International Journal of Food Science +Technology

Institute of Food Science +Technology ifst

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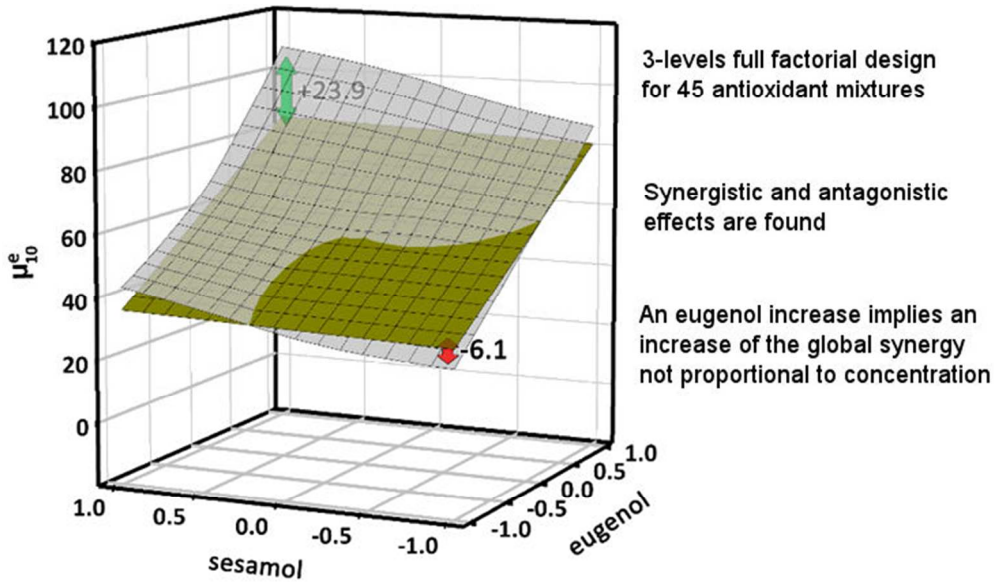
Journal:	*International Journal of Food Science and Technology*
Manuscript ID	IJFST-2016-21239.R3
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	n/a
Complete List of Authors:	Palma, Alberto; Universidad de Huelva Facultad de Ciencias Experimentales, Ingeniería Química, Química Física y Ciencia de los Materiales Ruiz Montoya, Mercedes; Universidad de Huelva Facultad de Ciencias Experimentales, Ingeniería Química, Química Física y Ciencia de los Materiales Díaz, Manuel Jesús; Universidad de Huelva Facultad de Ciencias Experimentales, Ingeniería Química, Química Física y Ciencia de los Materiales Arteaga, Jesús F.; Universidad de Huelva Facultad de Ciencias Experimentales, Departamento de Química Estévez Brito, Rafael; Universidad de Cordoba, Química Física y Termodinámica Aplicada Rodríguez Mellado, José Miguel; Universidad de Cordoba, Química Física y Termodinámica Aplicada
Keywords:	Antioxidant Activity, Antioxidants, Natural Products, Polarography and Voltammetry, Mathematical Model, Chemical Processes



Antioxidant capacity is measured from the decrease of the signal of H_2O_2 oxidation on a mercury electrode

259x182mm (72 x 72 DPI)

Evaluation of synergistic and antagonistic effects between some selected antioxidants by means of an electrochemical technique

A. Palma[a], M. Ruiz Montoya[a]*, M. J. Díaz[a], J. F. Arteaga[b], R. Estévez Brito[c] and J. M. Rodríguez Mellado[c]

[a]Departamento de Ingeniería Química, Química Física y Ciencias de los Materiales. Fac. Ciencias Experimentales. Centro de Investigación en Tecnología de Productos y Procesos Químicos (Pro2TecS). Campus El Carmen. Universidad de Huelva. E-21071- Huelva (Spain)

[b]CIQSO-Centro para la Investigación en Química Sostenible y Departamento de Química. Campus "El Carmen". Universidad de Huelva. E-21071- Huelva (Spain)

[c]Departamento de Química Física y Termodinámica Aplicada. Fac. Ciencias. CeiA3, Campus Rabanales, Universidad de Córdoba. E-14014-Córdoba (Spain)

*Corresponding author (Tel: +34 959218202; Fax: +34 959 21 99 83: E-mail: mmontoya@diq.uhu.es)

Keywords

Antioxidant Scavenging Capacity, Factorial Experimental Design, Synergistic Effects, Differential Pulse Voltammetry

Abstract

This work presents a systematic study of the possible interactions in mixtures of the natural antioxidants eugenol, thymol, sesamol and limonene. The antioxidant capacity is measured from the decrease of the DPV signal corresponding to the H_2O_2 oxidation on a mercury electrode in the presence of the antioxidants. A three levels full factorial design was used to analyze the antioxidant capacity of 45 mixtures. The higher synergistic effect was reached in the mixtures containing eugenol and sesamol (antioxidant capacity increases in a 28.5 %). The synergistic and antagonistic effects of the combinations having the lower antioxidant capacity (thymol, sesamol and limonene), ranged between 20.2% to 26.3%, and 8.6% to 11.8% respectively. An increase in the eugenol concentration implies a progressive increase of the global synergy, not proportional to the concentration. A possible explanation of the synergy, related to the oxidation mechanisms and to the possibility of interaction with the radicals is given.

Introduction

The composition-activity relation of natural antioxidants is of great interest (Sim and Sil 2008; Chan *et al.* 2016). Interactions may occur for juices (Bolling *et al.* 2013), mixtures of active compounds (Castro *et al.* 2006), anticancer and antibacterial activities (Cui *et al.* 2016, Ni *et al.* 2016) or off-flavours in water (Andersson *et al.* 2005).

The action of an antioxidant depends on its concentration and reactivity and is influenced by the interactions with other antioxidants. This can imply a combined effect different from the sum of the individual effects (Marinova *et al.* 2008). Even when the effects resulting from the combination of two antioxidants are known, the addition of a third compound can lead to an uncertain final effect, this making mandatory a continuous empirical study.

The antioxidant capacity of synthetic samples, food extracts, infusions and beverages has been determined by a variety of methods classified as HAT (hydrogen atom transfer), ET (non-competitive electron transfer) or mixed HAT-ET methods (Apak *et al.* 2016 a, b, c). There is not a clear correlation between activities determined by different assays for the same antioxidant or by the same assay in different laboratories (Niki 2011)

Reactive oxygen species (ROS) are radicals generated by H_2O_2, organic peroxides or water, which can induce the degradation of biological molecules. HAT methods measure the scavenging to ROS of an antioxidant by hydrogen atoms transfer. Total Antioxidant Capacity methods are usually non-competitive ET methods. The DPPH Radical Scavenging Assay is a HAT-ET method.

In vivo methods have been developed to evaluate antioxidant activities. Cellular antioxidant activity (CAA) measures the ability to prevent the formation of fluorescent

dichlorofluorescein peroxyl radicals by 2,2′-azobis(2-amidinopropane) dihydrochloride in hepatocarcinoma cells (Wolfe & Liu, 2007). This method is more biologically relevant than the chemical assays because it takes into account cell uptake, distribution, and metabolism of antioxidants. Microbial Test Systems (MTS) present the same advantage (Zhou *et al*., 2016).

Electrochemical methods were also used, e.g. by monitoring the polarographic oxidation of H_2O_2 that gives peroxide and hydroperoxide radicals (Palma *et al.* 2013; Estévez Brito *et al.* 2014a), which interact with antioxidants (Gorjanović *et al.* 2010). The use of the differential pulse voltammetry increased the accuracy of the measurements (Palma *et al.* 2014). Both the generation step and the detection of the interaction are made in the same voltammetric measurement (Palma et al., 2013). H_2O_2 is not active on carbon electrodes, ROS are generated in the Hg oxidation reaction in the presence of hydrogen peroxide (Estévez Brito *et al*. 2014a) and the use of Hg electrodes is necessary. The antioxidant capacities of antioxidants appearing in natural samples and some of their mixtures were obtained by this method (Palma *et al.* 2013, 2014) that has been selected in this work. It was found that the radicals produced in the electrooxidation of H_2O_2 interact with 3-hydroxycoumarin, carvacrol, vanillin and gallic acid (Palma *et al.* 2013). The studies were extended to aqueous extracts of spices and mixtures of antioxidants (Palma *et al.* 2014). The experimental antioxidant capacities were compared to those expected for each mixture, calculated by taking into account the concentration of each compound. An apparent linear correlation was found with a slope close to unity, which suggested that there are not synergistic or antagonistic effects in the mixtures, but this conclusion must be better established.

Differences in antioxidant activity of blueberries and strawberry extracts was attributed to differences in activities of phenolic compounds and their antagonistic and synergistic reactions with other antioxidants (Kaur and Kapoor 2001). Because spices are frequently used as food additives, it is important to establish if the antioxidant effect of a given combination of antioxidants is due only to the sum of individual antioxidant activities or if such effect is increased or decreased with respect to the total effect. Once stated the occurrence of antagonistic and/or synergistic effects, a next step must be to relate these interactions to the composition of the spices and the mechanisms of antioxidant activity of the components.

The aim of this work was to present a systematic study of the existence of interactions in mixtures of natural antioxidants found in spices, herbs and condiments (eugenol, thymol, sesamol and limonene) by means of a factorial experimental design.

MATERIALS AND METHODS

All chemicals (Merck analytical grade) were used without further purification. Antioxidants (Sigma-Aldrich) were dissolved in ethanol and stored in darkness at 277 K to avoid decomposition. Solutions of 0.1 M in both sodium carbonate and phosphoric acid were used as supporting electrolytes. The aqueous solutions were prepared with ultrapure water (resistivity 18.2 MΩ.cm at 298 K). Ionic strength was adjusted to 0.5 M with KNO_3.

Measurements were made at pH=10.50 to prevent interferences caused by the oxidation of mercury at lower pH values. Solutions were prepared with a fixed amount (6.9 mL) of supporting electrolyte, variable volumes, V_{AO}, of the stock solution of antioxidant in ethanol, (3–V_{AO}) mL of pure ethanol (final percentage in the cell 30%) and, after the solution was

purged with purified nitrogen, 100 µL of 0.05 M H_2O_2 (final concentration 0.5 mM). The reverse of the volume of antioxidant necessary to decrease the area of the H_2O_2 oxidation peak a 10%, μ_{10}, is a measurement of the antioxidant scavenging capacity (Palma *et al.*, 2014), this parameter increasing with the antioxidant capacity. In this work the adimensional μ_{10} parameter referred to 1 mM gallic acid was selected to express the antioxidant activity.

Measurements were made on a CHI650A electrochemical workstation from IJ Cambria coupled to an EF-1400 mercury electrode (BAS instruments) in the HMDE mode. The Hg drop area was 6.70×10^{-3} cm^2. The temperature was 298.0±0.1 K. Reference and counter electrodes were from BAS (MF-2052, Ag|AgCl|KCl_{sat} and MW-1034, Pt, respectively). The pulse parameters for differential pulse voltammetry (DPV) were: amplitude 0.05 V, width 0.05 s and period 0.2 s.

The experiments were repeated until the standard deviations of the data were less than 5%.

To relate the dependent (μ_{10}) and independent variables (limonene, eugenol, thymol, sesamol), a three levels factorial design was used (Akhnazarova and Kafarov 1982). Levels were “high” or +1, “medium” or 0, and “low” or –1, corresponding to the concentrations, in mM, 0.0, 2.0, 4.0 for limonene, 0.0, 0.5, 1.0 for eugenol, 0.0, 0.75, 1.5 for thymol, and 0.0, 0.75, 1.5 for sesamol.

Results and discussion

1. Experimental design and statistical analysis

The selection of the antioxidants and their concentrations was carried out by taking into account their antioxidant activities. Compounds found in spices, herbs and condiments with

high, moderate and low antioxidant activity were selected. Limonene, together carvacrol, thymol and eugenol, are essential oils with antioxidant activity (Charles, 2012). Eugenol is found in high amounts in clove (Shan *et al*., 2005) and in less extension in basil (Brewer, 2011) and other herbs. Thymol is an abundant component of thyme (Aeschbach *et al*., 1994; Shan *et al*., 2005), appearing also in other spices as cumin or ajowan (Charles, 2012). Limonene gives the characteristic aroma to citric peels, being also present in species as cumin, ajowan or cardamom or anise star (Charles, 2012). Sesamol is the main antioxidant of sesame oil. Higher concentrations were chosen for the less active compound (limonene) and lower for the most active compound (eugenol).

The data obtained in the factorial design are given in Table 1.

Table 1

A multiple analysis of variance (MANOVA) was used to obtain the significant independent parameter effects ($p<0.005$) indicated as second-order polynomials for each dependent variable. The polynomial model used was of the type shown in equation 1.

$$Z = a_0 + \sum_{i=1}^{n} b_i X_{ni} + \sum_{i=1;j=1}^{n} d_i X_{ni} X_{nj} \quad (i < j) \qquad \text{(Eqn. 1)}$$

where Z and X_{ni} denote dependent and normalized independent variables, respectively, and a_0, b_i, c_i and d_{ij} are unknown constants obtained from experimental data. Independent variables were normalized (X_n) by using the following equation:

$$X_n = \frac{(X - \overline{X})}{\left(\frac{X_{max} - X_{min}}{2}\right)} \qquad \text{(Eqn. 2)}$$

Where X is the absolute value of the independent variable, X_{med} is the average value and X_{max} and X_{min} are the maximum and minimum values, respectively.

The independent variables with statistically significant coefficients (those not exceeding a significance level of 0.05 in Student's t-test and that have a 95% confidence interval excluding zero) were used in the equations.

2. Results of antioxidant capacity measurements

The DP voltammograms of the oxidation of H_2O_2 decreases as the antioxidant concentration increases, as is shown in Figure 1 for thymol.

Figure 1

The decrease of the DPV peak is different for the different antioxidants due to the differences in scavenging activities; this applies also to the mixtures. The values of μ_{10} obtained for all mixtures are given in table 1.

3. Results of experimental design and statistical analysis

A Box-Behnken design was selected because it not contains an embedded factorial or fractional factorial design, and therefore it requires a fewer number of runs with respect to other response surface designs. The statistical model provided by the results obtained from this design is used to identify synergistic and antagonistic effects.

μ_{10} (experimental response) is related to the antioxidant capacity. Expected μ_{10} values were calculated for each mixture from the individual μ_{10} values and concentrations. An apparent

proportionality between experimental and calculated values is observed, being the slope of the correlation close to unity. This suggests that μ_{10} parameter is an additive measure of the scavenging activity, i.e., the overall μ_{10} value of a mixture is the sum of the μ_{10} individual values. Nevertheless, evident synergistic and antagonistic effects are observed in those mixtures presenting high deviations ($p<0.2$) between experimental and theoretical μ_{10} values.

The estimations of the coefficients and statistics parameters (R^2 and F) have been made by polynomial regression of equation 3, which can be used to estimate the variation of the dependent variable with changes in the independent variables, over the ranges considered, at constant values of the other variables. Only the terms with statistically significant coefficients are shown.

$$\mu_{10} = 63.99 + 30.08 \cdot EU + 11.40 \cdot TH + 11.30 \cdot LI + 9.06 \cdot SE - 8.68 \cdot LI \cdot TH \quad \text{(Eqn. 3)}$$

LI, EU, TH, SE are the normalized concentrations of limonene, eugenol, thymol, and sesamol, respectively.

Concerning the response equation, an acceptable (>0.95) R^2 and a high value for F= 33.69 have been obtained. A rather important difference between the values obtained by the simulation and those given by the statistical model can sometimes be observed. EU is the most influential variable in μ_{10}, followed by TH, LI and SE, in this order.

From the statistical analysis of the independent variables (antioxidants) on the dependent variable (μ_{10}) it is possible to weigh the relative influence for each compound, in percentage, on the mean variation of antioxidant capacity. The results show a relative influence of 45.1%

for eugenol, 12.6% for thymol, 12.4% for limonene, and 8.5% for sesamol. These values are in accord to the polynomial constants of eqn. 3.

The variation introduced on μ_{10} by each antioxidant at the same concentration reveals that the most contributing compound is eugenol. Each compound presents a different response to the selected variable.

4. Analysis of surface response. Synergistic and antagonistic effects.

The obtained model was analysed to determine the range of the operational variables giving the optimum values of the dependent variable. Figure 2 shows the response surface for the dependent variable at three levels of the most influential variable, in order to get a suitable visual observation of produced changes. The range of the operational variables that gives the optimum values of dependent variables is so determined.

Figure 2

The responses confirm that μ_{10} was much more sensitive to changes in eugenol ($p<0.1$) than for the other independent variables. In general, a high positive influence (higher μ_{10}) is found for eugenol.

Eugenol shows a similar trend at all tested concentrations. However, its statistical influence decreased ($p<0.15$) when the concentrations of the other components were increased. Similar (although lower) positive statistical influence is observed for thymol and limonene. The lowest μ_{10} value is obtained at the lowest eugenol, thymol and limonene concentrations.

Thymol was the second variable most strongly influencing μ_{10}. However, a slightly negative influence ($p<0.1$) of this parameter is found at high sesamol and eugenol concentrations,

and a synergistic effect on thymol and limonene is observed (Eqn. 3), which increases as the concentrations of the components increase.

The individual influence of the antioxidants in the mixture on the total antioxidant capacity, and the influence of an antioxidant over another, provide relevant information but poorly visual. The surface-response methodology, SRM (Box and Wilson 1951), allows the analysis of the influence of the presence of the different antioxidants on the antioxidant capacity of the mixtures. Using this method, it is possible to identify the synergistic and antagonistic effects in a visual manner.

From the theoretical antioxidant capacity (sum of the individual μ_{10} values), the following first degree polynomial was obtained:

$$\mu_{10} = 58.2 + 24.1 \cdot EU + 12.5 \cdot TH + 10.8 \cdot LI + 1.5 \cdot SE \qquad \text{(Eqn. 4)}$$

Figure 3 shows the superposition of the response surfaces originated from the theoretical and experimental polynomials for a combination of antioxidants (sesamol-eugenol).

Figure 3

The results show a similar tendency when eugenol is combined with the rest of antioxidants. The mixtures of eugenol at medium-high concentration with any concentration of thymol, and the mixtures of limonene and sesamol, also at medium-high concentrations, showed high synergistic effects. Antagonistic effects were not observed for the eugenol-thymol combination, but were evident for the combinations of low values of eugenol with low values of limonene or sesamol. The higher synergistic effect corresponded to the mixtures containing eugenol and sesamol at the higher values, the antioxidant capacity increasing a 28.5%. The higher antagonistic effect was obtained for the combination eugenol-limonene,

with a decrease of a 33.5%. The synergistic/antagonistic effects of the combinations having the lower antioxidant capacity (thymol, sesamol and limonene), ranged between 20.2-26.3%, and 8.6-11.8% respectively.

From the analysis of the surfaces corresponding to the theoretical and experimental responses for the combinations that include eugenol, the results were similar for the three normalized levels: for the lower and medium normalized eugenol values (0 and –1), synergistic and antagonistic effects were observed, whereas for the higher normalized eugenol value (+1), only synergistic effects were appreciated. An increase of the eugenol concentration implies an increase of the global synergy, not proportional to the concentration, accompanied by a decrease in antagonistic effects, which become negligible at the higher concentration values.

The structure of Eugenol allows the formation of more stable radicals than the rest, because the double bound in the lateral chain and the presence of one phenolic –OH and the methoxy group. Thymol and sesamol have only one phenolic –OH, though the dioxol ring of sesamol can be opened giving relatively stable radicals (Estévez Brito *et al*. 2014a). Thus, the synergy could be related to the oxidation mechanisms and the possibility of interaction between the radicals. The intermediate radicals generated in the eugenol oxidation could attack the rest of antioxidants, increasing the formation of radicals and this making easier the capture of ROS generated in the Hg/H_2O_2 oxidation. This could be the reason of other synergistic effects observed for eugenol (Braga *et al*. 2007).

Conclusions

It was found that the antioxidant capacity of mixtures of the antioxidants eugenol, thymol, sesamol and limonene present synergistic and antagonistic effects. The higher synergistic effect corresponded to the mixtures containing eugenol. The effects for the mixtures having the lower antioxidant capacity (thymol, sesamol and limonene), were significant, 20.2%-26.3%, for synergistic effects, and 8.6%-11.8%, for antagonistic effects. An increase in the eugenol concentration increase the global synergy in a way not proportional to the concentration. The synergy could be related to the oxidation mechanisms and to the possibility of interaction between the radicals generated in the eugenol oxidation with the rest of antioxidants that could promote the capture the ROS generated in the Hg/H_2O_2 oxidation. This could be the reason of other synergistic effects observed for eugenol.

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Table 1. Experimental domain and Box-Behnken design matrix used in the study. The values for the antioxidants are concentrations in mmol·L^{-1}.

Nº	Limonene	Eugenol	Thymol	Sesamol	μ_{10}
1	0	0	1.5	0	27.80
2	0	1	1.5	0	103.50
3	0	0.5	1.5	0.75	56.00
4	0	1	1.5	1.5	120.50
5	0	0	1.5	1.5	65.00
6	0	1	0.75	0.75	111.00
7	0	0.5	0.75	0.75	29.60
8	0	0.5	0.75	1.5	44.40
9	0	0.5	0.75	0	39.80
10	0	0	0.75	0.75	21.20
11	0	0	0	0	0
12	0	1	0	0	48.10
13	0	0.5	0	0.75	34.60
14	0	1	0	1.5	70.00
15	0	0	0	1.5	18.70
16	2	0	1.5	0	30.10
17	2	1	1.5	0	90.50
18	2	0.5	1.5	0.75	79.50
19	2	1	1.5	1.5	117.99
20	2	0	1.5	1.5	49.50
21	2	1	0.75	0.75	98.50
22	2	0.5	0.75	0.75	69.00
23	2	0.5	0.75	1.5	65.80
24	2	0.5	0.75	0	74.60
25	2	0	0.75	0.75	30.00
26	2	0	0	0	10.10
27	2	1	0	0	75.00
28	2	0.5	0	0.75	39.30
29	2	1	0	1.5	103.00
30	2	0	0	1.5	27.30
31	4	0	1.5	0	61.20
32	4	1	1.5	0	81.50
33	4	0.5	1.5	0.75	70.40
34	4	1	1.5	1.5	110.50
35	4	0	1.5	1.5	59.40
36	4	1	0.75	0.75	94.50
37	4	0.5	0.75	0.75	82.50
38	4	0.5	0.75	1.5	93.00
39	4	0.5	0.75	0	68.49
40	4	0	0.75	0.75	52.60
41	4	0	0	0	31.90
42	4	1	0	0	94.50
43	4	0.5	0	0.75	65.00
44	4	1	0	1.5	116.00
45	4	0	0	1.5	47.80

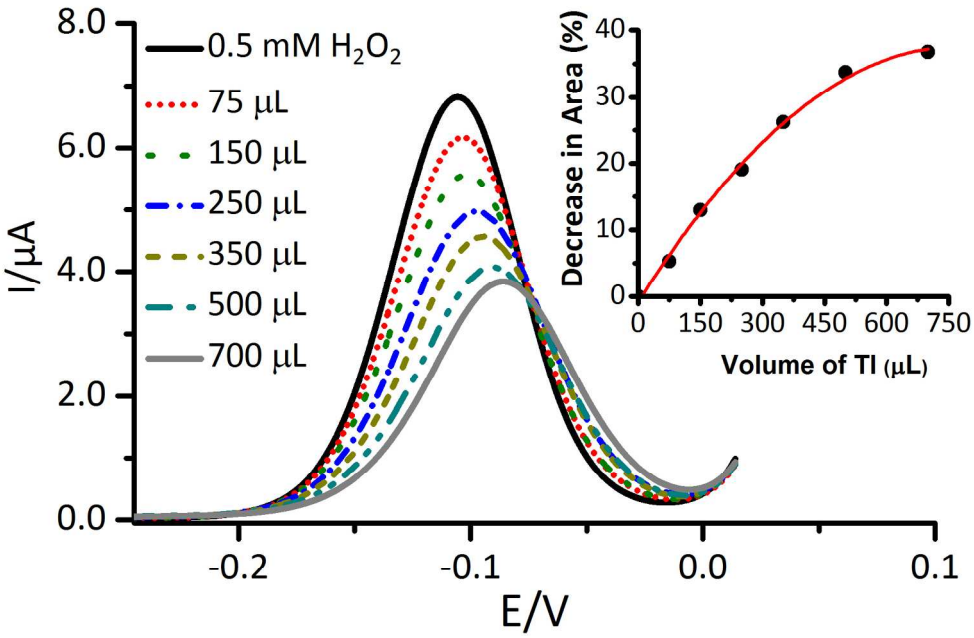

Figure 1. DPV voltammograms of 0.5 mM H_2O_2 at pH = 10.50, 30% ethanol, and different volumes of 5 mM thymol. Final volume 10 mL. Inset: Decrease of the DPV peak area with the volume.

180x119mm (300 x 300 DPI)

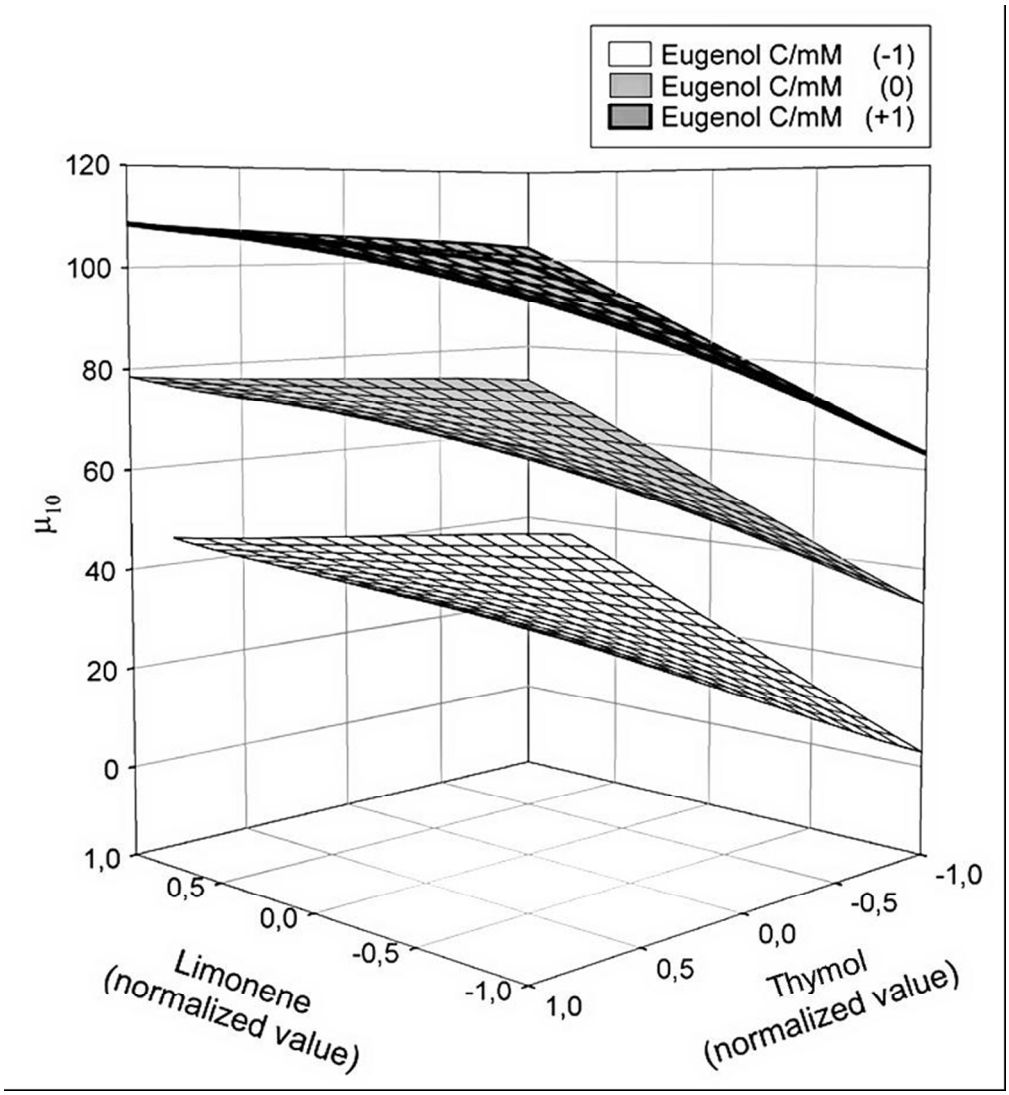

Figure 2. μ_{10} evolution with normalized values of Thymol and Limonene at three Eugenol concentrations.

227x246mm (96 x 96 DPI)

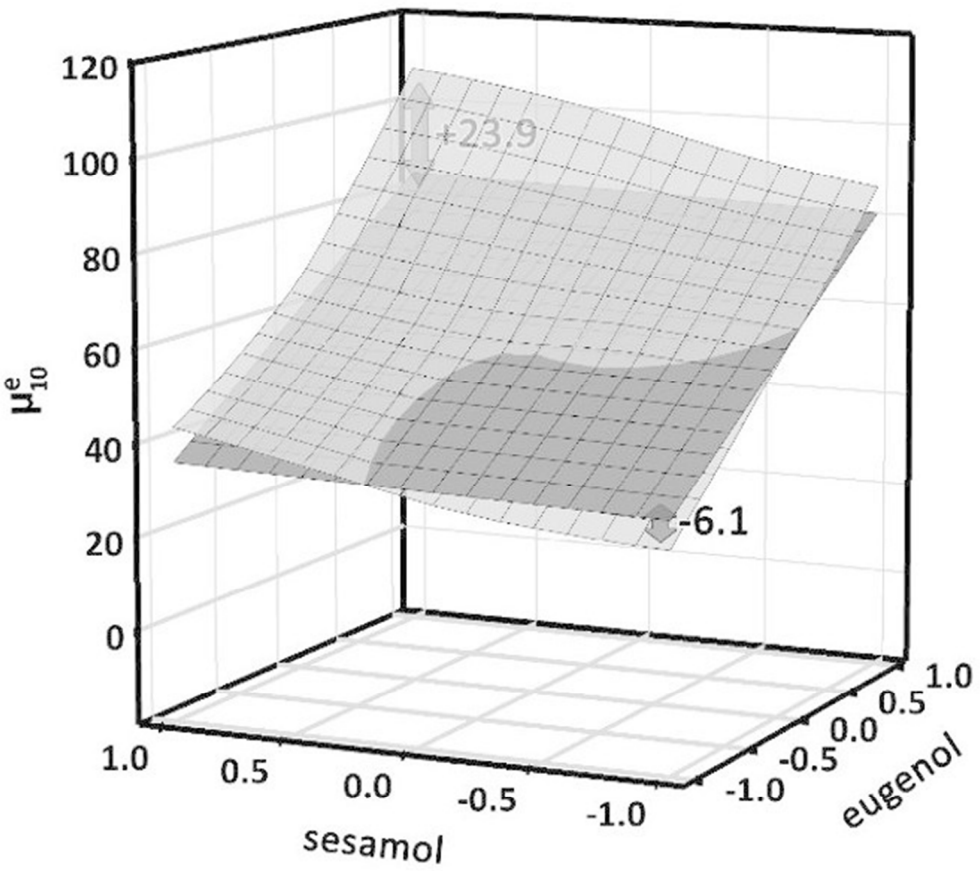

Figure 3. Synergistic and antagonistic effects for a combination of antioxidants in the mixtures (normalized values). Darker surface correspond to the theoretical polynomial.

236x220mm (72 x 72 DPI)