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REGULAR ARTICLE

ASSESSMEN OF THE REDUCING EFFECTS IN MIXTURES OF GRAPE (*VITIS VINIFERA*) SEED EXTRACTS WITH *α*-TOCOPHEROL USING RESPONSE SURFACE METHODOLOGY

Evangelia Karvela[1], *Dimitris P. Makris*[2,*]

Address: Dr. Dimitris P. Makris,

[1] Department of Science of Dietetics-Nutrition, Harokopio University, 70, El. Venizelou, 17671, Kallithea, Athens, GREECE

[2] Department of Food Science & Nutrition, University of the Aegean, 2, Mitr. Ioakim, 81400, Myrina, Lemnos, GREECE

*Corresponding author: dmakris@aegean.gr

ABSTRACT

The efficiency of grape seed extracts to express reducing power was assessed using two different approaches and the TPTZ as the chromophore probe. Further to that, the mixture effects when the extracts were combined with α-tocopherol (α-Tcp) were also evaluated. The approaches included a simple linear regression analysis between the response (reducing power) and concentration, but also a response surface methodology, which permitted the monitoring of the response upon simultaneous variation of both the concentration of the total polyphenols (TP) of α-Tcp. The outcome of the study indicated that the deployment of linear regression poses important constrains with regard to concentration ranges, whereas the response surface methodology might be a valuable statistical tool for similar assessments and credible modeling of binary mixtures of antioxidants. In all combinations tested it was found that an antagonism is manifested, presumably as a result of α-Tcp regeneration by the extract polyphenols, at the expense of the latter.

Keywords: Antagonism, antioxidants, grape seeds, polyphenols, response surface methodology

INTRODUCTION

Oxidative deterioration is a major concern with regard to consumer acceptability of foods, because of the generation of off-flavours, alteration / decomposition of essential nutrients and production of potentially toxic components (**Choe and Min, 2009**). The incorporation of exogenous antioxidants to food matrices, in addition to inherent protective substances, is believed to provide an effective shield against oxidation, by inhibiting or delaying the relevant reactions implicated. Lipophilic antioxidants, such as BHT, have been a tool of preference in this regard, owed to their efficiency and low cost. On the other hand, consumer demands for healthier foods with functional properties, as well as the strong evidence provided for plausible toxicity of synthetic additives, has shifted industrial interest into antioxidants of natural origin, including polyphenolic substances (**Pokorný, 2007**).

A great deal of work has been carried out on the antioxidant properties of polyphenols that can be recovered from abundant, inexpensive residual sources, such as wine industry by-products. Polyphenols and polyphenol-containing extracts deriving mainly from grape pomace and seeds have been proven to provide adequate protection of various foods against lipid peroxidation, including refined soybean oil (**Gámez-Meza *et al.*, 2009**), beef and pork (**Rojas and Brewer, 2008**), fish (**Sánchez-Alonso *et al.*, 2007**), and cooked chicken (**Shirahigue *et el.*, 2010**).

An issue of high importance, however, arising by the use of such compounds, is their actual efficiency in protecting the substrates occurring in foods, such as proteins and lipids. Phenomena embracing synergism and antagonism among various forms of polyphenolic substances are rather very common in real food matrices (**Choe and Min, 2009**) and therefore the antioxidant manifestations emerging by enhancing a given food in specific antioxidant additives might not be the anticipated ones. Such behaviours entail a multilateral assessment of the antioxidant activity, instead of simply evaluating the antioxidant status established in a food after addition of antioxidant(s).

That is, examinations of the antioxidant efficiency of a mixture of antioxidants should not be based solely on measurements of standard proportions (e.g. 1:1, 2:1 etc.) but the effects of varying simultaneously the content of both compounds should also be considered. This is important because the antioxidant effects exerted might be the consequence of an ideal ratio. Deviations from this ratio may bring about undesirable results, since an antioxidant system, under certain circumstances, might switch to prooxidant, with detrimental effects. However, since the antioxidants added are consumed in redox reactions occurring in foods with time,

such deviations are to be expected. Thus variations in the concentration of various antioxidants co-existing in foods could reveal an overall image of the antioxidant efficiency, even when one antioxidant is consumed at the expense of another.

On this conceptual basis, this investigation was undertaken with the scope of assessing possible effects of synergism / antagonism in mixtures of a well-known natural antioxidant, α-Tcp, with extracts deriving from grape seeds, a common and abundant vinification by-product, which exhibits significant antioxidant potential compared with other food by-products and wastes (**Makris *et al.*, 2007**). The implementation of response surface methodology in addition to a classic unilateral evaluation, revealed data that better illustrated the kind of interactions.

MATERIAL AND METHODS

Chemicals

Folin-Ciocalteu phenol reagent was from Fluka (Steinheim, Germany). 2,4,6-Tripyridyl-*s*-triazine (TPTZ) and α-tocopherol were from Sigma Chemical Co (St. Louis, MO, U.S.A.).

Vinification by-products

Seeds from two widely cultivated wine grape varieties were chosen; one red used for white wine production (Moschofilero) and one red used for red wine production (Agiorgitiko). All samples used were obtained from wineries within the prefecture of Attica (central Greece), located in the region of Megara and collected immediately after processing of grapes. All material was transferred within a few hours to the laboratory and stored at – 40 °C.

Preparation of extracts

Stems were lyophilised and ground to a fine powder using a domestic blender. An amount of approximately 0.5 g of material was placed in a 30-mL glass vial with 10 mL of solvent, composed of combinations of ethanol, as shown in Table 1. All solvent systems used contained citric acid (1 g L^{-1}) and were adjusted to the desired pH using 1N NaOH.

Extractions were carried out under magnetic stirring at 400 rpm, at room temperature (22±2 °C) for predetermined time periods. Both solvent composition and extraction time were chosen on the basis of previous investigations to afford extracts with maximum antiradical activity (**Karvela *et al.*, 2009**). Upon completion of extraction, the extracts were filtered through paper filter, and stored at – 20 °C until analysed. All extracts were also filtered through 0.45-μm syringe filters prior to determinations.

Following extraction, extracts were freeze-dried and a suitable amount of each freeze-dried extract was dissolved in ethanol/water (1/1). This extract solution was used for testing interactions with α-tocopherol. α-Tocopherol was dissolved in ethanol.

Table 1 Optimal conditions used for the recovery of seed extracts

Extract	Optimal conditions		
	EtOH (%)	pH	t (h)
Moschofilero	40	3.26	5
Agiorgitiko	60	4.56	5

Determination of total polyphenol (TP) concentration

Measurements were carried out according to a previously published protocol (**Arnous *et al.*, 2002**), employing the Folin-Ciocalteu methodology. Gallic acid was used as the reference standard, and results were expressed as mg gallic acid equivalents (GAE) L^{-1}.

Determination of the reducing power (P_R)

A previously published protocol was employed to estimate the reducing power of the extracts (**Makris *et al.*, 2007**), based on the TPTZ-ferric reducing assay. All samples were diluted appropriately before the analysis. Sample (0.05 mL) appropriately diluted with methanol was mixed thoroughly with 0.05 mL $FeCl_3$ solution (4.25 mM in 0.06 N HCl), and incubated for 30 min in a water bath at 37°C. Following this, 0.9 mL TPTZ solution (1.07 mM in 0.06 N HCl) were added, and the absorbance was recorded at 620 nm after exactly 5 min.

Determination of Mixture Effect (ME)

According to **Peyrat-Maillard *et al.*, 2003**, as mixture effect (ME) of two antioxidants could be defined the experimental value, divided by the calculated value, which is the sum of the effects of the two antioxidants obtained individually. If this ratio is > 1, then it can be said that synergism is observed, whereas a ratio < 1 would reveal antagonism. In the case of the reducing power assay, this could be mathematically expressed as:

$$ME = \frac{A_{620}^{AO/Extr}}{A_{620}^{AO} + A_{620}^{Extr}} \quad (1)$$

Where *AO*, is the antioxidant (α-Tcp) and *Extr* the stem extract.

Statistical analyses

Implementation of linear regression: For pure α-Tcp solutions, the response (A_{620}) was plotted against concentration and the linear equation, as well as the square correlation coefficient (R^2) drawn from simple linear regression analyses were calculated (Table 2). For the solutions of antioxidant / extract mixtures, responses were plotted against the total antioxidant concentration of the solutions, consisted of equal concentrations (mg L^{-1}) of α-Tcp and TP (extract). In all cases, the concentration ranges used were those within which linearity was best maintained ($R^2 > 0.99$). The concentration of the extracts was expressed as mg GAE L^{-1} TP, as determined by the Folin-Ciocalteu assay.

Table 2 Concentration ranges and statistical data generated after implementing simple linear regression of A_{620} against concentration of α-Tcp, seed extracts and α-Tcp / seed extract combinations

Extract	Concentration range (mg L^{-1})	Equation	R^2
α-Tcp	5 - 20	y = 0.004x + 0.052	1.000
Sd-Mf	5 - 20	y = 0.025x + 0.073	0.999
Sd-Ag	5 - 20	y = 0.042x + 0.046	0.998
St-Ag / α-Tcp	1 - 20	y = 0.057x + 0.018	1.000
St-Mf / α-Tcp	1 - 20	y = 0.042x + 0.022	1.000

Implementation of 3×3 factorial design: A 3 × 3 factorial experiment design was used to identify the relationship existing between the response function (A_{620}) and variables (concentration of α-Tcp and TP), as well as to determine those conditions that optimised the response. The two independent variables or factors used were i) α-Tcp concentration and ii) TP concentration of the extracts. Concentrations were coded at three levels, as shown in Table 3.

For each independent variable, the experimental range was based on the results of preliminary experiments. The independent variables were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i},\ \text{i} = 1, 2 \quad \textbf{(2)}$$

Where x_i and X_i are the dimensionless and the actual value of the independent variable i, X_0 the actual value of the independent variable i at the central point, and

Table 3 Experimental values and coded levels of the independent variables used for the 3×3 factorial design

Independent variables	Code units	Coded variable level		
		-1	0	1
[TP] / mg L^{-1}	X_1	5	55	105
[α-Tcp] / mg L^{-1}	X_2	5	55	105

ΔX_i the step change of X_i corresponding to a unit variation of the dimensionless value. Response function at each design point was recorded (Tables 4,5). One-way ANOVA permitted to check the statistical significance of the regression coefficients deriving from the model. Response surface plot was obtained using the fitted model, by keeping the independent variables simultaneous. All determinations were carried out at least in triplicate and values were averaged. For all statistics, SigmaPlot™ 11 and JMP™ 8 were used.

RESULTS

Linear regression approach

In Fig. 1 are given the linear regression graphs illustrating the relationship between the A_{620} and concentration of extracts and α-Tcp. From the equations describing the linear

regressions (Table 2) can be seen that Sd-Ag was a more effective reducing agent, followed by Sd-Mf and α-Tcp. Mixtures composed of equal concentrations of extracts and antioxidants were then tested in a similar manner (Fig. 2).

For reasons of identifying possible mixture effects (ME) resulting from interactions of the extracts with α-Tcp, a hypothesis was set up. By choosing an average concentration value of 25 mg L^{-1} and using the equation for α-Tcp in Table 2, it could be calculated that A_{620} = 0.152. Likewise, using the equation for Sd-Ag it was obtained a A_{620} = 1.096. In the same fashion, the equation corresponding to Sd-Ag / α-Tcp mixture would give A_{620}= 1.443. By replacing the A_{620} values to equation (1), it is calculated that ME = 1.16, which clearly points to synergism. However, the value of 25 mg L^{-1} chosen does not fall within the limits where linearity is obeyed for the equation for Sd-Ag / α-Tcp in Table 2. This might happen because beyond a concentration of 20 mg L^{-1} interactions of the polyphenols in Sd-Ag extract with α-Tcp might not follow a linear relationship and therefore linear regression cannot be implemented. To overcome this practical limitation, interactions could be recorded by simultaneously switching the concentration of both α-Tcp and Sd-Ag, by deploying a factorial design.

Table 4 Measured and predicted A_{620} values of St-Ag / α-Tcp mixtures, determined for individual design points

Design point	Independent variables		Response (A_{620})	
	X_1	X_2	Observed	Predicted
1	-1	-1	0.070	0.084
2	-1	1	0.695	0.739
3	1	-1	0.067	0.032
4	1	1	0.897	0.893
5	-1	0	0.488	0.430
6	1	0	0.442	0.481
7	0	-1	0.072	0.093
8	0	1	0.891	0.851
9	0	0	0.452	0.491
10	0	0	0.510	0.491

Table 5 Measured and predicted A_{620} values of St-Mf / α-Tcp mixtures, determined for individual design points

Design point	Independent variables		Response (A_{620})	
	X_1	X_2	Observed	Predicted
1	-1	-1	0.050	0.050
2	-1	1	0.540	0.540
3	1	-1	0.055	0.048
4	1	1	0.578	0.572
5	-1	0	0.301	0.300
6	1	0	0.301	0.314
7	0	-1	0.050	0.057
8	0	1	0.560	0.565
9	0	0	0.313	0.316
10	0	0	0.331	0.316

Factorial design approach

The experimental values of A_{620} were analysed by multiple regression to fit the second-order polynomial equations shown in Table 6 and the quality of fit was ascertained using the coefficients of determination (R^2). The experimental values showed a good fit with the equations, which were statistically acceptable at least at 99.9% significance level ($p < 0.001$). This fact indicated a highly satisfactory agreement between observed and predicted responses and that the equations found can adequately predict the experimental results. The utilisation of the predictive models enabled the theoretical calculation of the optimal sets of conditions, under which maximal A_{620} could be attained (Table 7), within predetermined concentration ranges. The trends revealed in each case were recorded in the form of three-dimensional plots (Figs. 3,4).

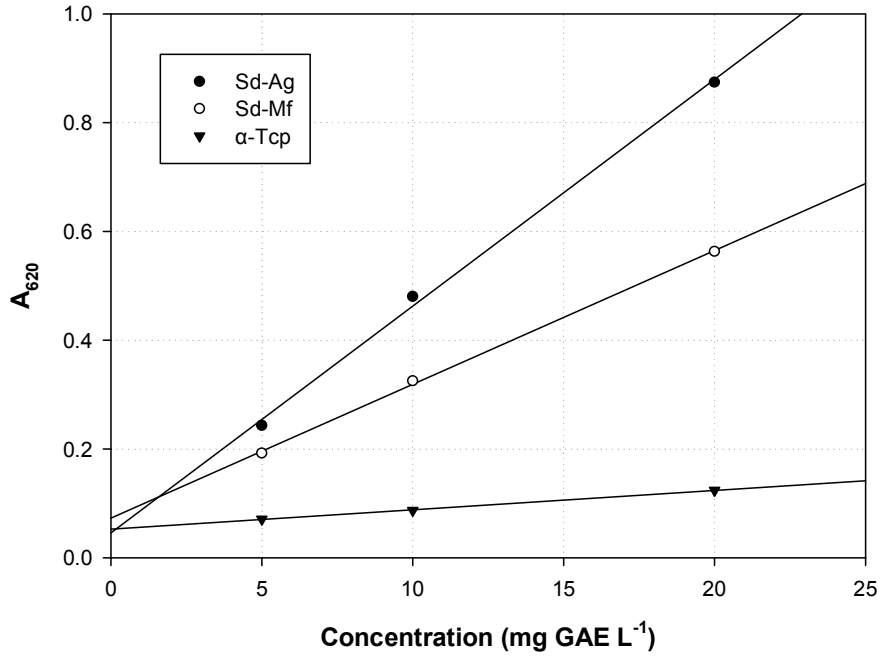

Figure 1 Linear regression showing the response (A_{620}) as a function of concentration of the seed extracts and α-Tcp

In order to test the validity of the models established, or to point out discrepancies with the linear regression approach, a similar hypothesis as above was used. By replacing in the equation obtained for the Sd-Ag / α-Tcp (Table 6) the coded values corresponding to a concentration of 12.5 mg L^{-1} for α-Tcp and 12.5 mg L^{-1} for Sd-Ag (total concentration = 25 mg L^{-1}), it was found a A_{620} = 0.145. Unlike in the linear regression approach, it is calculated that ME = 0.12, which clearly shows that the interactions resulted in antagonism. In the same manner and using the equation for Sd-Mf / α-Tcp, the ME was found 0.08. As can be seen, the interactions also manifested antagonistic effects.

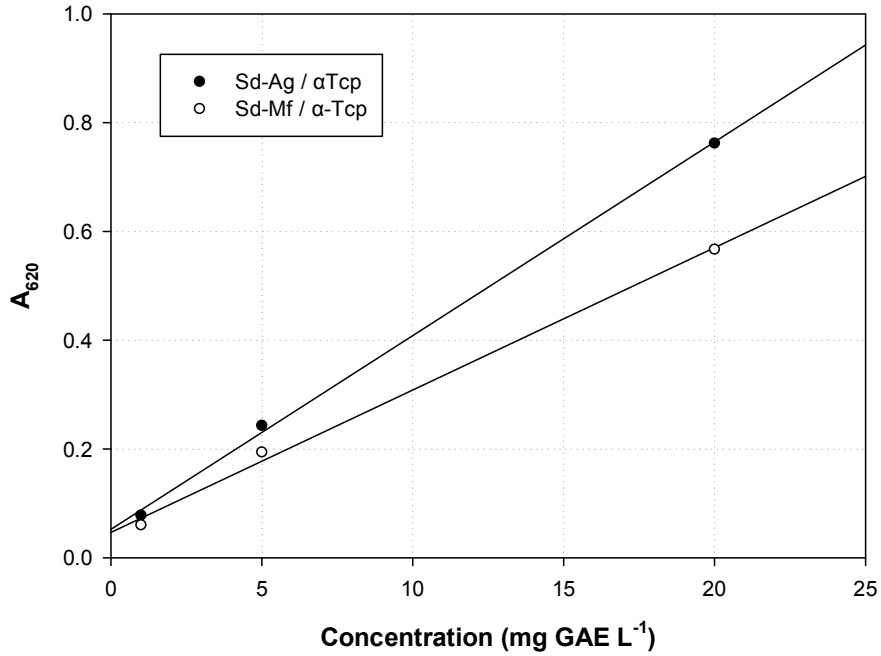

Figure 2 Linear regression showing the response (A_{620}) as a function of concentration of the seed extracts combined with equal concentration of α-Tcp

DISCUSSION

The "deactivation" of oxidant species by polyphenolic antioxidants (POH) is based, with regard to food systems that are deteriorated by peroxyl radicals ($R^{\bullet}$), on the donation of hydrogen, which actually interrupts chain reactions:

$$R^{\bullet} + POH \rightarrow R\text{-}H + PO^{\bullet}$$

Phenoxyl radicals ($PO^{\bullet}$) generated according to this reaction may stabilized through resonance and/or intramolecular hydrogen bonding, as proposed by **Bors *et al.*, 1990** for quercetin, or combine to yield dimerisation products, thus terminating the chain reaction:

$$PO^{\bullet} + PO^{\bullet} \rightarrow PO\text{-}OP$$

As pointed out by **Brand-Williams *et al.*, 1995**, and **Bondet *et al.*, 1997**, the efficiency of an antioxidant component to reduce $R^{\bullet}$ largely depends on its hydrogen-donating ability. It has been proposed that in binary mixtures of antioxidants several phenomena might occur, but

Table 6 Polynomial equations and statistical parameters describing the effect of the independent variables on the response (A_{620}) for all antioxidant mixtures tested, calculated after implementation of 3×3 factorial design

Antioxidant Mixture	**2nd order polynomial equations**	R^2	p
St-Ag / α-Tcp	$0.491 + 0.379X_1 + 0.026X_2 + 0.051X_1X_2 - 0.019X_1^2 - 0.035X_2^2$	0.99	0.0008
St-Mf / α-Tcp	$0.316 + 0.254X_1 + 0.007X_2 + 0.008X_1X_2 - 0.005X_1^2 - 0.009X_2^2$	1.00	< 0.0001

coupled reactions of regeneration could be taken into consideration to explain the ME observed (**Peyrat-Maillard *et al.*, 2003**). In this regard, the results anticipated could include (i) a synergistic effect if the less efficient antioxidant regenerates the more efficient one, (ii) an antagonistic effect if the more efficient molecule regenerates the less efficient one or (iii) no ME if both antioxidants have the same efficiency. Thus in a given antioxidant assay it is important to rank the substances used, to obtain an order of efficiency, as assumptions on their interactions are based on their relative antioxidant strength.

Considering the slopes of the equations describing the linear regressions in Table 2, the order of reducing power was as follows:

Sd-Ag > Sd-Mf > α-Tcp.

Table 7 Optimal, predicted concentration ratios and theoretically calculated maximal response (A_{620}) for all mixtures tested, obtained from the implementation of the 3×3 factorial design

Mixture	Maximal predicted response	Optimal ratio (mg L^{-1} / mg L^{-1})
St-Ag / α-Tcp	0.893±0.137	105 / 105
St-Mf / α-Tcp	0.572±0.028	105 / 99.85

That is, the extracts were the more powerful antioxidants than α-Tcp and therefore the antagonism observed by implementing response surface methodology was rather a consequence of regeneration of α-Tcp by the polyphenols contained in the extracts.

This theory can be rationalised by the concession that, as mentioned above, the the more efficient molecule regenerates the less efficient one. Taking into account the oxidation potentials, antagonism can be considered as the regeneration of a compound with higher oxidation potential, to the expense of another with lower oxidation potential, by donating H atoms. Hence regeneration of α-Tcp by the polyphenols resulting in antagonism could occur if the polyphenol-containing extract had, in total, lower oxidation potential than α-Tcp.

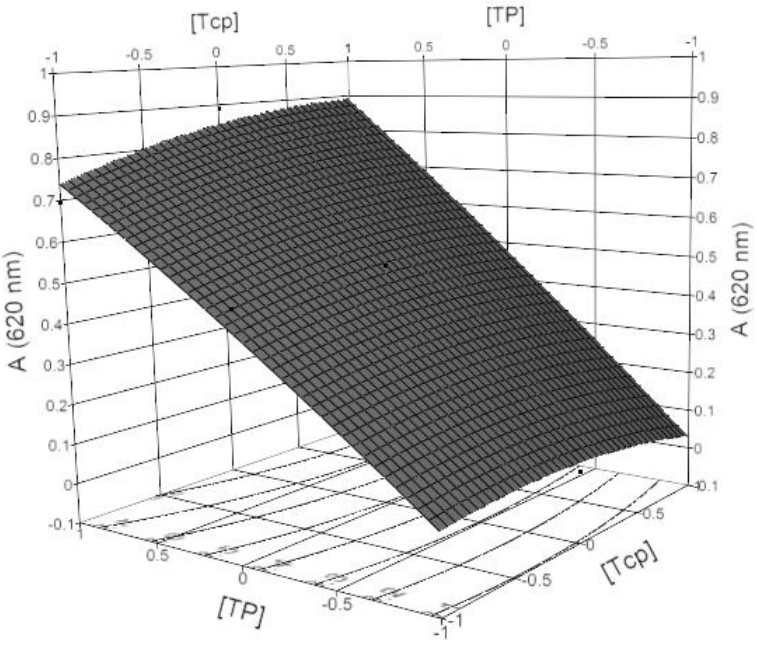

Figure 3 Three dimensional surface plots illustrating the response (A_{620}) upon simultaneous variation of TP and α-Tcp concentrations, for the St-Ag extract

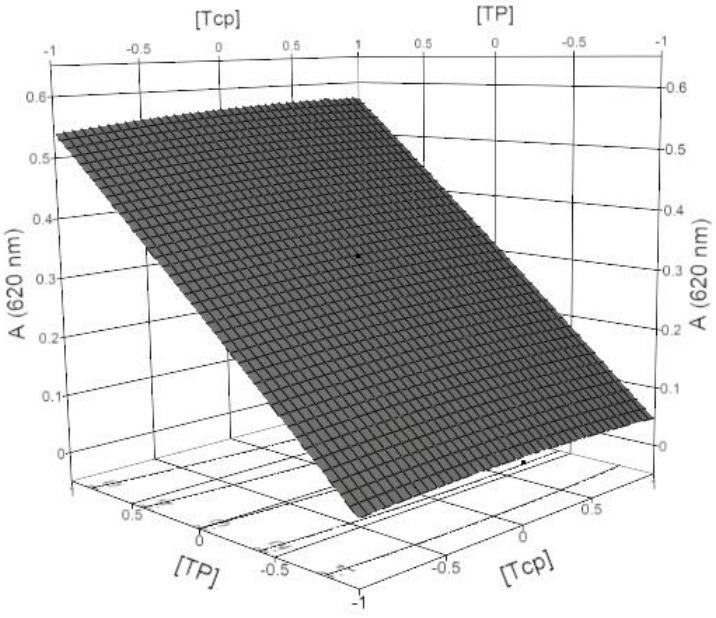

Figure 4 Three dimensional surface plots illustrating the response (A_{620}) upon simultaneous variation of TP and α-Tcp concentrations, for the St-Mf extract

Studies pertaining to flavanols / α-Tcp interactions provided sound evidence that various catechin derivatives can very efficiently regenerate α-Tcp, with the highest reaction rates being displayed by those derivatives possessing lower oxidation potentials (**Mukai *et***

al., **2005**). Data on regeneration of α-Tcp by flavanols in a phospholipid model system (**Pazos *et al*., 2009**), and tea flavanols during linoleic acid (JIA et al., 1998) or methyl lenoleate peroxidation (**Pedrielli and Skibsted, 2002**) are in agreement.

Although synergistic phenomena were revealed by using the equations extracted from the linear regressions, from studies pertaining to interactions in binary antioxidant mixtures there has been substantial evidence that the regenerating ability of an antioxidant towards another also depends on the relative amounts of the two antioxidants in the mixture. This has been demonstrated in a series of mixtures of flavonoids using FRAP assays (**Hidalgo *et al*., 2010**), combinations of α-Tcp and AA with various flavonols (**Hiramoto *et al*., 2002**), quercetin with α-Tcp and astaxanthin (**Becker *et al*., 2007**), and combinations of α-Tcp and myricetin (**Marinova *et al*., 2008**).

Therefore, by employing equal amounts of α-Tcp and TP, the antioxidant responses recorded might be misleading with respect to the effect observed (synergism or antagonism), as this could greatly depend on the relative amounts of the two components interacting. To overcome this unilateral assessment, a factorial design was implemented with the aim of detecting trends in the antioxidant response upon concomitant variation of concentrations of both α-Tcp and the polyphenolic concentration of the extracts.

The outcome of the factorial design approach indicated that the TP concentration had always statistically significant contribution in the expression of the reducing power in all mixtures tested (Table 8). On the other hand, in no case the concentration of α-Tcp was found significant, a phenomenon that could be attributed to the higher reducing power of the seed polyphenols compared with α-Tcp. It appears that there might be a threshold beyond which the concentration of an antioxidant becomes insignificant with respect to contributing to reducing effects, owed to profounder antagonism. This assumption, however, remains to be elucidated by more detailed studies involving model polyphenols.

CONCLUSIONS

A practical limitation arising from the use of linear regression to assess the efficiency of an antioxidant mixture is that beyond a certain point the linearity is not obeyed by the relationship between A_{620} and concentration. To overcome the lack of linearity, as well as to record the antioxidant behaviour by changing simultaneously the concentrations of both constituents in the mixtures tested, factorial design was deployed and provided the

appropriate mathematical tool to examine extract interactions with α-Tcp over a wider range of concentrations.

Table 8 *P*-values illustrating the significance of the concentration of [TP] of the extracts and [α-Tcp], in the mixtures tested by the 3x3 factorial design. Values were determined deploying one-way ANOVA

Extract	[TP]	[α-Tcp]
St-Ag / α-Tcp	< 0.0001	0.3206
St-Mf / α-Tcp	< 0.0001	0.2220

The response surface methodology used disclosed that polyphenol-containing extracts from a rich source, such as grape seeds, exhibit antagonism when combined with α-Tcp, a fact ascribed to the regenerating ability of polyphenols towards α-Tcp. It was also emphasised that maximal efficiency in the mixtures tested was a result of an ideal ratio of concentrations of TP and α-Tcp.

The approaches attempted in this study put in question investigations carried out by employing binary mixtures of antioxidants, combined at predetermined ratios. The evidence emerged from the investigations performed herein clearly suggests that to ascertain the behaviour of a system composed of two antioxidants, it is indispensable that a factorial design should be established, to enable reliable prediction of the response(s) within appropriate limits. This is particularly crucial for antioxidants that are destined to be added in food matrices, where maximal antioxidant protection is always sought.

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