Quantification, characterization and description of synergy and antagonism in the antioxidant response.

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

Grupo de Reciclado e Valorización de Materiais Residuais (REVAL), Instituto de Investigacións Mariñas (IIM), Consejo Superior de Investigaciones Científicas (CSIC), r/Eduardo Cabello, 6. Vigo-36208, Galicia, Spain.

*Corresponding author
E-mail: michaelumangelum@gmial.com
Tel.: +34986214469; +34986231930
Fax: +34986292762

ABSTRACT

This paper illustrates a methodological procedure to determine the synergistic and antagonistic effects of combining antioxidant agents. Current methods to determinate the interactive actions of antioxidants have been rejected, and we attempt to address this issue by incorporating well-established ideas from different existing fields. Two mathematical models are proposed, which provide explicit algebraic forms and generalize the classical hypothesis of independent action and concentration addition as they are defined in the dose-response relationships. In addition, a comprehensive index to summarize all the complex responses in one single value is proposed, which allows the extraction and identification of the relevant aspects. Although the approach could be directly expanded to other types of classical antioxidant methods, two complex scenarios were recreated using different but complementary well-known kinetic antioxidant methods, which are fairly representative of lipidic and hydrophilic oxidation processes. Meanings of synergy and antagonism concepts were found that describe and characterize the interactions between several pairs of commercial antioxidants in a statistically consistent form. The results also provided some evidence of a more basic character, which, if transferable to more realistic food matrices in the food industry, may guide the development and evaluation of food products and processes, as well as the study of mechanisms underlying different phenomena that may affect the quality of products.

Keywords: dose-response analysis; synergy and antagonism; mechanisms of interaction; antioxidant interaction; β-carotene and crocin bleaching assay

1. INTRODUCTION

An important and characteristic problem of any system (as defined in the Bertalanffy's theory: a set of interacting elements) is to determinate whether the joint effect of two or more elements on the system behavior is directly deducible from the individual effects of the elements. This issue has a long history of controversy whose first known attempt to solve it dates back to Aristotle, and it is frequently stated by replacing the expression "directly deducible from" with "the sum of", which significantly change the focus. Thus, in the field of antioxidant action, the concepts of synergy and antagonism are often characterized as those interactions of two (or more) antioxidants that are greater (synergy) or lesser (antagonism) than the sum of the individual effects (Jia et al., 1998; Marinova et al., 2008; Parker et al., 2010; Yang et al., 2009). Such a characterization is not acceptable for two reasons.

First, it postulates that the joint effect in the absence of interactions is the sum of the individual effects, which is an especially simplistic case and not applicable to asymptotic responses, such as those involved in the action of anti- and pro-oxidant agents. Indeed, the sum of two individual responses is meaningless if it exceeds the asymptotic response of the system. In fact, the referent of any phenomenon that perturbs the joint action of two agents is that joint action in the absence of

perturbation (not the individual actions), a situation that is often called as the "null interaction". Consequently, the first condition to decide the possible presence of synergistic or antagonistic effects is to define the null interaction. A second difficulty arises from the common tools applied to characterize the antioxidant action. Despite abundant criticism (Labuza & Dugan, 1971; Murado & Vázquez, 2010; Prieto et al., 2012; 2012a; Terpinc & Abramovič, 2010; Özilgen & Özilgen, 1990), such a characterization frequently disregards the kinetic aspects of the oxidation process and its inhibition. Although this objection has a less theoretical significance than the first one, its practical consequence is that the results may be poorly suited to discern the joint effect of two antioxidants.

This paper pursues a solution for each of these objections by using concentration-time response models applied to the β-carotene (βCM) (Marco, 1968; Miller, 1971) and crocin bleaching (CM) (Bors et al. 1984) methods –extensively used to quantify antioxidant and prooxidant activities- to assess the synergistic or antagonistic interactions between several pairs of well-known antioxidants. Their respective protocols have been repeatedly revised and improved, and they are optimized at present (Prieto et al., 2012; 2012a). They are appropriate for lipophilic and hydrophilic matrices and can provide useful complementary information in the study of complex natural extracts containing components with a variable degree of polarity (Prieto et al., 2013). β-carotene is a lipophilic oxidizable substrate that can join the system of lipidic micelles in which the oxidation reaction is accomplished. The method is especially sensitive to oxidation modifying agents in a lipidic environment, and it produces a very low response with hydrophilic antioxidants, even powerful ones (polar paradox). Complementarily, crocin is a hydrophilic oxidizable substrate, and lipophilic oxidation modifiers, even powerful ones, produce very low responses in the aqueous system that characterize the application of this method (apolar paradox). These assays were selected because they provide an optimized response system that is fairly representative of the lipidic and hydrophilic oxidation processes, especially accurate, reproducible and yields a low experimental error.

The first problem, which consists of distinguishing between null interaction and synergistic or antagonistic effects was studied by generalizing the classical approaches (Berenbaum, 1985a; 1985b; Bliss, 1937; 1939; Loewe & Muischnek, 1926; Greco et al., 1995) applied in the dose-response area (not free either of debate about the interactive effects) and others (Qin et al.,2011; Hewlett & Plackett, 1964; Gessner, 1988; Rovati & Nicosia, 1994; Baldwin & Roling, 2009). The second difficulty was solved by defining the response of the system to the simultaneous action of two antioxidants through a single value obtained from a kinetic description as previously discussed (Dávalos et al., 2004; Huang et al., 2008; Naguib, 2000; Prieto et al., 2012).

The proposed generalized procedures for the joint action of several well-known antioxidants produced consistent results in all cases. In addition, it provided some evidence of a more basic character, which could be transferable to the general field of the *in vivo* dose-response relationships.

2. MATERIAL AND METHODS

2.1. Methods to assess the antioxidant activity

2.1.1. ***Equipment and reagents***

- *Equipment:* Multiskan spectrum microplate photometer using polypropylene plates with 96 wells.
- *Antioxidants:* butyl-hydroxyanisole (BHA); ; propyl 3,4,5-trihydroxybenzoate (Propyl gallate; PG); butyl-hydroxytoluene (BHT); 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline (Ethoxyquin; ETO); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; TRO); and (2R)-2,5,7,8-tetramethyl-2-[(4R,8R)-(4,8,12-trimethyltridecyl)]-6-chromanol (α-tocopherol; TOC); manganese sulfate (Mn); (5R)-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5H)-one (Ascorbic acid; AA).
- *Crocin bleaching reagent:* 4 mg of Crocin and 75 mg of AAPH were dissolved in 25 and 5 mL, respectively, of 100 mM Briton buffer, pH=5.5, in Mili-Q water at 40ºC. Both solutions must be prepared and mixed just before use.

- *β-Carotene bleaching reagent:* 4 mg of β-Carotene, 0.5 ml of linoleic acid and 4 g of Tween-40 were dissolved in 20 ml of chloroform and the chloroform was evaporated in a rotary evaporator (40ºC/~15 min). One mililiter of the oily residue is added to 30 ml of Mili-Q water at pH=6.5 (Briton buffer 100 mM) at the assay temperature (45ºC).

2.1.2. ***Procedure***

Microplate assays were carried out based on a complete design (more details are provided in the appendix and Figure A1) that consisted of 8×8 arrays of two antioxidant mixtures at equally increasing concentrations, which were freshly prepared in water:ethanol (9:1). Thus, 25 µl of each antioxidant solution was added to each well containing 250 µl of the preheated reagent (CM: 37°C and βCM: 45°C). The apparatus was programmed for 200 min at 37°C / 450 nm (CM) and 45°C / 470 nm (βCM), with agitation at 660 cycles/min (1 mm amplitude), which was only interrupted for readings at 3 min intervals (covering initiation, propagation and asymptotic phases with a total of 64 independent kinetic measures per each of the 64 concentration combinations). By using the antioxidants listed in the materials section, 21 combinations were performed for each method, including those in which the pair of antioxidant is the same antioxidant (used simply as a control). The concentration range applied is presented in Table 3.

2.1.3. ***Selection of a single value to assess the response***

The usual methods were based on comparing the oxidation rate, half-life, lag phase and the area under the curve (*AUC*) of a given substrate in the presence of increasing concentrations of the studied antioxidant. All antioxidant combinations were first analyzed by comparing the four above mentioned parameters. Although the results showed that all of them lead to similar conclusions, the use of the area under the curve (*AUC*) proved to be a highly robust criterion, which summarizes in a single and direct datum the global feature of any kinetic profile, while avoiding some minor drawbacks (affecting mainly the smoothness of the values) that emerge when other parameters are used.

This criterion is frequently applied for a dose-time response of an antioxidant standardizing the responses in relation to *AUC* obtained for the control, which leads to the formulation of the relative area units (*RAU*), as defined by other authors (Dávalos et al., 2004; Huang et al. 2002; Naguib, 2000). To obtain the *RAU* values, the response first needs to be compute in terms of area units, which can be calculated by any numerical integration method. For example, if R_t is the response to a set of t times, the area units under the curve can be calculated using numerical methods of integration, such as the trapezoidal rule:

$$AUC = \frac{1}{2} h_t \left[R_{t=0} + R_{t=n} \right] + h_t \sum_{t=1}^{n-1} R_t \qquad (1)$$

in which h_t is the kinetic interval used (3 min). Consequently, if AUC_0 and AUC_c are the area units corresponding to the kinetic profiles found in the absence and presence of an antioxidant concentration c, respectively, the *RAU* value that increases with the concentration and the power of the antioxidant can be defined as follows:

$$RAU(A) = AUC_0 - AUC_c \qquad (2)$$

The AUC_0 is also the maximum response achievable (RUA_{max}). Consequently values of *RAU* obtained can be standardized in responses (R) over a scale [0,1], which facilitates comparisons:

$$R = RAU / RAU_{\max} \qquad (3)$$

The variation of *RAU* as function of any agent can be described satisfactorily using the Weibull cumulative distribution function (Weibull, 1951), thus the effect of increasing concentrations (A_i) of an antioxidant (*A*) can be described in general terms as follows:

$$R(A) = K\left\{1-\exp\left[-\ln 2\left(A/m\right)^{a}\right]\right\} \quad \text{briefly;} \qquad R = W\left(A;K,m,a\right) \tag{4}$$

where *K* is the asymptotic response, *m* is the concentration producing the half-maximal response and *a* is a shape parameter related to the slope. This equation is very versatile: if $a<1$, it fits the potential profiles produced by the model of (Terpinc & Abramovič, 2010), if $a=1$, it describes a first order kinetic model, and if $a>1$, it produces a variety of sigmoidal profiles that are the common solution for the system.

2.2. Dose-response theory

2.2.1. ***The null interaction forms***

Two basic types of null interaction are conventional considered in the dose-response field. For two agents, these interactions are defined as follows:

(a) Independent action

This mode of action assumes that the agents act via different mechanisms, each of which reaches an asymptotic maximum as a result of a statistically independent phenomenon. Under this premise, probability theory defines the response, as the sum of the probabilities of the individual phenomena minus the probability of their joint occurrence (Bliss, 1939). Consequently, if R_c is the response to the joint action of the concentrations c_1 and c_2, and R_{c1} and R_{c2} the individual responses at the same concentrations, the total response can be established:

$$R_c = R_{c1} + R_{c2}\left(1-R_{c1}\right) \tag{5}$$

An expression that is easily generalized to more than two agents can be obtained by writing R_{c1} as $1-(1-R_{c1})$ and substituting it in (5):

$$R_c = 1-\left(1-R_{c1}\right)+R_{c2}\left(1-R_{c1}\right) = 1-\left(1-R_{c1}\right)\left(1-R_{c2}\right) \tag{6}$$

(b) Concentration addition

The typical formulation (Berenbaum, 1985a; 1985b) does not define the null interaction as a relationship between individual responses, but uses the following criteria: the concentration (*c*) of an agent whose action obeys the equation $R=f(c)$ can be considered a fictitious combination of the concentrations c_1 and c_2 ($c=c_1+c_2$). Under these conditions, the response to *c* will be given by the equation $R=f(c)$ with $c=c_1+c_2$. If the response to a mixed dose of two agents behaves as the response to the "mixed" dose of the same agent, it is accepted that the interaction between them is null. This fact indicates that any agent concentration can be effectively substituted by the equivalent concentration of the other one.

2.2.2. ***The combined action of two antioxidants with and without interactions***

The simultaneous action of two antioxidants can occur according to any of both modes of action listed above, even in very simple processes (see Figure 1). Therefore, to propose explicit algebraic forms for these modes of action in the case of the action of antioxidant agents requires applying the equation (4) in the framework of the *IA* and *CA* hypotheses as follows:

(a) Independent action (IA)

The basic model (null interaction) is directly obtained by transferring the equation (4) into (6):

$$R = 1 - \left[1 - W\left(A_1; K_1, m_1, a_1\right)\right]\left[1 - W\left(A_2; K_2, m_2, a_2\right)\right] \quad (7)$$

Any interaction necessarily implies that the presence of an antioxidant alters the parameters of the response to the other in an effect that can be unidirectional or reciprocal. We have previously proven (Murado et al., 2011) that to change the response of Weibull's equation as function of the effect of another variable can be achieved by multiplying *K* and *m* parameters by a hyperbolic perturbation term that includes the variable responsible for the alteration. Because the variable that perturbs the response to an antioxidant A_i is another antioxidant A_j, the perturbation terms will have the following form:

$$\nu_{\theta i} = \left(1 + b_{\theta i} A_j\right) \Big/ \left(1 + c_{\theta i} A_j\right) \; ; \; (\mathrm{i} \neq \mathrm{j}) \quad (8)$$

where $\nu_{\theta i}$ is the factor that multiplies the θ parameter (*K* or *m*) of the response to A_i, and it is a function of the concentration of the antioxidant A_j with fitting coefficients $b_{\theta i}$ and $c_{\theta i}$. In the most complex scenario, assuming reciprocal perturbations in both parameters, the model (7) turns into the following:

$$R = 1 - \left[1 - W\left(A_1; K_1\nu_{k1}, m_1\nu_{m1}, a_1\right)\right]\left[1 - W\left(A_2; K_2\nu_{k2}, m_2\nu_{m2}, a_2\right)\right] \quad (9)$$

Expression (5) clarifies the meaning of an additional possible modification. Indeed, when the individual responses are denoted as W_i, it becomes evident that the W_1W_2 term of the joint response ($R=W_1+W_2-W_1W_2$) is associated with the hypothesis of statistical independence. Therefore, this term will be modified if this independence is altered by any global cooperative or competitive effect. Thus, a generalized *IA* model, in its most complex form, can be written as follows:

$$R = W\left(D_1; K_1\nu_{k1}, m_1\nu_{m1}, a_1\right) + W\left(D_2; K_2\nu_{k2}, m_2\nu_{m2}, a_2\right)\left[1 - s \times W\left(D_1; K_1\nu_{k1}, m_1\nu_{m1}, a_1\right)\right] \quad (10)$$

where the value of the coefficient *s* becomes greater or lesser than 1 depending on the predominance of competitive or cooperative effects, respectively. It should be noted that even though model (10) includes all the possible theoretical interactions, much simpler situations are normally found (because several $\nu_{\theta i}=1$).

(b) Concentration addition (CA)

The typical application of this hypothesis avoids the formulation of an explicit response surface model. This surface is indirectly analyzed through isoboles, or projections of equal response lines on the plane of the independent variables (Berenbaum, 1985a; 1985b; Sørensen et al., 2007; Vølund, 1992). Although the criteria used by Berenbaum to define the null interaction can also be used to formulate an explicit model (Murado et al., 2011), the response to a mixed dose of two agents can be postulated as the response of two fictitious "mixed" doses of the same agent as follows:

$$R = W\left[\left(A_1 + A_2\right); K, m, a\right] \quad (11)$$

Any interaction that is considered must preserve the key concept of the concentration addition, implying that the doses in equation (11) should act as an additive block within an algebraic expression with a single set of parameters (*K*, *m*, *a*). Accordingly, the possible perturbations are as described below:

- *Different antioxidant power*. The model is obtained by introducing a factor, *p*, to one of the doses (p<1 if the affected antioxidant is the most powerful):

$$R = W\left[\left(pA_1 + A_2\right); K, m, a\right] \quad (12)$$

Notably, this effect does not alter the condition of null interaction, and if a joint response can be described by the equation (12), the m_1 value of the individual response to A_1 is $m_1=m/p$.

- *Interactions modifying the effective dose.* If an antioxidant, A_1, reciprocally or non-reciprocally interacts with another A_2, in such a way that the effect of A_2 is equivalent to the effect due to an effective dose higher or lower than the nominal one, the different alternatives can be described by the following model, by using v_{0i} terms such as those (v_{Ai}) defined in (8):

$$R = W\left[\left(A_1\frac{1+b_1A_2}{1+c_1A_2} + A_2\frac{1+b_2A_1}{1+c_2A_1}\right); K, m, a\right] \quad (13)$$

- *Interactions modifying the sigmoidal parameters.* In general, the interactions in which each antioxidant specifically modifies the sigmoidal parameters (*K*, *m*, *a*) of the joint response can be considered according to a model as follows:

$$R = W\left[\left(A_1 + A_2\right); K\nu_{k1}\nu_{k2}, m\nu_{m1}\nu_{m2}, a\right] \quad (14)$$

Theoretically, this relationship implies that the individual responses increase non-asymptotically or decrease after a maximum (in the latter case with a similar profile to that produced by an enzymatic kinetic with substrate inhibition). Experimental evidence of this behavior has been found in the dose-response area (Cabo et al., 2000). However, either response is uncertain in the context of the interactive action of antioxidant agents. Nevertheless, the general model for *CA* in its more complex form is defined as follows:

$$R = W\left[\left(pA_1\nu_{A1} + A_2\nu_{A2}\right); K\nu_{k1}\nu_{k2}, m\nu_{m1}\nu_{m2}, a\right] \quad (15)$$

As noted with respect to the model (10), most practical situations should be resolved with simpler particular cases.

2.3. Numerical and statistical methods

2.3.1. ***Basic methods***

- *Fitting procedure:* simulated and experimental results were adjusted to the proposed models by non-linear least squares methods (quasi-Newton), using Solver complement.
- *Parametric estimations:* were performed by incorporating the 'SolverAid' macro (Prieto et al., 2012b; Prikler, 2009) for estimating the confidence intervals.
- *Model consistency tests*: student's *t* and Fisher's *F* tests, respectively, with α=0.05 in both cases.
- *Model selection criteria:* Because there were many possible combinations of parameters able to fit the combined effects of both antioxidants, a selection process needs to be applied to determine the model that best predicts the joint effect of the two variables in the interval studied. Therefore, different model selection criteria (MSC) were used to evaluate the appropriateness of the equations. For more details about the process of selection and its pitfalls a specific description is presented in the appendix section (Table A1 and Table A2).

2.3.2. ***Development of an automatic stepwise regression method for the analysis of the responses***

Although the initial number of parameter combinations (models with interactions (10) and (15)) is high, this number only signifies a high number of potential alternatives. The most complex cases that

were found involved a maximum of four interactive parameters plus those for the individual responses (6 and 4 for the *IA* and *CA*, respectively). However, in occasions when a large amount of data needs to be analyzed, the process of finding the most appropriate solution can be very laborious. Therefore, a stepwise regression method was developed by programming a routine in excel in which all possible parameter combinations are tested. The routine involved the following steps: 1) fitting the sigmoidal parameters from the individual responses (without interactions), using equation (7) and (11) for the *IA* and *CA* hypothesis, respectively; 2) these estimates were then used as the starting values for assaying all possible parameters combinations of the model (10) (*IA*, 9 parameters and 511 combinations) and (15) (*CA*, 13 parameters and 8.191 combinations); 3) rejecting the options that lead at least to a none statistically significant coefficient; and 4) selecting the most remarkable solutions, which are automatically ranked with several model selection criteria to differentiate the most "true solution".

3. RESULTS

3.1. Meaning of the synergy and antagonism notions

Once the equations (10) and (15) were accepted as generalized models for *IA* and *CA* hypotheses, respectively, an algebraic framework was established that characterizes synergy and antagonism through the specific variations imposed by the perturbations to the parameters and the response.

In a broad sense, an interaction is synergistic or antagonistic as it increases or decreases the expected response in the null interaction. In the *IA* model (10), a synergistic interaction raises at least one K_i parameter, reduces at least one m_i, reduces the *s* coefficient or imposes all these effects simultaneously, while antagonism determines the opposite effects. In the *CA* model (15), synergy and antagonism are translated into changes of the effective concentrations according to the equation (13), as well as, into variations of *K* and *m* parameters as in the *IA* model, at least theoretically. Notably, the modification of the effective concentration according to equation (13) is not mathematically possible in the *IA* model, in which the corresponding effect must be translated into variations of m_i parameters.

These definitions may be further restricted if the conventional analysis applied in toxicology to *CA* model is accepted. As already mentioned, this model is assessed by the isobole examination, accepting that straight, concave up and convex up isoboles indicate a null interaction, synergy and antagonism, respectively. Because this behavior only occurs in the perturbations described by the equation (13), synergy and antagonism could be limited to the interactions modifying the effective concentrations.

However, this restriction does not logically follow for two reasons: 1) other effects may increase or decrease the response corresponding to the null interaction without altering the effective concentration, and these effects should not be excluded from the synergy and antagonism definitions; 2) the isobole approach is only applicable in the context of the *CA* hypothesis, specifically, in cases that can be described by the equation (13). In fact, the complexity of the isoboles in the *IA* hypothesis prevents the use of the simple criterion of their concavity or convexity (cita Murado, PlosOne). Therefore, the concepts of synergy and antagonism will be used according to the broad sense defined before.

3.2. A step by step example of the methodological process

The methodological procedure and the mathematical models proposed in the previous sections yielded consistent results when combining all the antioxidants listed in the materials section for each of the methods. These results not only permitted the decision between the null interaction, synergy and antagonism, but also revealed some interesting aspects of the system used and the approach applied. To illustrate the methodological procedure of this approach, the joint action of TOC (A_1) and BHA (A_2) on the bleaching reaction of β-carotene will be described in detail (Figure 2, Figure 3 and Table 1).

3.2.1. ***Procedure to obtain the RAU values***

Figure 2 shows the procedure to obtain the *RAU* responses using the TOC (A_1) and BHA (A_2) antioxidant combination in the β-carotene reaction as an example. Figure 2A shows the remaining raw responses of the substrate (SH, in this case β-carotene in μM) for the reaction in the presence of TOC and BHA. In each single graph of the 8×8 array, the top line shows the response for the control, the bottom line shows the response for the corresponding combination of antioxidants and the shadow area shows the *RUA* values. In Figure 2B presents the obtained *RUA* data first in two separated 2D graphs that show the response in a non-standardized form as the individual effects caused for each antioxidant, and then as the response and antioxidant doses that are standardized to a scale of [0,1] presented in a single 3D graph.

Once the *RAU* responses were obtained, the modeling procedure to determine, characterize and quantify the interactive effects could be started. The procedure will be performed in different ways, first by analyzing intuitively the possible modes of action with and without interactions and afterwards applying the automatic stepwise regression method developed. The findings below demonstrate that both criteria converge into identical solutions.

3.2.2. ***Intuitive analysis of the hypothetical modes of action with and without interactions***

(a) TOC (A1) and BHA (A2) assuming independent action (IA)

The null interaction in the *IA* hypothesis implies that the joint action should be described by adjusting the individual responses to the model (7) and using the obtained parameters in the model (10), with all $v_{0i}=1$ ($b_{0i}=c_{0i}=0$) and $s=1$. By proceeding in this way, the r^2 and R^2_{adj} values, as well as the Student's t and Fisher's F test (both with $\alpha=0.05$) applied to the parametric estimations and to the explained variance, respectively, showed a statistically acceptable fit (Figure 3 and Table 1). However, the distribution between the observed and predicted results (OP) was biased, and the residuals showed that the computed response surface predicts lower values than those experimentally obtained, which suggests a synergistic interaction.

Indeed, a decrease in the OP bias and an improvement in the other fitting criteria were obtained by accepting a drop in the m parameter of the response to TOC due to the presence of BHA (increasing antioxidant potency: synergy in the strict sense). A further improvement could be obtained by accepting a similar drop in the K parameter (antagonism in the broad sense, less strong than the synergistic effect). Although the interactions producing simultaneous opposite effects on the response are in general neither formal nor mechanistically rejectable, the predicted individual responses in this case are statistically less correct than those corresponding to the simpler hypothesis of synergy. The decision would probably be clearer by slightly expanding the experimental domain, which could more precisely define the asymptotes of the individual responses. Nevertheless, the net effect of the interaction between TOC and BHA is synergistic.

(b) TOC (A1) and BHA (A2) assuming concentration addition (CA)

Under the *CA* hypothesis, the null interaction requires to set all $v_{Ai}=v_{0i}=1$ in model (15) . When the relative potency coefficient ($p\neq1$) was included under these conditions, the model produced a statistically significant description (r^2, R^2_{adj}, t and F). However, a biased OP distribution and residuals indicating a general underestimation of the predicted response with respect to the experimental results were again obtained (Figure 3 and Table 1). All fitting criteria improved significantly when a synergistic effect (strict sense) was included (one of the antioxidants increases the effective concentration of the other, a situation in which the *CA* model cannot distinguish directionality). If a hyperbolic variation of the effective concentrations was assumed, the fit was slightly higher than that corresponding to a linear variation. However, the correlation between the coefficients of the perturbation term penalizes their confidence intervals, which confines such coefficients close to the lack of statistical significance.

After considering these intuitive options, the description obtained by supposing linear variations of the effective doses under *CA* hypothesis was more accurate than those found under *IA* alternative. Therefore, it must be concluded that the model of the joint response to TOC and BHA obeys the CA mode with synergistic interaction.

3.2.3. ***Automatic analysis by a stepwise regression method***

When a large set of data needs to be analyzed, the intuitively process of finding the most appropriate solution can be very laborious. Therefore, an automatic procedure that integrates a set of statistical MSC to rank and selected the most appropriate solution has been developed by programming a routine in excel in which all possible parameter combinations were tested. For example, the top fitting results of TOC and BHA case for each mode of action after applying this automatic system to evaluate the results are shown in Table A2 (appendix section). The data demonstrates that the overall best model was the *CA* hypotheses, and within this hypothesis the case number 4 predicts accurately the data, being the most likely response to be correct. This selection was identical to that intuitively found above, which demonstrates the reliability of both ways for selecting the correct solution. However, because the automatic system is undoubtedly faster and reliable, it was the procedure used to assess all pairs of tested antioxidants.

3.3. Other findings drawn from the analysis of the joint action

Certainly, the solution can be directly determined in the majority of the cases analyzed. However, both models provide equal satisfactory results in occasions, such as when the solution is a continuum or mixed response of both hypotheses showing difficulties to choose any as the correct solution. This ambiguity has also been found in a recent and extensive revision (158 items) of experimental results carried out by Cedergreen et al. (2008). The consequences of our approach agree with those findings.

In addition to the statistically consistent detection of the interactive effects, for example, the analysis of TOC and BHA raises a more basic question related to the status of *IA* and *CA* hypotheses. The experimental results were better described by *CA* than by *IA* hypotheses. TOC and BHA could be accepted that act at the same point of a general oxidative pathway as summarized in Figure 1. Irrespective of this comparison, the description under *IA* hypothesis could not be rejected by applying common statistical criteria, which implies that the antioxidants act at different points of that pathway.

This ambiguity can be explained in terms of the relationships between the rate constants involved in the reaction sequence of the mentioned pathway. Considering Figure 1, it can be admitted that if the activity of the antioxidant act only through the k_1 and k_4 (k_2=k_3=0), or k_2 and k_3 (k_1=k_4=0) pathways, the model is *IA*, and if only acts through the k_1 and k_3 (k_2=k_4=0), or k_2 and k_4 (k_1=k_3=0) pathways, the model is *CA*. However, other less extreme situations clearly take place in which none of the rate constants equal zero. If the pathways k_1 and k_4 or k_2 and k_3 are simply dominant (k_1>k_2 and k_4>k_3, or k_1<k_2 and k_4<k_3), the model will be predominantly *IA*; and if the dominant mechanisms are k_1 and k_3 or k_2 and k_4 (k_1>k_2 and k_3>k_4, or k_1<k_2 and k_3<k_4), the model will be predominantly *CA*. Different mechanism of each antioxidant in the convergence points 1 and 2 of Figure 1 also serve as contributions to ambiguity.

Therefore, the joint antioxidant effect of TOC and BHA on the linoleic acid/β-carotene system strongly suggests a predominant action on the same point of the oxidative sequence, without implying that the antioxidants act only at one point.

A further achievement of these results is the presentation of the *IA* and *CA* hypotheses as the two ends of the same continuum, contrary to their usual presentation as mutually exclusive options. At the extremes, only one of the hypotheses will produce a statistically acceptable result; cases in which both

hypotheses are consistent exist within the continuum. In this case, the selection of the hypothesis that provides the best solution does not imply a lack of contribution of the other hypothesis.

If the relatively simple context of an *in vitro* antioxidant action provides ambiguous cases determined by the relationships between reaction rates of a schematic sequence, similar ambiguities necessarily and probably arise in the field of *in vivo* dose-response relationships (Murado et al., 2011). This last field has been noted (Jonker et al. 2005) to provide cases that do not follow any of the two classical hypotheses. We believe that the preceding results explain why reality exhibits cases in which both hypotheses are simultaneously obeyed.

3.4. Other examples of joint action that illustrate important aspects

By applying the methodological procedure to the joint action of several pairs of antioxidants, all solutions were described by one of the models or by both. However, not less important issues are occasionally found. Next, some of these aspects are confronted and discussed in detail.

3.4.1. ***Need for additional criteria to assist the selection process: AA (A_1) and ETX (A_2) in the crocin reaction***

This is a typical case in which the selection of the mode of action it was less questionable, but the selection of the interactive effects was complex, and it needs a deeper analysis that uses intuitive criteria to select the most correct solution. As in the case of TOC and BHA, the null interaction was not acceptable in any of the two modes of action, and the residuals suggested a synergistic effect. Contrary to what has been found in the previous case, the worst fitting solutions were obtained with *CA* model (15), and the best ones were provided by the *IA* model (10), in which three preliminarily acceptable possibilities can be found:

a) A_2 reduces the parameter m of the response to A_1 (synergy in the strict sense).
b) A_2 increases the parameter K of the response to A_1 (synergy in the broad sense).
c) Generic cooperative action ($s<1$) between antioxidants (synergy in the broad sense).

The confidence intervals (CI) of parameters yield to prefer the *a* option rather than the other two strict sense synergistic forms (unidirectional opposite and reciprocal), and the OP and R^2_{adj} criteria allowed the rejection of option *b*. The decision between *a* and *c* was uncertain, because *c* produces a better fit, but it generates an excessive effect, which produces responses higher than 1.03 in a small subdomain of simultaneous high concentrations of both antioxidants. Although the subdomain and deviation are of scarce importance, the less global option, *a*, does not create this problem, narrows the confidence intervals (CI) and reduces the fitting only slightly. Due to any combination of *a*, *b* and *c* did not produce acceptable results, option *a* seemed to be finally the best solution.

In other words, the results indicated a *predominantly* independent action that was clearly synergistic, which suggests the following: 1) at least one of the antioxidants acts at two different points in the oxidation sequence of the crocin reaction; 2) at one of these points, the action of the other antioxidant can be neglected; and 3) at the other point, where both antioxidants act through the same mechanism by adding their concentrations, the antioxidant effect is poor.

3.4.2. ***Antagonistic effects in the framework of the antioxidant action: Mn (A_1) and AA (A_2) in crocin reaction***

One important question in the join action of two or more antioxidants is related to the possibility of obtaining combinatory responses lower than the expected responses of their individual effects, or in other words antagonistic effects. The interactive activity between Mn (A_1) and AA (A_2) in the oxidation of crocin is a clear example of such a case. When both antioxidants are tested independently, they show a clear antioxidant character. However, when combined, the Mn significantly depressed the

effect of AA, which continuously decreased the maximum response (the parameter K_2). The joint response could be broadly described in a statistically significant way as an *IA* case with an antagonistic effect.

If transferable to real systems, such as the preservation of food and beverages in hydrophilic surroundings, these results indicate that the presence of Mn, a typical compound in plants, will diminish the activity AA, a typical antioxidant in hydrophilic environments, which would reduce the expected life of the system.

3.4.3. ***Cooperative effects: AA (A_1) and TRO (A_2) in crocin reaction***

Both models (*IA* and *CA*) indicated a synergistic (strict sense) joint response, with statistically higher results for the *IA* option. In this case, the fit improves if the synergy is complemented by a slight generic cooperative effect (s<1), which defines the response between AA and TRO as *predominantly IA* with antagonistic effects. As in the previous case, these results would indicate significant issues in different disciplines of food science in real systems.

3.4.4. ***Low response effects for one of the agents: TRO (A1) and BHT (A2) in β-carotene reaction***

This case represents an example of the "polar paradox" (Frankel et al., 1994; 2005; Koleva et al., 2002; Porter, 1993), a typical phenomenon in lipid emulsion systems, such as the β-carotene reaction. It favors the activity of the non-polar antioxidants over the activity of polar antioxidants, because the hydrophobic repulsion tends to concentrate the first non-polar antioxidants (*i.e.*, BHT), but not the polar ones (*i.e.*, Trolox) in the lipid environment where the oxidation occurs. In fact, the Trolox activity was very low in the concentration domain tested, showing a linear profile, an imperceptible contribution to the joint response at high levels of BHT. This linear relationship causes linear correlations between the coefficients of the perturbation terms (v_{0i}) and penalizes the CI of the parametric estimations, which increases as the experimental error increases and as the number of observations decreases. This low response is one weakness of models (10) and (15). Fortunately, accurate data are effortlessly obtained by working with microplate readers, and both problems are thus minimized.

The description of the system was statistically significant assuming *IA* when BHT reduces the m parameter of the response to Trolox. Therefore, the joint response was broadly typified as an independent action case with an antagonistic effect.

3.5. In search of a comprehensive index

If a single numerical value that summarizes the nature and the intensity of the synergistic or antagonistic interactions could be proposed, this clearly would help and become a useful index in different scientific fields. Once an explicit algebraic model for a response surface is settled, the definition of such an index seems to require only a comparison between the response corresponding to the null interaction hypothesis and the experimentally obtained response. The usefulness of this approach from a theoretical and practical perspective is questionable. In fact, neither the difference nor the quotient between the typical responses in null interactions and any interactive situation remains constant throughout the domain of the independent variables (see Figure 5). Thus, any index that is calculated at a specific point (*e.g.* for $A_1=m_1$ and $A_2=m_2$), or along a specific response (*e.g.* the half-maximal response), cannot account what happens in another region of the response surface. This fact is true even in a simple case as $s\neq1$ in model (10), and specific situations can exist (as opposite variations in K_i and m_i parameters) in which the net effect is synergistic in one subdomain of the response surface and antagonistic in another one.

However, in an effort to find a comprehensive index, the best alternative to summarize such a response could be to compute the percentage relative unit of volume (*RUV*) between the volume of the surface

produced by the null interaction (SV_{NI}) and the volume of the surface with interactions (SV_I) as follows:

$$RUV = \frac{SV_I - SV_{NI}}{SV_I} \times 100 \text{ ; being } SV = h_i h_j \sum_{i=0}^{n} \sum_{j=0}^{m} f\left(A_i A_j\right) \phi_{i,j} \tag{16}$$

in which A_i and A_j are the dependent variables that represent the n and m concentration of both antioxidants, h_i and h_j are the concentration interval sets and $\Phi_{i,j}$ is the product of the nested composite trapezoidal rule coefficients. Therefore, positive and negative values of *RUV* will describe the predominantly synergistic and antagonistic interaction effects between the antioxidants over the study range.

The variations in the parametric values of the response to an antioxidant as a function of the concentration of the other antioxidant (the structures of the perturbation terms) or the global approach of computing the *RUV* allow a brief reasonable description of the interactive effects. However, because the datum of practical interest is the possible difference between the null interaction and the experimental result in a given domain, only the "scenery" of these differences throughout the experimental domain allows effective and statistically founded statements.

The results for the *RUV* obtained for all 42 cases assessed are presented in Table 3. The full analysis of all the possible combinations is presented in the appendix (Figure A2, Figure A3, Table A3 and Table A4).

4. DISCUSSION

Synergy and antagonism are controversial characteristic behaviors of very diverse systems. Despite their importance, the common characterization of these phenomena in the context of the antioxidant action is often questionable due to some problematic definitions and the type of data used. The models proposed here showed a good ability to describe the joint action of several pairs of antioxidant under both aqueous and lipid emulsions. Their application allows to: 1) typify a joint antioxidant activity in terms of the two modes of joint action accepted in the field of dose-response relationships; and 2) led to the detection and quantification of synergistic and antagonistic effects by comparing, for each mode of action, the fitting of the experimental results to several formal models by describing different interaction scenarios, including null interaction.

Additionally, the results have proven that: 1) when synergy and antagonism are defined in the broad sense as interactions increasing or decreasing the response corresponding to the null interaction, several modalities of those effects arise, depending on the mode of action considered and the parameters of the response to an antioxidant which are modified by the presence of the other one; 2) synergistic and antagonistic consequences can vary along the response surface, even effects with the opposite signs in different subdomains of that surface are produced; 3) insofar as independent action and concentration addition models define –in very general terms– mechanisms, it is possible to connect the different forms of the models (10) and (15) with equally general aspects of the mechanisms involved in the oxidative pathways; 4) under this last perspective, *IA* and *CA* hypotheses arise –in opposition to the common idea of mutually exclusive possibilities– as the two extremes of a continuum. Such a continuum is characterized by the sites in which a given oxidative pathway is inhibited, and the relations between the rate constants of the inhibitory reactions (Figure 1).

5. CONCLUSIONS

In this paper, a methodological procedure has been developed for the joint action of several pairs of antioxidants in both aqueous and lipid emulsions, which enables the determination and quantification of the synergistic and antagonistic interactive effects. Although the approach could be directly

expanded to other types of classical antioxidant methods, the methods selected are fairly representative of the most complex scenarios that can be found in the oxidation process. Unfortunately, the proposed approach is a little more complex than some relatively common solutions appearing in the bibliography. However, we believe that it is free of the most controversial aspects of such solutions.

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FIGURES

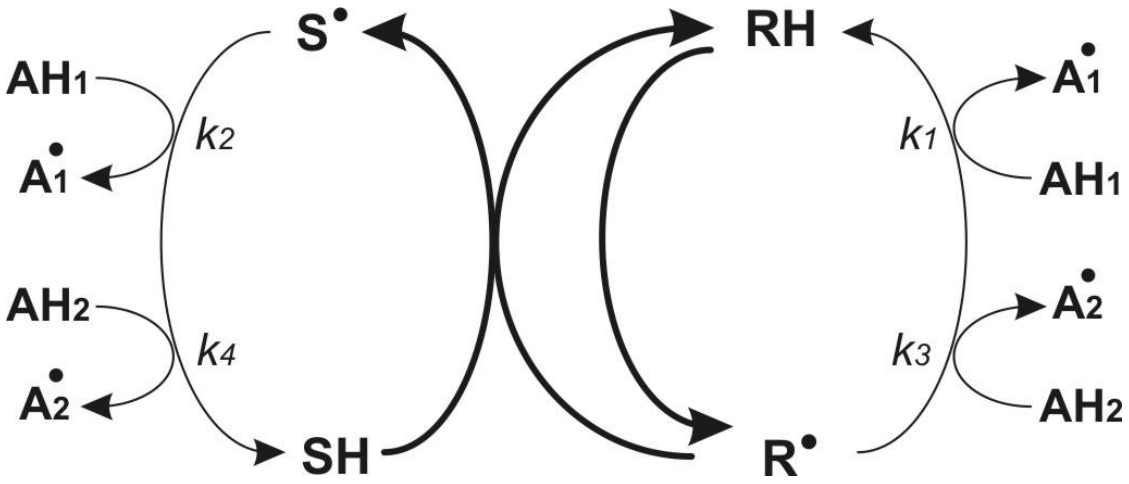

Figure 1: Oxidation of a SH substrate by R• radicals from a RH source, in the presence of AH_1 and AH_2 antioxidants. Reactions k_1 to k_4 hinder the main pathway (tick lines). It is supposed that reactions k_1 and k_2 have the same mechanism, which is different of the one of the reactions k_3 and k_4. Under these conditions, the appropriate model for the antioxidant joint action depends on the relative values of the rate constants k_1 to k_4 (see text).

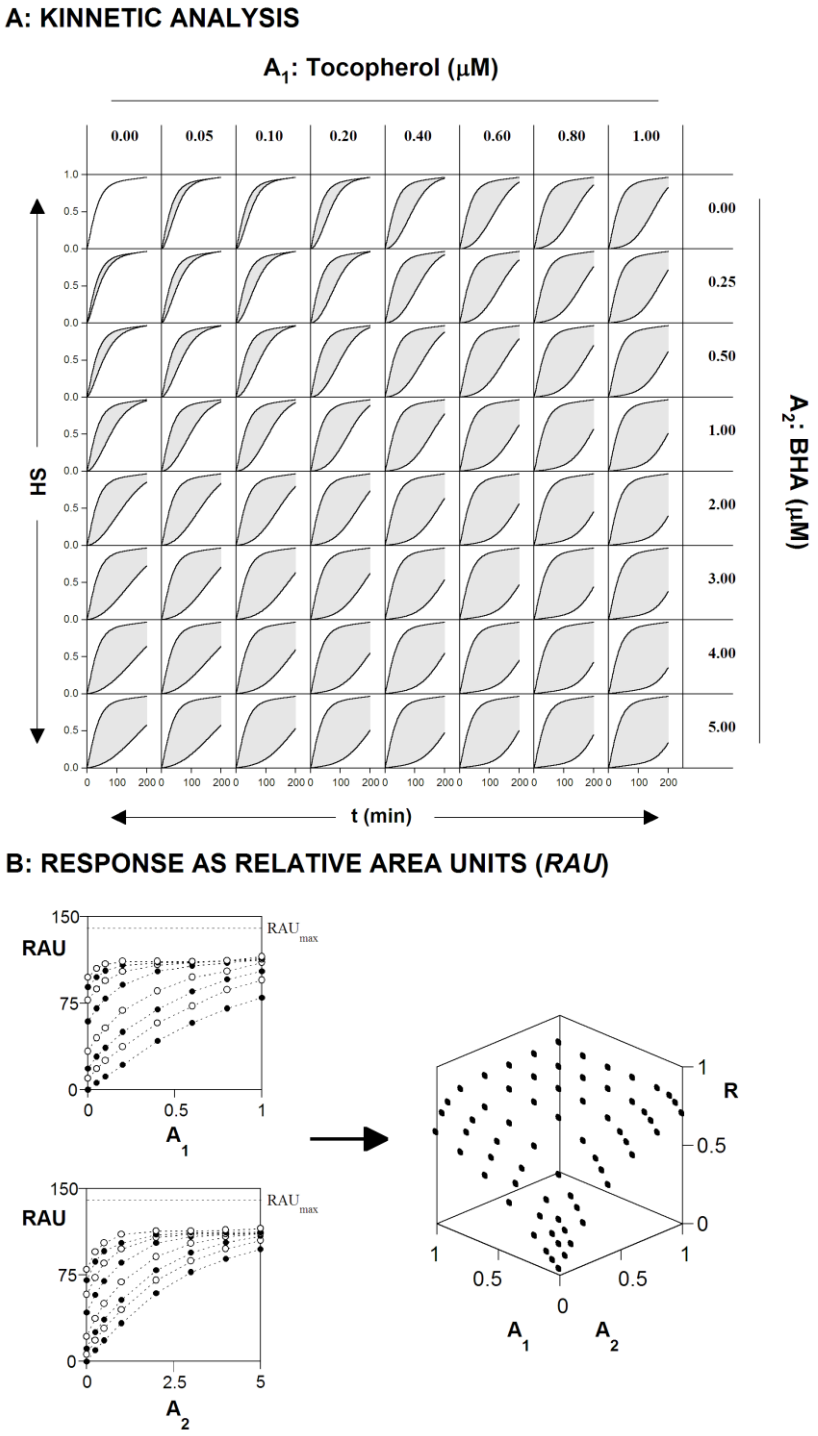

Figure 2: A descriptive example performed in stepwise mode, to show the process of obtaining the *RAU* responses. *A*: raw responses as remaining substrate (HS, in this case β-carotene in μM) of the reaction in the presence of TOC (A_1) and BHA (A_2). In each single graph for the 8×8 array, the top line shows the response for the control, the bottom line the response for the corresponding combination of antioxidants and the shadow area the *RUA* values. *B*: The obtained *RUA* data is presented, first, in two 2D graphs with non-standardized response, showing the individual effects caused for each antioxidant. Afterwards, response and antioxidant doses are standardized to a scale [0,1] and presented in a single 3D graph.

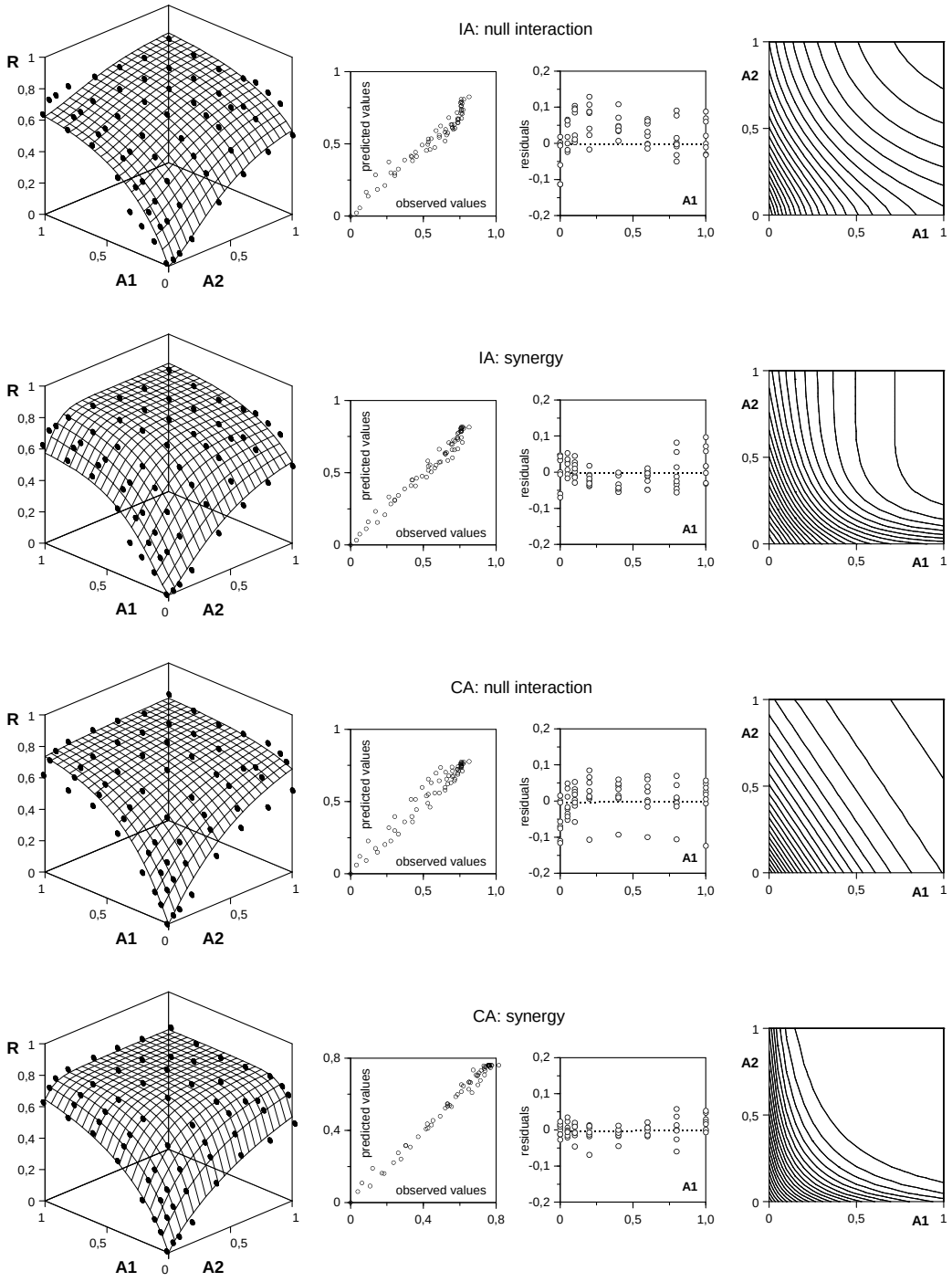

Figure 3: Joint effect of TOC (A_1) and BHA (A_2) on β-carotene oxidation under different hypotheses. Experimental results (points) and fittings to the models (10) and (15) (surfaces). Correlations between observed and predicted values, residuals and isobole projections of the response surfaces are also shown. See text for details. Numerical results in Table 1.

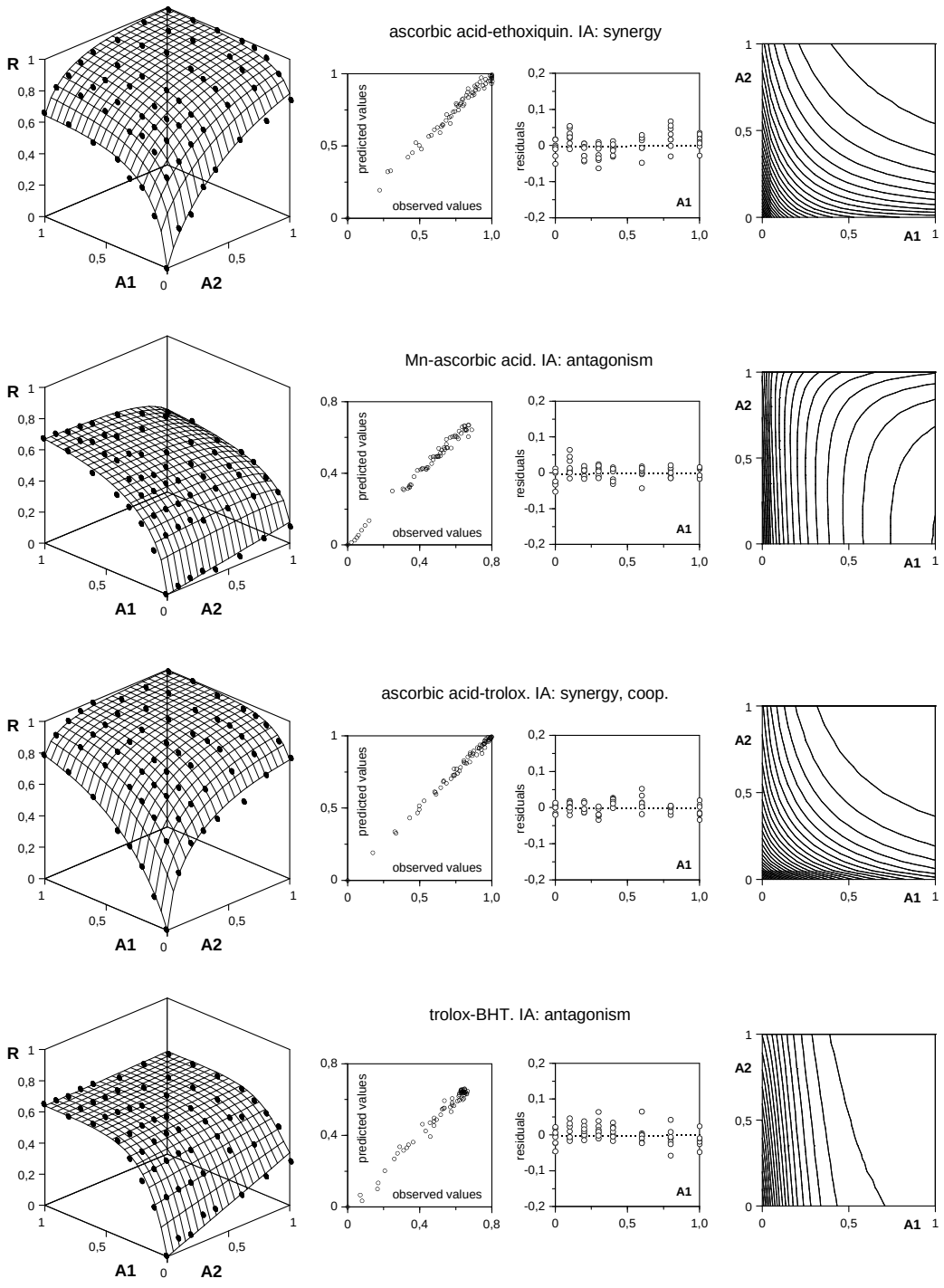

Figure 4: Characterization of the joint effect of the specified antioxidant pairs, using β-carotene (Trolox-BHT) and crocin (the rest) reactions. Graphic criteria and notations as in Figure 3. In ascorbic acid-Trolox, *coop*. means general cooperative action ($s<1$ in equation (10)). See text for details. Numerical results in Table 2.

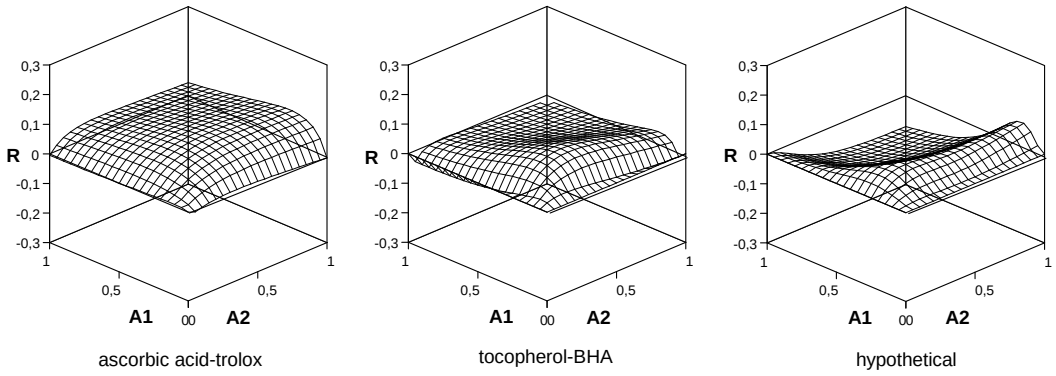

Figure 5: Differences between best-fit and null interaction responses in the specified cases. Hypothetical example was obtained by assuming independent action, with the following parametric values: K_1=K_2=0.7; m_1=m_2=0.25; a_1=a_2=1.5; c_{2m}=2; c_{1k}=1.

TABLES

Table 1: Joint action of TOC (A_1) and BHA (A_2) on β-carotene oxidation. The null interaction and synergy hypotheses are compared under the independent action and addition concentration suppositions, by fitting the experimental results to the (10) and (15) generalized models. θ: parametric estimations; *CI%*: confidence intervals (α=0.05) as % of the parametric estimations; R^2: adjusted coefficient of multiple determination. See Figure 3 and text for details.

independent action						**concentration addition**					
		null interaction		synergy				null interaction		synergy	
response to A_1	K_1	0.576±29.5		0.566±7.2		joint response	K	0.785±6.1		0.761±1.6	
	m_1	0.388±38.0		0.362±18.7			m	0.263±16.0		0.326±8.4	
	a_1	1.403±44.9		1.237±21.0			a	1.033±15.0		0.895±6.6	
response to A_2	K_2	0.677±22.2		0.589±10.7		relative potency	p	0.667±17.9		0.609±11.4	
	m_2	0.256±38.4		0.259±16.5		A_1 altering eff. conc. of A_2	b_{2D}	-	-	-	-
	a_2	0.958±36.2		1.244±23.2			c_{2D}	-	-	-	-
A_1 as perturbing factor for params. of the response to A_2	b_{2k}	-	-	-	-	A_2 altering eff. conc. of A_1	b_{1D}	-	-	12.24±22.8	
	c_{2k}	-	-	-	-		c_{1D}	-	-	-	-
	b_{2m}	-	-	-	-	A_1 as perturbing factor for params. of the joint response	b_{2k}	-	-	-	-
	c_{2m}	-	-	-	-		c_{2k}	-	-	-	-
A_2 as perturbing factor for params. of the response to A_1	b_{1k}	-	-	-	-		b_{2m}	-	-	-	-
	c_{1k}	-	-	-	-		c_{2m}	-	-	-	-
	b_{1m}	-	-	-	-	A_2 as perturbing factor for params. of the joint response	b_{1k}	-	-	-	-
	c_{1m}	-	-	4.006±44.5			c_{1k}	-	-	-	-
comp / coop	s	-	-	-	-		b_{1m}	-	-	-	-
							c_{1m}	-	-	-	-
	$\mathbf{R^2_{adj}}$	0.9139		0.9693			$\mathbf{R^2_{adj}}$	0.9411		0.9907	

1

Table 2: Parametric estimations for the joint action of the following particular cases. A_1 and A_2 are the first and second element, respectively, of each pair. Other notations as in Table 1. See Figure 4 and text for details.

A_1: **A_2:**		**AA** **ETX**	**AA** **Mn**	**AA** **TRO**	**BHT** **TRO**
response to A_1	K_1	0.700±39.1	0.568±13.2	0.668±16.2	0.597±22.1
	m_1	0.139±21.5	0.100±21.2	0.105±16.4	0.881±28.4
	a_1	0868±14.9	0.899±23.4	0.884±15.1	0.956±16.6
response to A_2	K_2	0.657±22.2	0.614±5.2	0.830±17.5	0.660±21.3
	m_2	0.100±19.2	0.305±14.1	0.153±16.2	0.117±21.6
	a_2	0.759±32.1	1.005±23.2	0.668±16.6	1.068±18.3
A_1 as perturbing factor for params. of the response to A_2	b_{2k}	-	-0.836	-	-
	c_{2k}	-	-0.759	-	0.314±8.8
	b_{2m}	-	-	-	-
	c_{2m}	-	-	-	-
A_2 as perturbing factor for params. of the response to A_1	b_{1k}	-	-	-	-
	c_{1k}	-	-	-	-
	b_{1m}	-	-	-	-
	c_{1m}	2.115±11.1	-	2.621±6.6	-
comp / coop	s	-	-	0.980±1.6	-
	$\mathbf{R^2_{adj}}$	0.9807	0.9876	0.9932	0.9942

2

Table 3: Effect of the combination of 42 different pairs of antioxidants for each reaction. In the cases one antioxidant is combined with itself is used simply as a control. For each case the *RUV* (%) is presented. Note, that the underline combinations are those that have been analyzed in detail in the text.

A: β-CAROTENE REACTION (LIPOPHILIC)

		BHA	TRO	TOC	ETO	PG	BHT
(0-5 µM)	BHA	*NI-CA (0.0%)*	*S-CA (12.2%)*	*S-CA (3.5%)*	*S-IA (2.4%)*	*A-IA (-9.6%)*	*S- IA (1.6%)*
(0-300 µM)	TRO	-	*NI-CA (0.0%)*	*A-IA (-0.5%)*	*A-IA (-3.7%)*	*A-CA (-2.1%)*	*A-IA (-11.7%)*
(0-1 µM)	TOC	-	-	*NI-CA (0.0%)*	*S-IA (5.3%)*	*S-CA (1.3%)*	*S- IA (1.9%)*
(0-2 nM)	ETO	-	-	-	*NI-CA (0.0%)*	*A-CA (-4.8%)*	*S- IA (20.3%)*
(0-80 µM)	PG	-	-	-	-	*NI-CA (0.0%)*	*S- IA (0.3%)*
(0-30 µM)	BHT	-	-	-	-	-	*NI-CA (0.0%)*

B: CROCIN REACTION (HYDROPHILIC)

		BHA	TRO	ETO	Mn	PG	AA
(0-350 µM)	BHA	*NI-CA (0.0%)*	*A-CA (-2.9%)*	*A-CA (-3.5%)*	*S-IA (6.3%)*	*S-CA (2.2%)*	*S-IA (0.7%)*
(0-150 µM)	TRO	-	*NI-CA (0.0%)*	*A-IA (-6.1%)*	*S-CA (5.8%)*	*S-IA (4.3%)*	*S-IA (7.7%)*
(0-60 µM)	ETO	-	-	*NI-CA (0.0%)*	*S-IA (7.3%)*	*S-IA (4.5%)*	*S-IA (9.4%)*
(0-10 µM)	Mn	-	-	-	*NI-CA (0.0%)*	*S-CA (4.7%)*	*A-IA (-4.2%)*
(0-300 µM)	PG	-	-	-	-	*NI-CA (0.0%)*	*S-CA (1.8%)*
(0-400 µM)	AA	-	-	-	-	-	*NI-CA (0.0%)*

NI: Null interaction / *S*: Synergy / *A*: Antagonism / *IA*: Independent action / *CA*: Concentration addition

APPENDIX SECTION

1. Model selection criteria.

In order to assist us select the best model, we have used different model selection criteria (MSC) to evaluate the multivariable fit and explanatory appropriateness of the equations. In the present work, the AIC, AICc, BIC, RIC, Cp, R^2_{adj}, FPE, and MSlC criteria (Table A1) were obtained directly using an Excel spreadsheet. The usefulness of MSC to choose the best solution and model is well-documented (Rivers & Vuong, 2002). A model should be complex enough to extract the regularities in data, but simple enough not to overfit it and thereby reduce predictiveness. MSC adjust the goodness of fit in order to penalize model complexity, overfitting and lack of generalizability. Currently, there are a variety of MSC available (Forster, 2000; Myung & Pitt, 2004), but there is no one criterion that can lead to a perfect choice (Roland T. Rust, Simester, Brodie, & Nilikant, 1995).

If the above solutions do no solve completely the selection, other criteria more intuitively can be used, such as the asymmetric, kurtosis and distribution of the residuals. The residuals should be randomly scattered around zero to avoid autocorrelation (Roland T. Rust et al., 1995). These residuals should not be grouped and should not increase or decrease as a function of the independent variables. Hereafter, we will call OP the point's distribution that correlates, with a coefficient r^2, observed and predicted results, and R^2 the adjusted coefficient of multiple determination.

Model selection criteria help to differentiate the most "true solution". In general, all statistical MSC merge into similar solutions. Such a conclusion, can be explained, because once the solutions that do not present significant parameters are excluded, any of the MSC presented will solve similar and precisely the selection most appropriate.

In Table A2, an illustrative summary of the application of the different MSC used to evaluate the results obtained for the case study of BHA and tocopherol presented in the manuscript is shown.

Table A1: Comparison of different model selection criteria (MSC) typically used to compare the models based in their complexity, goodness of fit, overfitting providing criteria to choose the most "true" solution. n: number of independent measurements considered in the fit. k: number of fitted parameters. RSS: residual sum of squares. ESS: explained sum of squares.

Criterion	Ranking	Claim	Formula	Additional information	References
Akaike Information Criterion (AIC)	Smaller value	complexity (efficient)	$AIC = n\ln\left(\frac{RSS}{n}\right)+2k$	It favors models with many variables.	(Gang & George, 1988; Shi & Tsai, 2002)
Akaike Information Criterion Corrected (AICc)	Smaller value	complexity (efficient)	$AIC_C = n\ln\left(\frac{RSS}{n}\right)+\left(\frac{2(k+1)}{n-k-2}\right)$	It favors models with many variables, but penalizes the complexity of the models in larger way than the AIC.	(Gang & George, 1988; Shi & Tsai, 2002)
The Schwartz or Bayesan Information Criterion (BIC or SIC)	Smaller value	complexity (consistent)	$BIC = n\ln(RSS)+\ln(n)k$	The BIC is Bayesian because it is designed as an index of the evidence in favor of a given model being "true".	(Schwarz, 1978)
Akaike's Final Prediction Error (FPE)	Smaller value	goodness of fit	$FPE = n\frac{RSS(n+k)}{(n-k)}$		(Shi & Tsai, 2002)
Mallows' criteria (Cp)	Smaller value	goodness of fit / overfitting	$C_p = n\left[RSS/(ESS/n-1)\right]-n+2k$		(Gang & George, 1988; Shi & Tsai, 2002)
Adjusted Coefficient of determination (R^2_{adj})	Highest value	goodness of fit / complexity	$R^2_{adj} = \frac{(n-1)R^2-k}{n-1-k}$	The proposed adjusted coefficients correct the overestimation problem of the unadjusted coefficients.	(Shi & Tsai, 2002)
Residual Information Criterion (RIC)	Smaller value	goodness of fit / overfitting	$RIC = (n-k)\ln(RSS)+k\left[\ln(n)-1\right]+\frac{4}{n-k-2}$	Performs well except when the sample size is small and the signal-to-noise ratio is weak. RIC's large penalty function allows it to perform better than BIC.	(Shi & Tsai, 2002)
Model Selection Criterion (MSlC)	Highest value	goodness of fit	$MSC = \ln\left(\frac{ESS}{RSS}\right)-\frac{2k}{n}$		(Schwarz, 1978)

Table A2: Model ranking (Rk) obtained for each MSC for the TOC and BHA case (β-carotene bleaching reaction). Two different rankings are shown, one taking into account the results of both modes of action, and another (in brackets) that only considers the results for each hypothesis. For each mode of action the C-1 is the statistical results found for the null interaction, and the other four cases are the top cases that best fit the joint action of TOC and BHA.

CASES		STATISTICS					MODEL SELECTION CRITERIA													
		k	RSS	R^2_{adj}	ESS	S^2	AIC		AICc		BIC		FPE		R^2_{adj}		RIC		Cp		MSC	
							Value	Rk	Value	Rk	Value	Rk	Value	Rk	Value	Rk	Value	Rk	Value	Rk	Value	Rk
IA	**C-1**	6	0.310	0.9139	3.20	0.0409	-353.9	*10(5)*	-365.7	*10(5)*	-74.8	*10(5)*	16.25	*10(5)*	0.9455	*9(5)*	-71.4	*10(5)*	261.7	*10(5)*	2.37	*10(5)*
	C-2	6	0.120	0.9627	2.80	0.0445	-389.9	*8(4)*	-401.6	*8(4)*	-110.8	*7(3)*	9.27	*8(4)*	0.9588	*8(4)*	-104.0	*7(3)*	120.6	*8(4)*	2.96	*7(3)*
	C-3	7	0.085	0.9628	3.14	0.0499	-409.3	*6(2)*	-423.0	*6(2)*	-128.0	*6(2)*	6.84	*6(2)*	0.9694	*6(2)*	-117.8	*6(2)*	60.2	*6(2)*	3.38	*6(2)*
	C-4	8	0.110	0.9667	2.73	0.0435	-391.3	*7(3)*	-406.9	*7(3)*	-107.8	*8(4)*	9.08	*7(3)*	0.9618	*7(3)*	-98.1	*8(4)*	114.4	*7(3)*	2.96	*8(4)*
	C-5	8	0.080	0.9693	2.76	0.0486	-474.0	*4(**1**)*	-489.6	*4(**1**)*	-190.5	*5(**1**)*	2.49	*4(**1**)*	0.9888	*4(**1**)*	-170.5	*5(**1**)*	-8.1	*5(**1**)*	4.37	*4(**1**)*
CA	**C-1**	4	0.165	0.9411	2.91	0.0463	-373.3	*9(5)*	-381.1	*9(5)*	-98.5	*9(5)*	12.00	*9(5)*	0.9431	*10(5)*	-95.2	*9(5)*	172.6	*9(5)*	2.75	*9(5)*
	C-2	6	0.029	0.9906	3.02	0.0481	-479.6	*2(2)*	-491.3	*2(2)*	-200.5	*2(2)*	2.28	*2(2)*	0.9896	*2(2)*	-185.3	*2(2)*	-12.7	*3(3)*	4.44	*3(3)*
	C-3	6	0.029	0.9905	3.05	0.0485	-479.2	*3(3)*	-491.0	*3(3)*	-200.1	*3(3)*	2.29	*3(3)*	0.9895	*3(3)*	-184.9	*3(3)*	-12.8	*2(2)*	4.45	*2(2)*
	C-4	4	0.028	0.9907	3.04	0.0482	-481.2	***1(1)***	-491.4	***1(1)***	-204.3	***1(1)***	2.22	***1(1)***	0.9899	***1(1)***	-191.6	***1(1)***	-14.6	***1(1)***	4.47	***1(1)***
	C-5	5	0.033	0.9892	3.03	0.0482	-473.4	*5(4)*	-483.2	*5(4)*	-196.5	*4(4)*	2.51	*5(4)*	0.9883	*5(4)*	-184.4	*4(4)*	-9.4	*4(4)*	4.35	*5(4)*

k: number of fitted parameters; RSS: residual sum of squares; ESS: explained sum of squares; S^2: standard deviation

2. Experimental design

In any design, a convenient practice is to code the doses (dividing them by the maximum ones) in such a way that both individual series include the same values (D_i) within the [0, 1] interval. Together with the encoding of the response in the same interval, this facilitates the fitting process and provides standardized parametric estimates. Once the D_i series is defined, there are several reasonable modes to establish the mixed doses covering the experimental domain (Figure A1).

Simple radial design

Besides the individual series D_{1i}, 0 and D_{2i}, 0 (D_{1i}=D_{2i}=D_i), this option includes several additional sets of mixed doses (d_{1i}, d_{2i}), each set defined by a constant ratio (d_{1i}/d_{2i}=Q) between the concentrations of both effectors. Thus, the mixed dose set located along the radius defined by Q_n is:

If $Q_n \le 1$: $d_{1i[Q_n]} = D_i$; $d_{2i[Q_n]} = D_i \times Q_n$

If $Q_n > 1$: $d_{1i[Q_n]} = D_i / Q_n$; $d_{2i[Q_n]} = D_i$

Concentric radial design

Similar to the preceding one, but with mixed doses defined from the angle (φ_n) that each radius makes with the variable representing the D_{1i} series:

$d_{1i[\varphi_n]} = D_i \times \cos\varphi_n$; $d_{2i[\varphi_n]} = D_i \times \sin\varphi_n$

Number of radii and values of φ_j (or Q) can be freely fixed, taking into account that high (~75º) and low (~15º) values of φ_j favor the detection of interactions.

Equiadditive design

Mixed doses are grouped in series defined by a constant sum (d_{1i}+d_{2i}=S). Thus, v being the desired number of doses per series:

If $S_n \le 1$: $d_{1i[S_n]} = S_n - h_v\left(\frac{S_n}{v-1}\right)$; $d_{2i[S_n]} = S_n - d_{1i[S_n]}$; (h_v=0, 1,…v–1)

If $S_n > 1$: $d_{1i[S_n]} = (S_n - 1) + h_v\left(\frac{1 - S_n + 1}{v-1}\right)$; $d_{2i[S_n]} = S_n - d_{1i[S_n]}$; (h_v=0, 1,…v–1)

Radial equiadditive design: mixed doses fulfill simultaneously the conditions d_{1i}/d_{2i}=Q_n and d_{1i}+d_{2i}=S_n, therefore:

$d_{1i} = S_n/(1+Q_n)$; $d_{2i} = S_n\left[1-\left(1/(1+Q_n)\right)\right]$

Complete design

It is the most intuitive experimental plan, combining simply all the doses of an effector with all doses of the other.

In principle, each design offers specific advantages for identifying concrete modes of action and interaction by comparing, through an appropriate statistical criterion, the observed responses at certain dose series with the expected ones under IA or CA null interaction hypotheses. However, in our

experience the response surface properties in joint actions imply: 1) numerous indistinguishable situations as analyzed by means of radial or equiadditive series; 2) responses whose behavior in a given region of the experimental domain does not represent necessarily what takes place in other regions.

In fact, the most discriminative tool is the explicit model, and in order to simulate such a conditions, the complete design is the most advisable. Even if one wants to disregard doubtful auxiliary functions, the responses to a same dose set of an effector in the presence of increasing doses of the another form very specific systematic sequences. These sequences are more informative than radial or equiadditive ones, and can be advantageously subjected to the comparative criteria above mentioned. Additionally, a good coverage of the experimental domain (complete design) is more efficient than an increase of the number of replicates to minimize the effects of the experimental error.

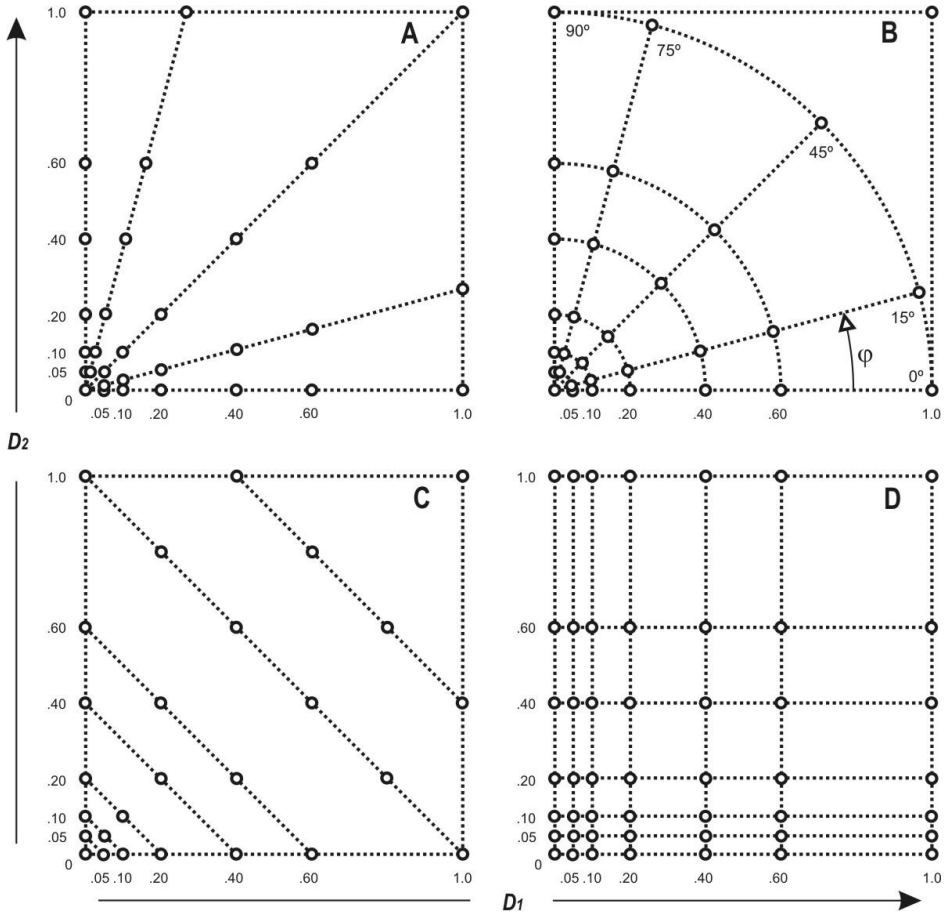

Figure A1: simple radial (A), concentric radial (B), equiadditive (C) and complete (D) designs.

3. Full antioxidant pairs combination analysis

3.1.1. ***Crocin bleaching reaction***

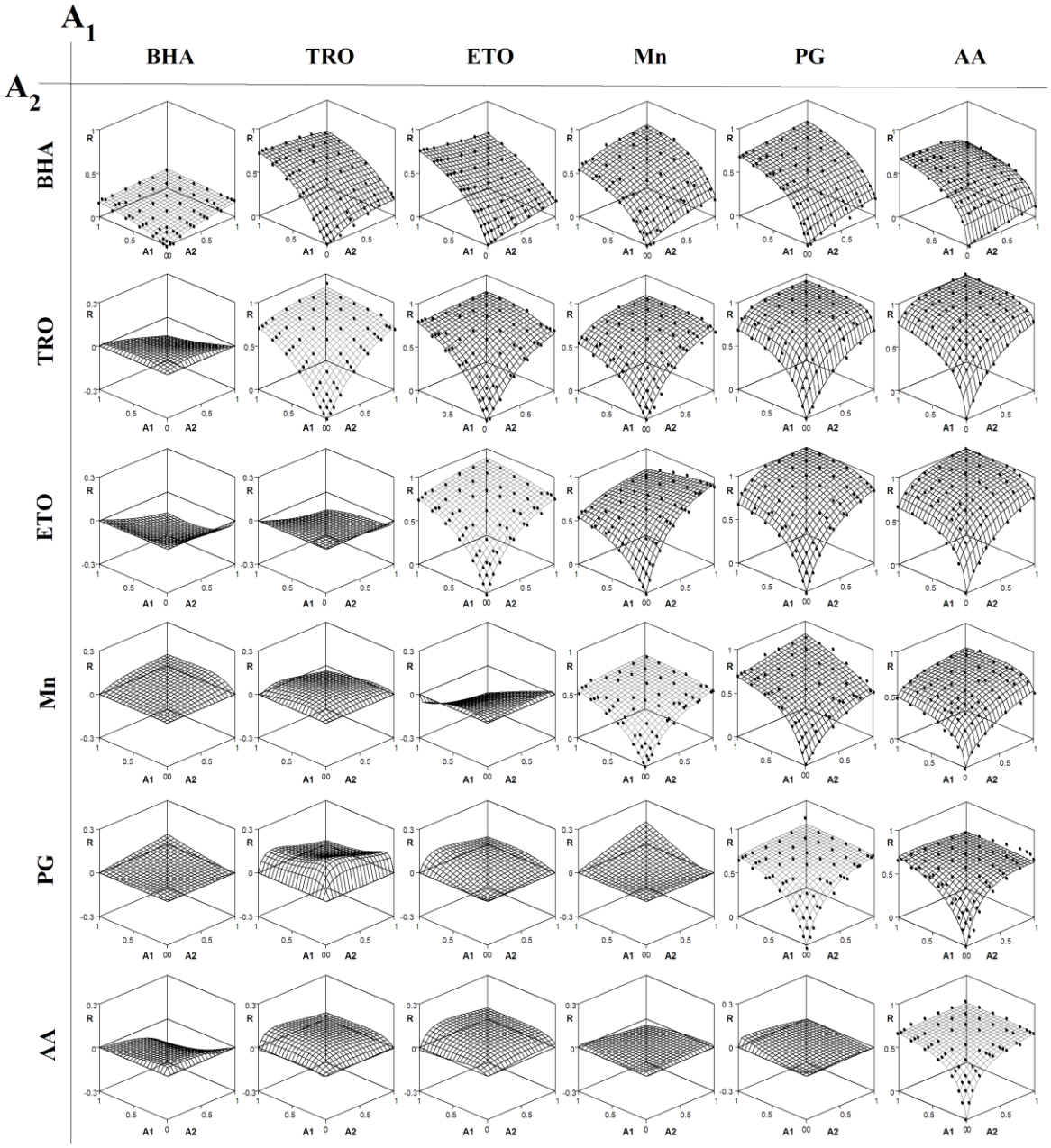

Figure A2: Matrix combination responses for the crocin bleaching reaction, which is organized as follows: a) in the diagonal it can be seem the results obtained for the controls; b) in the top part of the diagonal the surface responses for each pair antioxidant combination is presented; and c) in the bottom diagonal part the differences "scenery" between their respective null interaction form and the obtained response is presented. Numerical results in Table A3.

Table A3: Parametric values of the joint action of six different antioxidants in the crocin oxidation reaction. The null interaction and synergy hypotheses are compared under the independent action and addition concentration suppositions, by fitting the experimental results to the (10) and (15) generalized models. See Figure A2 and text for details. In all the presented results the parameters estimations are significant.

INDEPENDENT ACTION (IA)

A_1 **A_2**		*ETX* *TRO*	*Mn* *BHA*	*Mn* *ETX*	*PG* *TRO*	*PG* *ETX*	*AA* *BHA*	*AA* *TRO*	*AA* *ETX*	*AA* *Mn*
	K_1	0.904	0.606	0.614	0.722	0.701	0.730	0.668	0.700	0.568
response to A_1	m_1	0.369	0.302	0.305	0.197	0.187	0.149	0.105	0.139	0.100
	a_1	1.041	0.985	1.005	0.905	0.959	0.669	0.884	0868	0.899
	K_2	0.710	0.308	0.997	0.804	0.992	0.302	0.830	0.657	0.614
response to A_2	m_2	0.380	0.457	0.248	0.386	0.319	0.657	0.153	0.100	0.305
	a_2	1.322	0.994	0.922	1.065	0.885	0.988	0.668	0.759	1.005
A_1 as perturbing factor for params. of the response to A_2	b_{2k}	-0.903	-		-	-	2.801	-	-	-0.836
	c_{2k}	-	-	-0.771	-	-	-	-	-	-0.759
	b_{2m}	-	-	-	-	-	-	-	-	-
	c_{2m}	-	-	-	2.804	2.338	-	-	-	-
A_2 as perturbing factor for params. of the response to A_1	b_{1k}	-	-	-	-	-	-	-	-	-
	c_{1k}	-	-	-	-	1.090	-	-	-	-
	b_{1m}	-	-0.305	-	1.682	5.358	-	-	-	-
	c_{1m}	-	-	-	11.049	-	-	2.621	2.115	-
comp / coop	s	0.613	-	-	1.034	0.886	-	0.980	-	-
	$\mathbf{R^2_{adj}}$	0.9756	0.9991	0.9994	0.9997	0.9998	0.9798	0.9932	0.9807	0.9876

CONCENTRATION ADDITION (CA)

A_1 **A_2**		*BHA* *BHA*	*TRO* *TRO*	*ETX* *ETX*	*Mn* *Mn*	*PG* *PG*	*AA* *AA*	*TRO* *BHA*	*ETX* *BHA*	*Mn* *TRO*	*PG* *BHA*	*PG* *Mn*	*AA* *PG*
	K	0.249	0.868	0.950	0.647	0.739	0.689	0.851	0.942	0.808	0.233	0.548	0.792
joint response	m	0.599	0.437	0.372	0.336	0.208	0.129	1.720	4.181	0.344	0.393	0.292	0.270
	a	0.965	1.117	0.844	0.915	0.865	0.758	1.118	0.857	1.030	0.968	1.034	0.856
relative potency	p	1.035	1.001	1.000	1.000	1.006	1.000	4.616	11.372	0.961	5.808	2.161	1.596
A_1 altering eff. conc. of A_2	b_{2D}	-	-	-	-	-	-	-	-	0.843	-	-0.5571	-
	c_{2D}	-	-	-	-	-	-	-	-	-0.493	-	-	-
A_2 altering eff. conc. of A_1	b_{1D}	-	-	-	-	-	-	-	-	-	-	-	-
	c_{1D}	-	-	-	-	-	-	-	0.818	-	0.201	-	-
A_1 as perturbing factor for params. of the joint response	b_{2k}	-	-	-	-	-	-	-	-	-0.051	8.321	0.266	-
	c_{2k}	-	-	-	-	-	-	-	-	-	2.148	-	0.191
	b_{2m}	-	-	-	-	-	-	-	-	0.599	-	-	-
	c_{2m}	-	-	-	-	-	-	-	-	-	-	-	-
A_2 as perturbing factor for params. of the joint response	b_{1k}	-	-	-	-	-	-	-0.194	-	-		0.270	-
	c_{1k}	-	-	-	-	-	-	-	-	-	-0.096	-	-
	b_{1m}	-	-	-	-	-	-	-	-	-	-	-	-
	c_{1m}	-	-	-	-	-	-	-	-	-	-	-0.452	-
	$\mathbf{R^2_{adj}}$	0.9985	0.9995	0.9998	0.9994	0.9985	0.9984	0.9937	0.9995	0.9998	0.9992	0.9989	0.9958

3.1.2. ***β-carotene bleaching reaction***

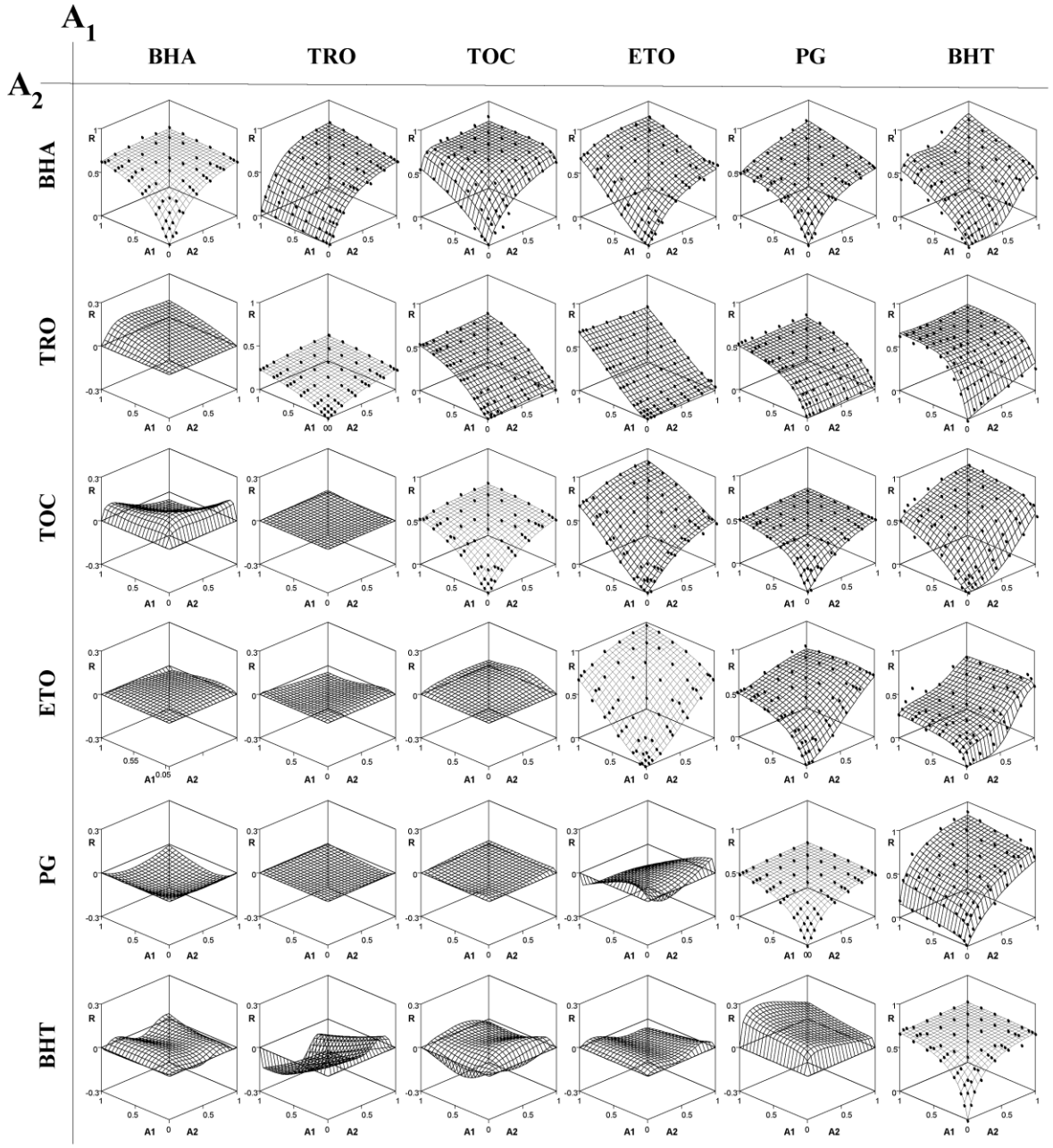

Figure A3: Matrix combination responses for the β-carotene bleaching reaction, which is organized as follows: a) in the diagonal it can be seem the results obtained for the controls; b) in the top part of the diagonal the surface responses for each pair antioxidant combination is presented; and c) in the bottom diagonal part the differences ("scenery") between their respective null interaction form and the obtained response is presented. Numerical results in Table A4.

Table A4: Parametric values of the joint action of six different antioxidants in the β-carotene oxidation reaction. The null interaction and synergy hypotheses are compared under the independent action and addition concentration suppositions, by fitting the experimental results to the (10) and (15) generalized models. See Figure A3 and text for details. Note, that in all the presented results the parameters estimations are significant.

INDEPENDENT ACTION (IA)

$\mathbf{A_1}$ $\mathbf{A_2}$		*TOC* *TRO*	*ETX* *BHA*	*ETX* *TRO*	*ETX* *TOC*	*PG* *BHA*	*BHT* *BHA*	*BHT* *TRO*	*BHT* *TOC*	*BHT* *ETX*	*BHT* *PG*
	K_1	0.583	1.000	1.000	1.000	0.514	1.000	0.597	0.597	0.154	0.374
response to A_1	m_1	0.376	0.754	0.758	0.754	0.201	0.881	0.881	0.468	0.080	0.211
	a_1	1.184	1.629	1.567	1.584	0.960	0.837	0.956	1.236	1.090	0.395
	K_2	0.410	0.594	0.100	0.576	0.591	0.528	0.660	1.000	0.861	0.621
response to A_2	m_2	6.669	0.276	1.696	0.389	0.255	0.679	0.117	0.912	0.394	0.700
	a_2	0.926	1.039	0.781	1.135	0.973	4.031	1.068	2.522	0.655	4.456
	b_{2k}	-	-	-	-0.447	0.498	-	-	-	-	-
A_1 *as perturbing factor for*	c_{2k}	-	-	-	-	-	-0.367	0.314	-	-	-
params. of the response to A_2	b_{2m}	-	-0.235	-	-	-0.747	-	-	-0.475	-0.669	-
	c_{2m}	-0.901	-	12.772	-0.744	-	-	-	-	-	-0.136
	b_{1k}	0.097	-0.245	-	-	-0.630	-0.711	-	-	-	-
A_2 *as perturbing factor for*	c_{1k}	-	-	-	-	-	-	-	-	-	-
params. of the response to A_1	b_{1m}	-	-	-	-	-	-	-	-	-	-
	c_{1m}	-	-	-	0.711	-	5.423	-	-	-	20.241
comp / coop	s	-	-	-0.991	1.251	-	-	-	-	-	1.434
	$\mathbf{R^2_{adj}}$	0.9987	0.9997	0.9996	0.9988	0.9976	0.9923	0.9942	0.9892	0.9818	0.9785

CONCENTRATION ADDITION (CA)

$\mathbf{A_1}$ $\mathbf{A_2}$		*BHA* *BHA*	*TRO* *TRO*	*TOC* *TOC*	*ETX* *ETX*	*PG* *PG*	*BHT* *BHT*	*TRO* *BHA*	*TOC* *BHA*	*PG* *TRO*	*PG* *TOC*	*PG* *ETX*
	K	0.674	0.995	0.599	1.000	0.508	0.694	0.843	0.761	0.572	0.624	1.000
joint response	m	0.261	15.043	0.408	0.756	0.205	0.140	0.241	0.326	6.993	0.373	5.041
	a	0.978	0.894	1.166	1.558	0.971	0.806	1.199	0.895	1.067	1.151	0.869
relative potency	p	1.010	0.998	1.016	0.999	0.997	1.005	0.038	0.609	36.116	1.975	10.091
	b_{2D}	-	-	-	-	-	-	0.531	-	-	1.682	-
A_1 *altering eff. conc. of* A_2	c_{2D}	-	-	-	-	-	-	-	-	8.381	1.399	-0.680
A_2 *altering*	b_{1D}	-	-	-	-	-	-	-	12.24	-	-	-
eff. conc. of A_1	c_{1D}	-	-	-	-	-	-	13.320	-	-	-	7.262
	b_{2k}	-	-	-	-	-	-	-	-	-	-	-
A_1 *as perturbing factor for*	c_{2k}	-	-	-	-	-	-	-	-	-	-	0.445
params. of the joint response	b_{2m}	-	-	-	-	-	-	-	-	0.912	1.588	-
	c_{2m}	-	-	-	-	-	-	-	-	-	-	-
	b_{1k}	-	-	-	-	-	-	-0.038	-	0.098	-	-
A_2 *as perturbing factor for*	c_{1k}	-	-	-	-	-	-	-	-	-	-	-
params. of the joint response	b_{1m}	-	-	-	-	-	-	1.248	-	0.249	-	-
	c_{1m}	-	-	-	-	-	-	-	-	-	-0.236	8.211
	$\mathbf{R^2_{adj}}$	0.9987	0.9981	0.9994	0.9996	0.9976	0.9981	0.9998	0.9881	0.9991	0.9987	0.9975

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