response ($K/2$) in τ seems a too high value as a reference, the reciprocal form of [1] can be used to calculate the time corresponding to any response R representing any lower fraction of K :

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

Some algebra of [1] allows to define the slope at time τ (or rate v_τ in the inflection point), the maximum slope (v_m , which coincides with v_τ if the function is symmetrical), time at which the maximum diene of the first formation phase is achieved (t_K) and the lag time (λ) as:

$$v_\tau = \frac{Ka \ln 2}{2\tau} ; \text{ therefore } R(t) = K \exp \left[-(\ln 2)^{1-a} \left(\frac{2v_\tau t}{Ka} \right)^a \right] \quad [3]$$

$$v_m = \frac{Ka \ln 2}{\tau} G^G e^{-G} ; \text{ where: } G = \frac{a-1}{a} ; \text{ therefore } R(t) = K \exp \left[-(\ln 2)^{1-a} \left(\frac{e^G v_m t}{K a G^G} \right)^a \right] \quad [4]$$

$$t_K = \tau \left(\frac{a-1}{a \ln 2} \right)^{1/a} + \frac{K}{2v_\tau} ; \text{ therefore} \quad [5]$$

$$\lambda = \frac{\tau}{\sqrt[a]{\ln 2}} \left[G^{1/a} + \frac{e^{-G} - 1}{a G^G e^{-G}} \right] ; \text{ therefore } R(t) = K \exp \left[- \left(\left(G^{1/a} + \frac{e^{-G} - 1}{a G^G e^{-G}} \right) \frac{t}{\lambda} \right)^a \right] \quad [6]$$

The reader should note, that we have selected Eq. [1] as the principle formula to perform the analysis of the effector agent. Thus, we have selected the form that makes explicitly the τ parameter and avoided the other possible reparametrizations that provide other parameters of interests (v_m , v_τ , t_K and λ). Such a preference, shared by many other authors in many different fields, should not be a restriction. The confidence intervals of v_m , v_τ , t_K and λ could be estimated by means of the reparametrization of Eq. [1] to make such values explicit (Eq. [3], [4], [5] and [6]), but the result is less operative than the robust estimation of the τ parameter.

3.2.2. Joint description of the time and dose response: bivariate approach

Knowledge of dose-time effect would be particularly important in the establishment of reference levels and to assess appropriately the effect of *OM*. Additionally, optimal efficient data analysis should involve simultaneous description of all curves, rather than fitting each one individually because, if necessary information to describe a parameter of the function is missing in one or various curves, this can be completed with the information from the other curves. Also, because when the mathematical behaviors of the responses are unlocked, this information may provide the base for classification systems that could reveal *OM* mechanistic patterns. Therefore, the method gains characterizing capability and statistical consistence if the two perspectives are combined in a single algebraic explicit model. Since any *OM* alters necessarily at least one parameter θ (K , τ or a) of Eq. [1], its concentration can be included in such a equation admitting that any value θ_0 in the absence of the agent varies in its presence as a function H_θ of the *OM* level. On a purely empirical, but highly versatile basis, it can be decided that, in the more complex case, H_θ should be a factor able to model situations in which the parametric value increases –or decreases– linearly, or with increasing or decreasing slope. This is achieved with a hyperbolic form as:

$$\theta(OM) = H_\theta \theta_0 ; H_\theta = \frac{(1 + b_\theta OM)}{(1 + c_\theta OM)} ; (\theta = K, \tau, a) \quad [7]$$

In practice the solution is usually simpler, since the effects of the modifier are in general directly ($c_{\theta}=0$) or inversely ($b_{\theta}=0$) related to its concentration. In fact, the examination of numerous anti- and pro-oxidant agents (Prieto, Murado, Vazquez, et al., 2013; Prieto et al., 2012) revealed only the following behaviors: (1) In open systems (without oxygen limitation) K do not vary, whereas τ increases linearly or with decreasing slope in the presence of antioxidants, and decreases with decreasing slope in the presence of pro-oxidants; (2) In closed systems (with possible oxygen limitation) the only difference is the possible decrease of K , with decreasing slope, in the presence of antioxidants.

Thus, without prejudging the anti- or pro-oxidant character of the modifier, the bivariate model can be stated as follow:

$$R(t, OM) = H_k K \left\{ 1 - \exp \left[-\ln(2) (t/H_{\tau} \tau)^{H_{a,a}} \right] \right\} \quad [8]$$

Therefore, this model provides the simultaneous description of any set of kinetic profiles resulting from the oxidation of a substrate in the presence of different concentrations of a modifier.

3.2.3. Application to describe the dose-response of anti- and pro-oxidant responses produced in the LDL oxidation method induced by copper

Figure 2, shows the application of the univariate (Eq. [1]) and bivariate (Eq. [8]) alternatives to describe the reduction or inhibition of the LDL oxidation, by pro- and anti-oxidant cases, obtained from bibliographical references (Table 1, C1 and C7). Figure 2A (C1), describes the dose-response of the antioxidant properties of peanut protein hydrolysates (EHPP: 0, 10, 20, 50 and 100 $\mu\text{g/ml}$) after a treatment with esperase for 2 h (Hwang, Shyu, Wang, & Hsu, 2010). The LDL induced by copper ion was isolated from 3 donors to reduce the variability of the results. Plots A1 and A2 show the results of applying the univariate and bivariate alternatives (Eq. [1] and [8], respectively). In those graphs, the white dots are the ones used for the fitting analysis and the black dots are the ones that are excluded. Plots A3-5, show the more interesting analytical parameters and their dose-relations. In those plots, the dots shows the parametric estimates obtained with the univariate approach and the lines for the bivariate one. The parameters τ and λ show a linear increasing relation, while v_m shows an asymptotic decreasing one. Finally, the plot A6 shows the correlation between the observed and predicted data for the bivariate analysis ($r^2=0.9954$). Note that the differences between v_m and v_{τ} are narrow, and the analysis has been avoided here and in the subsequent results. Accordingly, the dose-responses of λ and t_K are equivalent and the analysis has also been avoided.

In similar terms, Figure 2B (C7), describes the dose-response of a pro-oxidant case (0.5, 0.7 1.0, 1.6 and 5.0 μM of Cu^{+2} in the final solution) obtained from the work of Gieseg & Esterbauer (1994). Accordingly to the previous case, plots B1-6 describe the same actions for a pro-oxidant agent, in which the parameters τ and λ show an asymptotic decreasing relation, while v_m an exponentially increasing one.

Table 2 shows the parametric estimates and statistical results for the univariate and bivariate analysis of the anti- and pro-oxidant responses presented in Figure 2. Statistically significant results were found continuously (Student's t-test, $\alpha = 0.05$), the equation was consistent (Fisher's F-test) and the goodness of fit coefficient of determination was higher than 0.98. In general, the dose-response relation described for the relevant parameters derived are in agreement with those found by other authors (Devasagayam et al., 2003; Gieseg & Esterbauer, 1994; Pinchuk et al., 1998; Raveh, Pinchuk, Schnitzer, & Fainaru, 2000; Yoshida & Kisugi, 2010).

3.3. A new, complete and meaningful alternative to characterize the oxidation of LDL

The diene formation/decomposition response typically obtained (ups and downs in the curve present in Figure 1) could provide more insights than the incomplete description of fitting the first part of the response, and in consequence, the conclusions that can be derived. In this sense, by merging the previous analysis with the mechanistic reactions that take place in the LDL oxidation, we believe a new, complete and meaningful criterion to characterize the responses can be developed. Next, at first, the univariate and the bivariate of this new criterion will be presented and validated with bibliographic results, and then, an illustration of the variations that can take place will be performed. Afterwards, the model will be tested with abundant pro- and anti-oxidant data from bibliographic references. Finally, the new outcomes of the developed solution will be discussed.

3.3.1. Univariate model for Cu^{2+} induced LDL oxidation and its validation

If the oxidizable substrate (LH) was exhaustively transformed into dienes ($\text{LOO}^\bullet$), and these dienes were stable, the LH drop (from the initial level K to exhaustion) and the $\text{LOO}^\bullet$ increase (from a negligible initial level to the asymptotic level K) would follow strictly complementary sigmoidal profiles. However, the actual profile of the diene concentration does not show this behavior, because it reflects other two phenomena: (1) A diene consumption through the $\text{LOO}^\bullet \rightarrow \text{LOOH}$ process, as a consequence of which the increasing slope is less pronounced and does not lead to an asymptote, but to a maximum value lower than K , followed by a transitory decrease; (2) A secondary diene production, either in radicalized form ($\text{LO}^\bullet$) or as part of decomposition products (DP, such as aldehydes, ketones and epoxides). This secondary process –which actually is coexistent with the primary one from the initiation phase– is responsible for the subsequent increase to the transitory decrease mentioned above. In an open system, its limit is again K .

As a result, the real diene time course can be explained in its entirety (not only up to the first maximum) as the resultant from three asymptote-sharing sigmoidal kinetics. Using the brief notation of Eq. [1] we can write:

$$R(t) = K \left[W(\tau_0, a_0) - W(\tau_1, a_1) + W(\tau_2, a_2) \right] \quad [9]$$

Moreover, since in a reaction chain as the studied one the half-lives of the successive substrates are interdependent, τ_1 and τ_2 can be expressed as products of τ_0 by two specific constants (ϕ_1 and ϕ_2). This implies a parameter reduction which is additional to the one derived from the identity of the asymptotes, and it facilitates sensibly the use of the model, which in its expanded form is:

$$R(t) = K \left\{ \left[1 - \exp \left[-\ln 2 \left(\frac{t}{\tau_0} \right)^{a_0} \right] \right] - \left[1 - \exp \left[-\ln 2 \left(\frac{t}{\phi_1 \tau_0} \right)^{a_1} \right] \right] + \left[1 - \exp \left[-\ln 2 \left(\frac{t}{\phi_2 \tau_0} \right)^{a_2} \right] \right] \right\} \quad [10]$$

This novel model was validated using the results obtained by Manach et al. (1998) (C2 from Table 1) in the absence of any OM with 10 μM Cu^{2+} in the final concentration. Figure 3 shows the experimental data of the diene kinetics ($\circ$) and the fittings to the data by Eq. [10] (continuous black line). In addition, the three independent kinetic profiles for the formation and decomposition of $\text{LOO}^\bullet$ (green and red lines, respectively) and the formation of DP (blue line) contributing to the definition of the diene time-course are presented. An interesting additional validation of Eq. [10] was derived from its capability for modeling the peroxide time course as well, through the difference between its first (green line) and second (red line) components, as shown in Figure 3 with a dotted black line. Table 3 shows the statistical significance ($\alpha=0.05$) in all the parametric estimates, as well as good sensitivity, high correlation between the observations and predictions

($r^2=0.9991$) and unbiased residuals. The variations of those phases are easily controlled by the values of the parameters ϕ_1 and ϕ_2 , which defines the times that these phases are extended/reduced with respect the first one (τ_0). Theoretically, these distances can expand the previous definition of the actions of *OM* over the oxidation of LDL.

3.3.2. Bivariate model for Cu^{2+} induced LDL oxidation and its validation

Once the accuracy of our approach was proven in its univariate form, it was necessary to validate it as a simultaneous (bivariate) description of series of kinetic profiles in the presence of increasing concentrations of anti- and pro-oxidant agents. The development of a theoretical model is greatly facilitated by the possibility of combining all experimental data into a single master curve that is able to account for the important variables simultaneously. Such a solution allows to control most factors that affect the system, provide the key knowledge to understand the governing mechanisms, and helps to uncover the essential information that otherwise will remain hidden.

The general bivariate model was obtained simply by multiplying, in Eq. [10], K and τ_0 by the corresponding hyperbolic factors H_k and H_τ as defined in Eq. [7]. Although the expected null coefficients b_θ and c_θ were not suppressed *a priori*, fittings revealed again the simple behaviors (direct and inverse proportionalities) previously observed in other systems. Thus, making $H_K \times K = K^\bullet$ and $H_\tau \times \tau_0 = \tau^\bullet$, the bivariate form can be written as:

$$R(t, OM) = K^\bullet \left\{ \left[1 - \exp \left[-\ln 2 \left(\frac{t}{\tau^\bullet} \right)^{a_1} \right] \right] - \left[1 - \exp \left[-\ln 2 \left(\frac{t}{\phi_1 \tau^\bullet} \right)^{a_2} \right] \right] + \left[1 - \exp \left[-\ln 2 \left(\frac{t}{\phi_2 \tau^\bullet} \right)^{a_3} \right] \right] \right\} \quad [11]$$

Indeed, when this procedure was applied to the experimental results of Pinchuk et al. (1998) (C8 from Table 1), the accuracy of the fittings was equivalent or higher than that achieved by the complex mechanistic interpretations proposed by these authors and somehow validate its applicability. These authors followed the accumulation of reaction products in the time course of copper-induced peroxidation of LDL at multiple wavelengths analysis (typical profile oxidation of LDL, hydroperoxides formation and the first phase of dienes formation) for different concentrations of LDL affected by a dose-response of a copper agent (pro-oxidant effector). Figure 4 shows the fittings to one of the dose-response of copper/LDL relations. Figure 4A shows the fittings to the oxidation of LDL by the bivariate Eq. [11], the white dots ($\circ$) are the kinetic diene formation (at 234 nm) as a function of four copper concentrations (5.00, 2.50, 1.00, 0.50 and 0.25 μM). Figure 4B and C show the kinetic sub-products concentration and their correlations between the data measured by the authors and those predicted by Eq. [11] once it was fitted to the whole diene formation phases in Figure 4A.

Our proposal represents an alternative for the dose-time-response behavior and provides two kinetic parameters which jointly defines the capacities of the *OM* to extend or shorten the maximum protection as a function of the concentration. In fact, it is able to describe accurately different rate-dose tendencies. It allows quantification of the variations of the kinetic profiles which characterize the different types of *OM* in a useful way that can provide even indications concerning modes of action. Independently of the mechanistic interpretation that can be inferred by analyzing the specific behavior of both characterizing parameters, it produces consistent and meaningful criteria for comparative characterization and quantification of the oxidation of LDL. Next, we will illustrate some possible LDL oxidation patterns that can occur.

3.3.3. Effects of oxidation modifiers illustrating the main characteristics of the expectable kinetic behavior of the LDL oxidation

By means of simulations, Figure 5 shows 7 possible cases of typical families of curves (S1-5 antioxidants and S6-7 pro-oxidants), which are obtained by assigning realistic parametric values to Eq. [11]. The parametric values can be seen at the top of each simulated case. The idea is to exemplify clearly how relevant are the other phases of the typical oxidation LDL profile. Each case is built over five different plots. The top plot, shows the parametric patterns of the three half-life parameters ($\bullet$ τ_0 , $\blacksquare$ τ_1 and $\blacktriangle$ τ_2) and the asymptotic K ($\circ$) one, as a function of OM effectors. The bottom plot, shows the typical dose-response of the diene accumulative of LDL oxidation profile at 234 nm. The thick line of the kinetic dose-response curves corresponds to the response without modifier agent. Finally, the three plots in the middle, correspond to the three compound reactions predicted by the Eq. [11]. The characteristics of the expected results, according to the proposed model, are thus highlighted.

3.3.4. Effects of oxidation modifiers of the bivariate model of LDL oxidation

Experimental data to validate Eq. [11] were taken from the abundant bibliographic information with *Get Data Graph Digitizer*, trying to test the model against a broad spectrum of oxidation-modifying agents, methods and time and concentration domains. Figure 6 and Figure 7 show the application of this model to a selection of real anti- and pro- oxidation cases, whose sources and conditions are described in Table 1 (we omit a number of other real examples in which the model produced equally satisfactory fittings). For each case eight different graphs are presented (plots from A to H). Plot A shows the individual fittings of the univariate Eq. [10] (lines) to the data (dots). The black dots are those for the kinetic profiles obtained for the absence of an agent (control), while the white dots are those in the presence of agent (pro- or anti-oxidants) in a dose-response way. Plot B shows the global fitting analysis of the bivariate Eq. [11] (surface) to the data (dots). Plots C, D, E and F show the parametric estimations (K , τ_0 , τ_1 , τ_2 , v_m and λ) found from the univariate (dots) and bivariate (lines) analysis. Plot G shows the correlation between the data obtained and the prediction from the bivariate Eq. [11]). Finally, plot H shows the contour plot produced by the bivariate Eq. [11]. Numerical results and their statistical information are specified in Table 3. The overall consideration of these results proves the accuracy and high statistical consistence provided of the new model developed either in its univariate or bivariate form, for anti- and pro-oxidant effects.

From this analysis, it is clear that the effects increasing a parametric value θ_0 are linear because, in theory, such a value could cover the range $[\theta_0, \infty)$, whereas the effects decreasing θ_0 are hyperbolic with decreasing slope (in general inversely proportional to the modifier concentration) simply because the domain of the parametric variation is here $[0, \theta_0]$. The hyperbolic decreases of τ and λ due to pro-oxidants (with linear increase of v_m ; Figure 7) are examples of this fact, which also took place in the K decrease of the preceding case.

3.4. Other considerations. Usefulness from the proposed model for in vivo studies

The conventional *in vitro* and *in vivo* assessment of oxidation-modifying agents is based on the variations they induce in the lag time (λ) and –less usually– the maximum rate (v_m), as they can be deduced from the diene kinetic profile up to its first maximum. Undoubtedly, this information is useful, but it omits other characterizing elements, uses a relatively problematic parameter (λ) and excludes the subsequent events to the first maximum, which are not strictly irrelevant, as shown in Figure 1. Regarding the subsequent course to the maximum, actual examples –as depicted in Figure 3, Figure 4, Figure 6 and Figure 7– prove that the secondary diene formation is a phase that should be taken into account, and the simulation in Figure 5 suggests that such a phase can even be very important. This seems of special interest when clinical implications are considered. Therefore, apart from the robust fitting solution to the particular diene formation profile produced during the LDL oxidation, other useful considerations derived from the proposed approach (in its univariate [10] or

bivariate [11] forms) can be obtained. Next, some specific ones that are opportune to be discussed are highlighted.

3.4.1. Rapid tool to discriminate potential antioxidant compounds for application for prevention and therapy of oxidative stress diseases

There are several coefficients that can be used to identify and compare the power of an antioxidant agents. When testing the activity of antioxidant compounds, it is crucial to understand its pattern as a function of a dose-response. Because the information provided by testing its activity at one single concentration can be very tricky. When a dose-response is considered, as discussed, the values of τ_0 (half-life value of the peroxide formation phase, time units), the dose-response coefficient b_τ (1/agent units) and the extension coefficients ϕ_1 and ϕ_2 —corresponding to the decomposition of peroxides and formation of DP phases—are probably the most relevant ones. They provide the inhibitory times and concentration capacity of agents. However, others such as the K (asymptote) or the changes in the slopes of the different phases (a_1 , a_2 and a_3) could also be very relevant. As example decreasing effects of K can be related with an inhibition of products derived at any phase of diene formation, which would indicate ability to block partially or totally one or several reactions. Decreases in the slopes would reflect a rate deceleration process of one of the different reactions involved in diene formation.

As an illustrative experimental case, C6 (Figure 6) refers to the dose-response action of the simvastatin antioxidant, which besides the expectable half-life extension, requires to accept a drop of the asymptotic value. Treatment with statins are known to lower plasma and low-density lipoprotein (LDL) cholesterol levels in humans with resultant prevention and regression of atherosclerosis. Besides, the effect of simvastatin antioxidant on the LDL oxidation, the authors also studied the effect of this antioxidant over the kinetic of hexanal formation, because there were evidences that indicated that the treatment with statins clearly reduced their formation. Accordingly, those authors found that simvastatin produces a decrease in hexanal concentrations, and in general terms, the reduction on the production of the major apolar cytotoxic aldehydes (2-Hexenal; 2,4-Heptadienal; 2-Octenal; Octanal; 2-Nonenal; 2,4-Decadienal; Decanal) derived from LDL polyunsaturated fatty acid oxidation. Clearly, such a detail conclusion would not be possible to conclude by applying our analytical tool to the LDL oxidized by copper assay (based in diene formation), because it cannot differentiate which DP reaction could be blocked. However, by applying our proposed analytical tool, in general terms, with a simple dose-response (Figure 6, C6), authors would be able to detect major capacities of compounds to prevent the formation of cytotoxic products. Because, such a drop on the asymptotic value of K as a function of the concentration of the antioxidant simvastatin is only explainable by supposing a reduction on the formation of DP.

All parameters together translate and provide the potential applicability of compounds for the treatment of oxidative stress diseases. Our proposal could be very useful to explore quickly and effortless compounds that can act as primary antioxidants inhibiting the first phase of the diene formation in the LDL oxidation and as secondary ones inhibiting the formation of DP. Such a compounds are currently the target of pharmacology research for developing new drugs to reduce the effects of oxidative stress diseases (Chatterjee, Poduval, Tilak, & Devasagayam, 2005).

3.4.2. A possible assisting tool for defining clinically the risk of oxidative stress diseases in humans

As previously suggested by Esterbauer et al. (1992), the oxidation behavior of human LDL against an increasing concentration of Cu^{2+} could be used as a tool for defining the risk of oxidative stress diseases: (a) For specific patients would serve as an analytical test to follow their state and response

to the treatment; and (b) For healthy individuals would serve as an analytical test to prevent and predict major complications.

Unfortunately, rarely is used as a clinical test –probably due the complexity of the assay– and its typical application is for evaluating treatment in pharmacology and medicine fields of research. The resistance to Cu^{2+} -induced oxidation of LDL is periodically checked in experiments –using subject groups according to their clinical state– which were submitted for a given period to a given treatment. The treatment could vary from drugs, habits, exercise, diets, etc. As example, in diets experiments, results show that the LDL resistance to the oxidation is increased by such diets, whose effects are revealed by higher λ and lower v_m values with regard to control subjects trials (Esterbauer, Puhl, Dieber-Rotheneder, Waeg, & Rabl, 1991; Li, Devaraj, Fuller, & Bucala, 1996; Parks et al., 1998; Princen et al., 1992; Ujhelyi et al., 1998). Both variations are dependent on the dietary level of antioxidants, according to hyperbolic relations reflecting the general phenomenon of the decreasing yields. Since the asymptotic values of these relations have been found to be characteristic of the subject, it is admitted they constitute predictive indexes of the probability of many oxidative stress diseases.

In this context, Eq. [11] offers a richer information, which is potentially suitable for refining these predictions. Indeed, the resistance to the primary and secondary phases of the diene formation are respectively quantified by the values of ϕ_1 and ϕ_2 , and –the rest of the parameters being equal– an increase of the ϕ_2/ϕ_1 ratio indicates strictly the degree of predominance of the second phase. If λ and v_m values of the first phase are characteristic of the subject, it is reasonable to suppose that the ϕ_2/ϕ_1 ratio also is, reflecting specific features of the metabolic incorporation of dietary antioxidants to the lipidic body of LDL particles. It could even be speculated that the apo B-100 conformational changes (which are responsible for the particle recognition by the target tissues, as affected by the electrical charges of the lipidic body), are not equally sensitive to the characteristic products of the two phases of the oxidative process.

CONCLUSIONS

Metabolic dynamics of LDL particles offers, without doubt, numerous difficulties outside the formal description of the oxidative processes in which such particles are involved. However, since their copper-induced oxidation is considered the basic model for their response to oxidation-modifying agents, we believe that a complete formal description of their peculiar kinetic profile can constitute a useful tool to investigate in depth other aspects of this phenomenon, starting from an unambiguous basis.

The formal model we propose avoids the need to know the concentrations of the intermediate chemical species, as it would be demanded by a more rigorous –but also more complex– kinetic approaches through rate equations and mass balances. Nevertheless, the model produces parametric values with clear kinetic meanings. It proved its capability to describe, with high accuracy and statistical consistence, experimental results obtained by other authors under very diverse conditions, both regarding diene –the main objective– or peroxide time courses.

The complexity of our approach, regarding the conventional one, is only apparent, since it concerns merely a calculation step that is trivial with the current informatic tools. In fact, the fitting of a data set derived from the studied experimental context to any of the hypothesis considered by the model is achieved with an immediate convergence, providing in return a more informative and accurate information. Even if the study is limited to the period up to the first diene maximum, the unified treatment of the kinetic profiles due to any concentration series of a oxidation-modifying agent produces, with Eq. [8], a more complete and compact characterization than the usual one, and less random or systematic error-sensitive results.

More interestingly, Eq. [11] conserves the advantages of Eq. [8], enabling, in addition, the description of the entirety of the diene kinetic profile beyond its first maximum. The kinetic differences between the two categories of compounds that are involved in the two phases of the diene formation, as well as the specific effects of different oxidation modifiers on each of them are entirely described. This detailed characterization can refine the prediction of the oxidative stress diseases risks. It suggests the possibility of connecting the relationships between both oxidative phases with conformational changes on the apo B-100 component which favor the capture of LDL particles by the arterial endothelium.

In conclusion, this approach offers a robust quantification alternative to investigate the oxidation of LDL induced by copper. The criteria could be valid for: (1) *in vitro* routine oxidation method when testing the activity anti- and pro-oxidant compounds; (2) clinical studies for many different purposes (testing drugs, natural antioxidants, different diet habits, etc.); or (3) a tool for defining the risk of oxidative stress diseases in patients to monitor their clinical condition and in healthy individuals to prevent and predict major complications.

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FIGURE CAPTIONS

Figure 1: Criteria for identifying the parameters of the copper-induced oxidative kinetics of LDL by the conventional graphical method. A: diene concentration profile; B: rate of the diene formation profile (numeric derivative of A). See text for details.

Figure 2: Kinetic analysis quantify the response of the copper-induced oxidative LDL method by fitting it partially to the univariate (Eq. [1]) and bivariate (Eq. [8]) alternatives to describe the action of anti- and pro-oxidant cases, obtained from bibliographical references (C1 and C7 in Table 1). Plots 1 and 2 show the results of applying the univariate and bivariate alternatives (Eq. [1] and [8], respectively). In those graphs, the white dots are the ones used for the fitting analysis and the black dots are the ones that are excluded. Plots 3-5, show the more interesting analytical parameters and their dose-relations. Finally, the plot 6 shows the correlation between the observed and predicted data for the bivariate analysis. Table 2 shows the parametric estimates and statistical results.

Figure 3: Diene kinetics in the copper-induced oxidation of LDL, according to the experimental data ($\circ$) from Manach et al. (1998) as described by the Eq. [10] (continuous black line). In addition, the three independent kinetic profiles for the formation and decomposition of peroxides (green and red lines, respectively) and the formation of DP (blue line) contributing to the definition of the diene time-course are presented. Dotted line represents the kinetic profile of peroxides. Table 3 shows the statistical significance ($\alpha=0.05$) in all the parametric estimates.

Figure 4: shows the fittings to one of the dose-response of copper/LDL relations presented in the work of Pinchuk et al. (1998). Figure 4A shows the fittings to the oxidation of LDL by the bivariate Eq. [11] for the kinetic diene formation (at 234 nm) as a function of four copper concentrations (2.50, 1.00, 0.50 and 0.25 μM). The black dots ($\bullet$) are the lower cooper concentrations and white dots ($\circ$) the increasing ones. Figure 4B and C show the kinetic sub-products concentration and their correlations between the data measured by the authors and those predicted by Eq. [11] once it was fitted to the whole diene formation phases in Figure 4A.

Figure 5: Some typical parametric families illustrating the main characteristics of the expectable kinetics of the LDL oxidation in the presence of pro- and anti-oxidant agents. Simulations by assigning the specified parametric values to Eq. [11].

Figure 6: Kinetics of the LDL oxidation induced by copper in the presence of increasing concentrations of antioxidant agents. Plots A and B: experimental results (points) and their fittings to the Eq. [10] and [11], respectively. Plots C to F show the: effects of the antioxidant concentration on the kinetic parameters of the oxidative process. Plot G shows the observed *vs.* expected results and plot H the projections of isolines response on the time-concentration plane. Data sources in Table 1 and numerical results in Table 3.

Figure 7: Kinetics of the LDL oxidation induced by copper in the presence of increasing concentrations of three pro-oxidant agents obtained from bibliographical references (Table 1). Graphical notations as in Figure 6. Data sources in Table 1 and numerical results in Table 3.

TABLE CAPTIONS

Table 1: Description of the references used to collect data to be analyzed in this study, the figures, agent involved and some relevant conditions of the assays.

Table 2: Parametric estimates and confidence intervals from fittings of the specified cases to the univariate (Eq. [1] and in the other reparametrized forms [3], [4], [5] and [6]) and bivariate (Eq. [8]) models. Sources of the used experimental results of C1 (antioxidant) and C7 (pro-oxidant) are indicated at Table 1. The confidence intervals (for $\alpha=0.05$) are presented as a percentage of the parameter value. Note that confidence intervals and R_{adj}^2 (linear correlation coefficient adjusted) were computed without taking into account the black dots showed in Figure 2.

Table 3: Parametric estimates and confidence intervals (as % of the estimated value) from fittings of the specified cases to Eq. [10] and [11]. Sources of the used experimental results of C1 to C8 are indicated at Table 1. The confidence intervals (for $\alpha=0.05$) are presented as a percentage of the parameter value. Graphical responses are presented in Figure 6 (form antioxidants) and Figure 7 (for pro-oxidants).

REFERENCIAS

- Albertini, R., Ramos, P., Giessauf, A., Passi, A., De Luca, G., & Esterbauer, H. (1997). Chondroitin 4-sulphate exhibits inhibitory effect during Cu²⁺-mediated LDL oxidation. *FEBS Letters*, 403, 154–158.
- Baty, F., & Delignette-Muller, M.-L. (2004). Estimating the bacterial lag time: which model, which precision? *International Journal of Food Microbiology*, 91, 261–277.
- Chatterjee, S., Poduval, T. B., Tilak, J. C., & Devasagayam, T. P. a. (2005). A modified, economic, sensitive method for measuring total antioxidant capacities of human plasma and natural compounds using Indian saffron (*Crocus sativus*). *Clinica chimica acta; international journal of clinical chemistry*, 352, 155–63.
- Comuzzi, C., Polese, P., Melchior, A., Portanova, R., & Tolazzi, M. (2003). SOLVERSTAT: a new utility for multipurpose analysis. An application to the investigation of dioxygenated Co(II) complex formation in dimethylsulfoxide solution. *Talanta*, 59, 67–80.
- Devasagayam, T. P. a, Bloor, K. K., & Ramasarma, T. (2003). Methods for estimating lipid peroxidation: an analysis of merits and demerits. *Indian journal of biochemistry & biophysics*, 40, 300–8.
- Diekman, T. (1998). Increased Oxidizability of Low-Density Lipoproteins in Hypothyroidism. *Journal of Clinical Endocrinology & Metabolism*, 83, 1752–1755.
- El-Saadani, M., Esterbauer, H., El-Sayed, M., Goher, M., Nassar, A., & J"urgens, G. (1989). A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. *Journal of Lipid Research*, 30, 627.
- Esterbauer, H., Gebicki, J., Puhl, H., & Jürgens, G. (1992). The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radical Biology and Medicine*, 13, 341–90.
- Esterbauer, H., Puhl, H., Dieber-Rotheneder, M., Waeg, G., & Rabl, H. (1991). Effect of antioxidants on oxidative modification of LDL. *Annals of Medicine*, 23, 573–581.
- Esterbauer, H., & Striegl, G. (1989). Continuous Monitoring of in Vitro Oxidation of Human Low Density Lipoprotein. *Free Radical Biology and Medicine*, 6, 67–75.
- Ghaffari, M., & Ghiasvand, T. (2010). Kinetic study of low density lipoprotein oxidation by copper. *Indian Journal of Clinical Biochemistry*, 25, 29–36.
- Ghaffari, M., & Ghiasvand, T. (2011). The study of kinetic inhibition of copper (II)-induced oxidation of low-density lipoprotein by lycopene. *Journal of Food Biochemistry*, 35, 228–240.
- Gieseg, S. P., & Esterbauer, H. (1994). Low density lipoprotein is saturable by pro-oxidant copper. *FEBS letters*, 343, 188–94.
- Girona, J., Ville, A. La, & Plana, N. (1999). Simvastatin decreases aldehyde production derived from lipoprotein oxidation. *The American journal of cardiology*, 83, 846–51.

- Gutteridge, J. M. C., & Halliwell, B. (2010). Antioxidants: Molecules, medicines, and myths. *Biochemical and biophysical research communications*, 393, 561–4.
- Huang, D., Ou, B., Prior, R. L., & Ronald, L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, 53, 1841–56.
- Hur, I. Y., & Reicks, M. (2012). Relationship between whole-grain intake, chronic disease risk indicators, and weight status among adolescents in the National Health and Nutrition Examination Survey, 1999–2004. *Journal of the Academy of Nutrition and Dietetics*, 112, 46–55.
- Hwang, J.-Y., Shyu, Y.-S., Wang, Y.-T., & Hsu, C.-K. (2010). Antioxidative properties of protein hydrolysate from defatted peanut kernels treated with esperase. *LWT - Food Science and Technology*, 43, 285–290.
- Kemmer, G., & Keller, S. (2010). Nonlinear least-squares data fitting in Excel spreadsheets. *Nature Protocols*, 5, 267–81.
- Kleinveld, H. A., Hak-Lemmers, H. L., Stalenhoef, A. F., & Demacker, P. N. (1992). Improved measurement of low-density-lipoprotein susceptibility to copper-induced oxidation: application of a short procedure for isolating low-density lipoprotein. *Clinical chemistry*, 38, 2066–72.
- Laguerre, M., Lecomte, J., & Villeneuve, P. (2007). Evaluation of the ability of antioxidants to counteract lipid oxidation: existing methods, new trends and challenges. *Progress in lipid research*, 46, 244–82.
- Lee, Y. T., Chiang, L. Y., Chen, W. J., & Hsu, H. C. (2000). Water-soluble Hexasulfobutyl[60]fullerene inhibit low-density lipoprotein oxidation in aqueous and lipophilic phases. *Proceedings of The Society for Experimental Biology and Medicine*, 224, 69–75.
- Lezerovich, A. (1986). Derivative UV spectra of lipid conjugated dienes. *Journal of the American Oil Chemists' Society*, 63, 883–888.
- Li, D., Devaraj, S., Fuller, C., & Bucala, R. (1996). Effect of alpha-tocopherol on LDL oxidation and glycation: in vitro and in vivo studies. *Journal of lipid research*, 37, 1978–1986.
- López-Alarcón, C., Speisky, H., & Lissi, E. (2007). Antioxidant effect of 5-amino salicylic acid on copper-mediated LDL oxidation. *Biological research*, 40, 155–62.
- Manach, C., Morand, C., Crespy, V., Demigné, C., Texier, O., Régéat, F., & Rémésy, C. (1998). Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties. *FEBS letters*, 426, 331–6.
- Mertens, a, & Holvoet, P. (2001). Oxidized LDL and HDL: antagonists in atherothrombosis. *FASEB journal* : official publication of the Federation of American Societies for Experimental Biology, 15, 2073–84.
- Mosinger, B. J. (1997). Human low-density lipoproteins: oxidative modification and its relation to age, gender, menopausal status and cholesterol concentrations. *European journal of clinical*

- Murado, M. A., & Vázquez, J. A. (2010). Mathematical model for the characterization and objective comparison of antioxidant activities. *Journal of Agricultural and Food Chemistry*, 58, 1622–9.
- Osakabe, N., Yasuda, A., & Natsume, M. (2002). Catechins and their oligomers linked by C4→C8 bonds are major cacao polyphenols and protect low-density lipoprotein from oxidation in vitro. *Experimental*, 227, 51–56.
- Özilgen, S., & Özilgen, M. (1990). Kinetic Model of Lipid Oxidation in Foods. *Journal of Food Science*, 55, 498–498.
- Parks, E. J., German, J. B., Davis, P. a, Frankel, E. N., Kappagoda, C. T., Rutledge, J. C., ... Schneeman, B. O. (1998). Reduced oxidative susceptibility of LDL from patients participating in an intensive atherosclerosis treatment program. *The American journal of clinical nutrition*, 68, 778–85.
- Patterson, R. A., Horsley, E. T. M., & Leake, D. S. (2003). Prooxidant and antioxidant properties of human serum ultrafiltrates toward LDL: important role of uric acid. *Journal of lipid research*, 44, 512–21.
- Pettersen, R. J., Muna, Z. A., Kuiper, K. K., Svendsen, E., Müller, F., Berge, R. K., & Nordrehaug, J. E. (2001). Sustained retention of tetradecylthioacetic acid after local delivery reduces angioplasty-induced coronary stenosis in the minipig. *Cardiovascular research*, 52, 306–13.
- Pinchuk, I., Schnitzer, E., & Lichtenberg, D. (1998). Kinetic analysis of copper-induced peroxidation of LDL. *Biochimica et Biophysica Acta*, 1389, 155–72.
- Prieto, M. A., Murado, M. A., & Vázquez, J. A. (2013). Quantification, characterization and description of synergy and antagonism in the antioxidant response. *Food Research International*, (In press). doi:10.1016/j.foodres.2013.09.033
- Prieto, M. A., Murado, M. A., Vazquez, J. A., Anders, Y., & Curran, T. P. (2013). A new microplate procedure for simultaneous assessment of lipophilic and hydrophilic antioxidants and pro-oxidants, using crocin and β -carotene bleaching methods in a single combined assay: Tea extracts as a case study. *Food Research International*, 53, 836–846.
- Prieto, M. A., Rodríguez-Amado, I., Vázquez, J. A., & Murado, M. A. (2012). β -Carotene assay revisited. Application to characterize and quantify antioxidant and prooxidant activities in a microplate. *Journal of Agricultural and Food Chemistry*, 60, 8983–8993.
- Prikler, S. (2009). Robert de Levie: Advanced Excel for scientific data analysis, 2nd ed. *Analytical and Bioanalytical Chemistry*, 395, 1945.
- Princen, H. M., Van Poppel, G., Vogelesang, C., Buytenhek, R., & Kok, F. J. (1992). Supplementation with vitamin E but not beta-carotene in vivo protects low density lipoprotein from lipid peroxidation in vitro. Effect of cigarette smoking. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 12, 554–562.

- 1 Raveh, O., Pinchuk, I., Schnitzer, E., & Fainaru, M. (2000). Kinetic analysis of copper-induced
2 peroxidation of HDL, autoaccelerated and tocopherol-mediated peroxidation. *Free Radical*
3 *Biology and Medicine*, 29, 131–146.
- 4 Rikitake, Y., Hirata, K., Kawashima, S., Akita, H., & Yokoyama, M. (1998). Inhibitory effect of
5 inducible type nitric oxide synthase on oxidative modification of low density lipoprotein by
6 vascular smooth muscle cells. *Atherosclerosis*, 136, 51–7.
- 7
8 Seccia, M., Albano, E., & Bellomo, G. (1997). Suitability of chemical in vitro models to investigate
9 LDL oxidation: study with different initiating conditions in native and alpha-tocopherol-
10 supplemented LDL. *Clinical chemistry*, 43, 1436–41.
- 11
12
13 Staprans, I., Pan, X.-M., Rapp, J. H., & Feingold, K. R. (2003). Oxidized cholesterol in the diet is a
14 source of oxidized lipoproteins in human serum. *Journal of lipid research*, 44, 705–15.
- 15
16
17 Stocker, R., & Keaney, J. F. (2004). Role of oxidative modifications in atherosclerosis.
18 *Physiological reviews*, 84, 1381–478.
- 19
20 Ujhelyi, L., Balla, J., Muszbek, L., Kakuk, G., Belcher, J., Jacob, H. S., ... Balla, G. (1998). A
21 microassay to assess the oxidative resistance of low-density lipoproteins. *Clinical chemistry*,
22 44, 1762–4.
- 23
24
25 Wang, X., Greilberger, J., Ledinski, G., Kager, G., Paigen, B., & Jürgens, G. (2004). The
26 hypolipidemic natural product Commiphora mukul and its component guggulsterone inhibit
27 oxidative modification of LDL. *Atherosclerosis*, 172, 239–46.
- 28
29
30 Weibull, W., & Sweden, S. (1951). A statistical distribution function of wide applicability. *Journal*
31 *of applied mechanics*, 18, 293–297.
- 32
33
34 Yoshida, H., & Kisugi, R. (2010). Mechanisms of LDL oxidation. *Clinica chimica acta;*
35 *international journal of clinical chemistry*, 411, 1875–82.
- 36
37
38
39
40
41
42
43
44
45
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2 **FIGURES**
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Figure 1: Criteria for identifying the parameters of the copper-induced oxidative kinetics of LDL by the conventional graphical method. A: diene concentration profile; B: rate of the diene formation profile (numeric derivative of A). See text for details.

Figure 2: Kinetic analysis quantify the response of the copper-induced oxidative LDL method by fitting it partially to the univariate (Eq. [1]) and bivariate (Eq. [8]) alternatives to describe the action of anti- and pro-oxidant cases, obtained from bibliographical references (C1 and C7 in Table 1). Plots 1 and 2 show the results of applying the univariate and bivariate alternatives (Eq. [1] and [8], respectively). In those graphs, the white dots are the ones used for the fitting analysis and the black dots are the ones that are excluded. Plots 3-5, show the more interesting analytical parameters and their dose-relations. Finally, the plot 6 shows the correlation between the observed and predicted data for the bivariate analysis. Table 2 shows the parametric estimates and statistical results.

Figure 3: Diene kinetics in the copper-induced oxidation of LDL, according to the experimental data ($\circ$) from Manach et al. (1998) as described by the Eq. [10] (continuous black line). In addition, the three independent kinetic profiles for the formation and decomposition of peroxides (green and red lines, respectively) and the formation of DP (blue line) contributing to the definition of the diene time-course are presented. Dotted line represents the kinetic profile of peroxides. Table 3 shows the statistical significance ($\alpha=0.05$) in all the parametric estimates.

A: TYPICAL LDL OXIDATION PROFILE

B: PEROXIDES PROFILES

C: HYDROPEROXIDES FORMATION PROFILES

Figure 4: shows the fittings to one of the dose-response of copper/LDL relations presented in the work of Pinchuk et al. (1998). Figure 4A shows the fittings to the oxidation of LDL by the bivariate Eq. [11] for the kinetic diene formation (at 234 nm) as a function of four copper concentrations (2.50, 1.00, 0.50 and 0.25 μM). The black dots ($\bullet$) are the lower cooper concentrations and white dots ($\circ$) the increasing ones. Figure 4B and C show the kinetic sub-products concentration and their correlations between the data measured by the authors and those predicted by Eq. [11] once it was fitted to the whole diene formation phases in Figure 4A.

ANTI-OXIDANT

PRO-OXIDANT

Figure 5: Some typical parametric families illustrating the main characteristics of the expectable kinetics of the LDL oxidation in the presence of pro- and anti-oxidant agents. Simulations by assigning the specified parametric values to Eq. [11].

C1: **C3:** **C4:** **C5:** **C6:**

Figure 6: Kinetics of the LDL oxidation induced by copper in the presence of increasing concentrations of antioxidant agents. Plots A and B: experimental results (points) and their fittings to the Eq. [10] and [11], respectively. Plots C to F show the: effects of the antioxidant concentration on the kinetic parameters of the oxidative process. Plot G shows the observed vs. expected results and plot H the projections of isolines response on the time-concentration plane. Data sources in Table 1 and numerical results in Table 3.

C7:

C8:

Figure 7: Kinetics of the LDL oxidation induced by copper in the presence of increasing concentrations of three pro-oxidant agents obtained from bibliographical references (Table 1). Graphical notations as in Figure 6. Data sources in Table 1 and numerical results in Table 3.

TABLE CAPTIONS

Table 1: Description of the references used to collect data to be analyzed in this study, the figures, agent involved and some relevant conditions of the assays.

CASE FIG.	CONDITIONS	AGENT	REFERENCES
A: ANTIOXIDANT EFFECTOR ANALYSIS			
C1 Fig. 1	LDL (100 µg/ml) humans donors, pH 7.4 (PBS), 25 µM Cu ²⁺ , 37 °C, 234 nm.	EHPP (0, 10, 20, 50 and 100 µg/ml).	(Hwang et al., 2010)
C2 Fig. 3B	LDL (50 µg/ml) humans donors, pH 7.4 (PBS), 10 µM Cu ²⁺ , 37 °C, 234 nm.	Isorhamnetin Glucoronides (0.5 µM).	(Manach et al., 1998)
C3 Fig. 1	LDL healthy humans 0.1 µM, pH 7.4 (PBS), 1.6 µM Cu ²⁺ , 37 °C, 234 nm.	Chondroitin 4-sulphate (0, 0.25, 0.5, 1 and 2 mg/ml).	(Albertini et al., 1997)
C4 Fig. 1A	LDL (50 µg/ml) humans donors, pH 7.4 (PBS), 10 µM Cu ²⁺ , 37 °C, 234 nm.	<i>Commiphora mukul</i> extract (0, 1, 2, 10 and 25 µg/ml).	(Wang et al., 2004)
C5 Fig. 3	LDL (25 µg/ml) humans donors, pH 7.4 (PBS), 10 µM Cu ²⁺ , 37 °C, 234 nm.	Tetradecylthioacetic acid (0, 5, 10, 15 and 20 µM).	(Pettersen et al., 2001)
C6 Fig. 1A	LDL (50 µg/ml) normolipidemic humans, pH 7.4 (PBS), 6 µM Cu ²⁺ , 30 °C, 234 nm.	Active simvastatin (0, 5, 10, 20, 30, 40, 50, 75and 100 µM).	(Girona, Ville, & Plana, 1999)
B: PROOXIDANT EFFECTOR ANALYSIS			
C7 Fig. 2	LDL (91 µM) humans donors, pH 7.4 (PBS), 30 °C, 234 nm.	Cu ²⁺ (0.5, 0.7, 1.0, 1.6 and 5.0 µM)	(Gieseg & Esterbauer, 1994)
C8 Fig. 1	LDL (0.1 µM) humans donors, pH 7.4 (PBS), 37 °C, 234 nm.	Cu ²⁺ (0.25, 0.5, 1, 2.5 and 5 µM, middle curves).	(Pinchuk et al., 1998)

Table 2: Parametric estimates and confidence intervals from fittings of the specified cases to the univariate (Eq. [1] and in the other reparametrized forms [3], [4], [5] and [6]) and bivariate (Eq. [8]) models. Sources of the used experimental results of C1 (antioxidant) and C7 (pro-oxidant) are indicated at Table 1. The confidence intervals (for $\alpha=0.05$) are presented as a percentage of the parameter value. Note that confidence intervals and R^2_{adj} (linear correlation coefficient adjusted) were computed without taking into account the black dots showed in Figure 2.

A: UNIVARIATE APPROACH FITTING THE OXIDATION OF LDL PARTIALLY								
agent (μM)	<i>main kinetic parameters</i>			<i>other computed parameters</i>				R^2_{adj}
	K	τ	a	ν_m	ν_τ	t_K	λ	
<i>A1: univariate approach for describing the antioxidant agent of C1</i>								
0.0	0.865 $\pm$ 15.5	97.07 $\pm$ 4.0	4.63 $\pm$ 16.5	0.0144 $\pm$ 1.3	0.0148 $\pm$ 2.5	127.82 $\pm$ 3.0	31.79 $\pm$ 12.3	0.9988
10.0	0.865 $\pm$ 8.4	120.52 $\pm$ 3.7	5.01 $\pm$ 26.5	0.0126 $\pm$ 6.2	0.0115 $\pm$ 0.5	164.49 $\pm$ 9.6	39.47 $\pm$ 6.8	0.9987
20.0	0.865 $\pm$ 4.9	157.62 $\pm$ 5.7	5.15 $\pm$ 14.7	0.0099 $\pm$ 9.9	0.0094 $\pm$ 2.2	201.15 $\pm$ 12.0	51.62 $\pm$ 44.7	0.9987
50.0	0.865 $\pm$ 14.7	233.37 $\pm$ 6.6	4.54 $\pm$ 8.4	0.0059 $\pm$ 1.5	0.0061 $\pm$ 3.3	311.15 $\pm$ 8.1	76.43 $\pm$ 23.4	0.9980
100.0	0.865 $\pm$ 18.8	389.30 $\pm$ 7.7	3.70 $\pm$ 21.7	0.0029 $\pm$ 0.6	0.0038 $\pm$ 13.6	494.47 $\pm$ 10.7	127.50 $\pm$ 17.7	0.9941
<i>A2: univariate approach for describing the pro-oxidant agent of C7</i>								
0.50	0.865 $\pm$ 3.7	219.65 $\pm$ 4.1	3.46 $\pm$ 5.2	0.0045 $\pm$ 0.3	0.0045 $\pm$ 9.0	310.37 $\pm$ 16.7	95.22 $\pm$ 40.1	0.9922
1.00	0.865 $\pm$ 1.5	193.89 $\pm$ 1.2	3.40 $\pm$ 5.1	0.0054 $\pm$ 2.1	0.0054 $\pm$ 3.3	276.08 $\pm$ 26.3	91.79 $\pm$ 40.4	0.9960
1.75	0.865 $\pm$ 9.6	165.87 $\pm$ 2.3	3.64 $\pm$ 14.1	0.0068 $\pm$ 0.7	0.0068 $\pm$ 2.2	236.04 $\pm$ 5.7	86.83 $\pm$ 0.3	0.9982
3.50	0.865 $\pm$ 1.9	124.85 $\pm$ 4.9	4.24 $\pm$ 8.8	0.0106 $\pm$ 3.4	0.0106 $\pm$ 3.2	174.71 $\pm$ 42.5	76.55 $\pm$ 34.2	0.9951
6.00	0.865 $\pm$ 0.3	102.01 $\pm$ 2.4	6.64 $\pm$ 13.3	0.0177 $\pm$ 7.0	0.0175 $\pm$ 2.8	125.90 $\pm$ 33.0	64.95 $\pm$ 45.0	0.9926
B: BIVARIATE APPROACH FITTING THE OXIDATION OF LDL PARTIALLY								
agent	<i>main kinetic parameters</i>			<i>modifiers of the kinetic parameters</i>				R^2_{adj}
	K	τ	a	b_τ	c_τ	b_a	c_a	
<i>B1: bivariate approach for describing the antioxidant agent of C1</i>								
EHPP	0.865 $\pm$ 9.1	96.01 $\pm$ 0.9	4.75 $\pm$ 7.9	0.029 $\pm$ 1.3	--	--	--	0.9954
<i>B2: bivariate approach for describing the pro-oxidant agent of C7</i>								
Cu²⁺	0.865 $\pm$ 10.1	240.98 $\pm$ 0.4	3.02 $\pm$ 12.4	--	0.242 $\pm$ 0.2	0.159 $\pm$ 7.3	--	0.9934

Table 3: Parametric estimates and confidence intervals (as % of the estimated value) from fittings of the specified cases to Eq. [10] and [11]. Sources of the used experimental results of C1 to C8 are indicated at Table 1. The confidence intervals (for $\alpha=0.05$) are presented as a percentage of the parameter value. Graphical responses are presented in Figure 6 (form antioxidants) and Figure 7 (for pro-oxidants).

		ANTIOXIDANTS						PRO-OXIDANTS	
Parameters		C1	C2	C3	C4	C5	C6	C7	C8
Asymptote	K	0.885±9.5	0.923±1.5	0.758±18.7	0.846±16.9	0.978±8.6	1.407±10.0	1.070±13.2	1.152±12.3
Asymptote modifiers	b_K	--	--	--	--	--	--	--	--
	c_K	--	--	--	--	--	0.011±2.7	--	--
Half-life	τ	100.7±0.4	102.1±0.3	102.1±1.5	52.0±2.9	74.3±0.6	151.0±1.0	269.9±0.6	174.9±0.9
Half-life modifiers	b_τ	0.029±2.2	--	0.776±0.1	0.128±0.1	0.052±1.2	0.008±2.1	0.278±0.1	1.387±0.0
	c_τ	--	--	--	--	--	--	--	--
Extension coefficients	ϕ_1	1.751±14.8	1.683±3.3	1.436±3.6	1.491±3.5	1.578±16.5	1.342±3.8	1.448±3.6	1.640±3.1
	ϕ_2	2.025±19.9	2.115±4.1	1.842±11.9	3.095±7.1	1.518±26.6	1.946±11.2	2.123±10.3	2.849±7.7
Slope controllers	a_1	5.215±16.1	6.811±6.8	5.911±5.4	2.939±10.9	10.53±8.0	4.837±6.5	4.158±7.6	3.425±9.3
	a_2	2.716±40.2	2.791±4.1	2.057±10.4	2.027±10.6	3.661±29.8	2.577±8.2	2.359±9.0	1.513±14.1
	a_3	1.721±56.6	2.249±5.2	1.470±4.9	1.192±6.0	2.965±32.9	1.378±5.2	1.359±5.2	1.100±6.5
Fitting statistics	R_{adj}^2	0.9979	0.9991	0.9960	0.9876	0.9961	0.9982	0.9876	0.9960