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Evaluation of mixture effects in binary solutions of ascorbic acid with grape (*Vitis vinifera*) seed extracts using Response Surface Methodology

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Abstract

Extracts from seeds from three different varieties of grape were combined with ascorbic acid (AA) to evaluate the effects on the antiradical behaviour, using the DPPH assay. The investigations aimed at illustrating possible mixture effects, manifested either as synergism or as antagonism. By employing a simple linear regression approach, combinations of all extracts exhibited antagonism when combined with AA at concentration ratio of 1:1. Furthermore, to thoroughly investigate the role of the relative concentrations of the antioxidants, a 3×3 factorial design was implemented. This approach enabled the recording of the responses upon simultaneous variation of both the polyphenol concentration of the extracts and AA in the mixtures. In this case too, it was demonstrated that combinations of grape seed extracts with AA resulted in antagonism. This effect was discussed on the basis of grape seed composition and plausible antioxidant interactions.

Introduction

Oxidation is a multilateral process that brings about deleterious phenomena in food quality, affecting the organoleptic characters, safety and nutritional value (Choe and Min, 2009). As a result, foods undergone extensive oxidation may suffer major defects and have no consumer acceptability. Oxidation in foods is manifested mainly through discoloration and generation of off-flavours, while alteration of principal substrates, such as vitamins, lipids and proteins, is not always apparent. However, since oxidation is unequivocally associated with significant loss of nutritionally precious ingredients, as well as the formation of potentially toxic substances, the shielding of food matrices against oxidative deterioration is of undisputed importance.

Oxidation can be minimised to some extent by removing factors contributing to catalysis and initiation, such as free fatty acids and trace metal ions, or by excluding direct exposure to light. However, the incorporation of antioxidants to delay or even inhibit oxidation is the commonest practice and antioxidants such as butylated hydroxytoluene (BHT) and similar analogues 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.

The efforts for using naturally occurring antioxidant compounds as food additives for effective protection of various foods, has almost exclusively been expended on the application of polyphenols or polyphenol-containing extracts (Yanishlieva *et al*., 2006; Kiokias *et al*., 2008), whereas the utilisation of other substances with proven antioxidant ability, such as carotenoids, is extremely limited, most likely because, in addition to their higher instability, their recovery from various sources is more laborious, time-consuming and of higher cost. The antioxidants added in foods protect susceptible matrices from oxidation, but they are consumed in redox reactions occurring in foods with time. 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.

Based on such a concept, this investigation was undertaken with the scope of assessing possible effects of synergism / antagonism in mixtures of a well-known natural antioxidant of wide occurrence, ascorbic acid (AA) 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

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of response surface methodology in addition to a classic unilateral evaluation, revealed data that better illustrated the kind of interactions.

Materials and Methods

Chemicals

Folin-Ciocalteu phenol reagent was from Fluka (Steinheim, Germany). Gallic acid, 2,2´-diphenyl-picrylhydrazyl (DPPH•) stable radical and ascorbic acid were from Sigma Chemical Co (St. Louis, MO, U.S.A.).

Vinification by-products

Seeds from three widely cultivated wine grape varieties were chosen; one white (Savatiano), 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. Seeds were collected immediately after processing of grapes, with the exception of Agiorgitiko, where seeds were obtained from pomace disposed after being in contact with the fermenting must for 12 days. Following collection, seeds were transferred within a few hours in the laboratory and stored at –40°C.

Preparation of extracts

Seeds were lyophilised and ground to a fine powder using a domestic blender. The powder was defatted with repeated extractions with hexane and the solvent after the final extraction was removed in vacuo. An amount of approximately 0.5 g of defatted 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 1 N 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 freeze-dried. A suitable amount of each freeze-dried extract was dissolved in ethanol/water (1/1). This extract solution was used for testing interactions with AA. AA was dissolved in ethanol.

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}.

Table 1. Optimal conditions used for the recovery of seed extracts with the highest $\%\Delta A_{515}$

Extract	Optimal conditions		
	EtOH (%)	pH	t (h)
Moschofilero	50	4	3
Savatiano	40	6	5
Agiorgitiko	40	2	5

Determination of the antiradical activity (%ΔA$_{515}$)

The absorbance of a DPPH• solution (100 μM in methanol) was obtained at 515 nm ($A_{515}^{t=0}$). An aliquot of sample (0.025 mL) was added to 0.975 mL DPPH• solution and the absorbance at 515 nm was read after exactly 30 min ($A_{515}^{t=0}$). The % decrease in DPPH• absorbance was calculated as

$$\%\Delta A_{515} = \frac{A_{515}^{t=0} - A_{515}^{t=30}}{A_{515}^{t=0}} \times 100$$

Determination of mixture effects (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 DPPH assay employed in this study, this could be mathematically expressed as:

$$ME = \frac{\%\Delta A_{515}^{AO/Extr}}{\%\Delta A_{515}^{AO} + \%\Delta A_{515}^{Extr}} \qquad (1)$$

Where *AO*, is the antioxidant (AA) and *Extr* the seed extract.

Statistical analyses

Implementation of linear regression

For pure AA or pure extract solutions, the response ($\%\Delta A_{515}$) 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 AA / extract mixtures, responses were plotted against the total antioxidant concentration of the solutions, which consisted of equal concentrations (mg L^{-1}) of AA and extract (Table 2). In all cases,

Table 2. Concentration ranges and statistical data generated after implementing simple linear regression of $\%\Delta A_{515}$ against concentration of solutions of GSE, AA and combinations thereof

Compound	Concentration range (mg L^{-1})	Equation	R^2
AA	5-80	0.252x – 0.378	0.998
Mf	5-40	1.221x + 4.325	0.997
Ag	5-40	1.390x + 3.544	0.999
St	80-320	0.393x + 1.375	0.999
Mf + AA	40-320	0.211x + 24.080	0.999
Ag + AA	20-120	0.623x + 16.442	0.998
St + AA	80-320	0.200x – 0.583	0.995

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
[AA] / mg L^{-1}	X_2	5	55	105

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} total polyphenols (TP), as determined by the Folin-Ciocalteu assay.

Implementation of 3×3 factorial design

A 3 x 3 factorial experiment design was used to identify the relationship existing between the response function ($\%\Delta A_{515}$) and variables (concentration of AA and TP), as well as to determine those conditions that optimised the response. The two independent variables or factors used were i) AA 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:

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

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 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 - 6). 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.

Table 4. Measured and predicted $\%\Delta A_{515}$ values of Ag / AA mixtures, determined for individual design points

Design point	Independent variables		Response ($\%\Delta A_{515}$)	
	X_1	X_2	Observed	Predicted
1	-1	-1	12.04	11.82
2	-1	1	93.72	93.92
3	1	-1	38.59	37.85
4	1	1	95.50	95.18
5	-1	0	75.24	75.26
6	1	0	87.85	88.91
7	0	-1	23.67	24.62
8	0	1	94.22	94.34
9	0	0	82.42	81.87
10	0	0	82.40	81.87

Table 5. Measured and predicted $\%\Delta A_{515}$ values of St / AA mixtures, determined for individual design points

Design point	Independent variables		Response ($\%\Delta A_{515}$)	
	X_1	X_2	Observed	Predicted
1	-1	-1	4.00	3.68
2	-1	1	38.90	39.73
3	1	-1	31.00	30.06
4	1	1	64.20	64.41
5	-1	0	22.00	21.49
6	1	0	46.30	47.02
7	0	-1	16.50	17.76
8	0	1	54.00	52.96
9	0	0	36.00	35.14
10	0	0	34.50	35.14

Table 5. Measured and predicted $\%\Delta A_{515}$ values of St / AA mixtures, determined for individual design points

Design point	Independent variables		Response ($\%\Delta A_{515}$)	
	X_1	X_2	Observed	Predicted
1	-1	-1	4.00	3.68
2	-1	1	38.90	39.73
3	1	-1	31.00	30.06
4	1	1	64.20	64.41
5	-1	0	22.00	21.49
6	1	0	46.30	47.02
7	0	-1	16.50	17.76
8	0	1	54.00	52.96
9	0	0	36.00	35.14
10	0	0	34.50	35.14

Table 6. Measured and predicted $\%\Delta A_{515}$ values of Mf / AA mixtures, determined for individual design points

Design point	Independent variables		Response ($\%\Delta A_{515}$)	
	X_1	X_2	Observed	Predicted
1	-1	-1	6.50	7.11
2	-1	1	76.00	77.21
3	1	-1	33.50	32.21
4	1	1	85.40	84.71
5	-1	0	52.10	50.27
6	1	0	64.60	66.57
7	0	-1	20.50	21.17
8	0	1	83.00	82.47
9	0	0	59.30	59.93
10	0	0	60.70	59.93

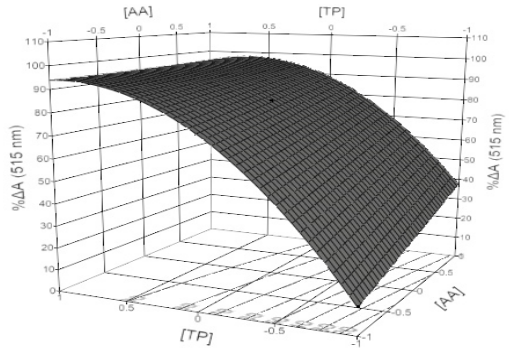

Figure 1. Three dimensional surface plots illustrating the response (%ΔA_{515}) upon simultaneous variation of TP and AA concentrations, for the Ag extract

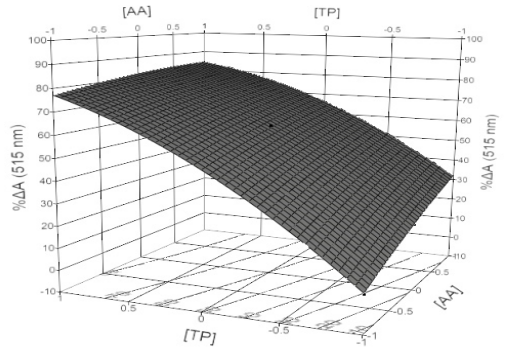

Figure 2. Three dimensional surface plots illustrating the response (%ΔA_{515}) upon simultaneous variation of TP and AA concentrations, for the Mf extract

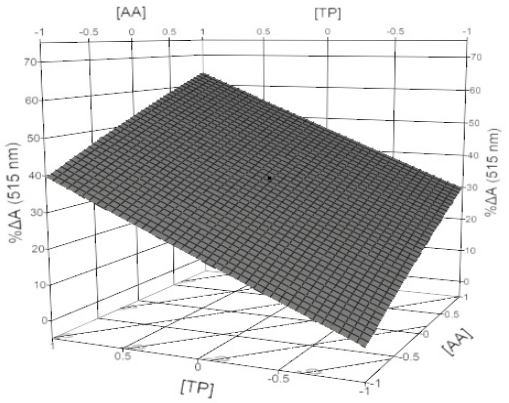

Figure 3. Three dimensional surface plots illustrating the response (%ΔA_{515}) upon simultaneous variation of TP and AA concentrations, for the St extract

All determinations were carried out at least in triplicate and values were averaged. For all statistics, SigmaPlot™ 11 and JMP™ 8 were used.

Results and Discussion

Considering the slopes of the equations describing the linear regressions in Table 2, the order of antiradical activity was as follows: Ag > Mf > Sv > AA. To identify possible mixture effects (ME) resulting from interactions of the extracts with AA, a hypothesis was set up. By choosing an average concentration value of 50 mg L^{-1}, based on the concentration ranges used for all extracts, and using the equation for AA in Table 2, it could be calculated that %ΔA_{515} = 12.22. Likewise, using the equation for Mf, it was obtained a %ΔA_{515} = 65.38. In the same fashion, the equation corresponding to Mf / AA mixture would give %ΔA_{515} = 34.63. By replacing the %ΔA_{515} values to equation (1), it was calculated that ME = 0.45, which clearly pointed to antagonism. However, the value of 50 mg L^{-1} chosen does not fall within the limits where linearity is obeyed between polyphenol concentration in Mf extracts and %ΔA_{515}. This does not happen because the value obtained for %ΔA_{515} is higher that the theoretical upper limit, which is 100, but because beyond a concentration of 50 mg L^{-1} radical scavenging by Mf extract might not follow a linear relationship with polyphenol concentration and therefore linear regression cannot be implemented.

To overcome this practical limitation, interactions for all combinations tested could be recorded by simultaneously switching the concentration of both the antioxidant and the extract polyphenols, deploying a factorial design. The experimental values of %ΔA_{515} were analysed by multiple regression to fit the second-order polynomial equations shown in Table 7 and the quality of fit was ascertained using the square correlation coefficients (R^2). The experimental values showed a good fit with the equations, which were statistically acceptable at least at 99.99% significance level ($p < 0.0001$). 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 %ΔA_{515} could be attained (Table 8), within predetermined concentration ranges. The trends revealed in each case were recorded in the form of three-dimensional plots (Figures 1, 2 and 3).

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 Mf / AA (Table 7) the coded values corresponding to a concentration of 25 mg L^{-1} for AA and 25 mg L^{-1} for Mf (total concentration = 50 mg L^{-1}), it was found a %ΔA_{515} = 32.15. Just like in the linear regression approach, it was calculated that ME = 0.47 and can be argued that the interactions also resulted in antagonism. In the same manner, the ME for Ag / AA and St / AA were 0.52 and 0.37, respectively.

Table 7. Polynomial equations and statistical parameters describing the effect of the independent variables on the response ($\%\Delta A_{515}$) for all antioxidant mixtures tested, calculated after implementation of 3×3 factorial design

Antioxidant Mixture	2nd order polynomial equations	R^2	p
Ag	$81.87+34.86X_1+6.82X_2-6.19X_1X_2-22.39X_1^2$	1.000	<0.0001
St	$35.14+17.60X_1+12.77X_2$	0.998	<0.0001
Mf	$59.93+30.65X_1+8.15X_2-4.4X_1X_2-8.11X_1^2$	0.998	<0.0001

Table 8. Optimal, predicted concentration ratios and theoretically calculated maximal response ($\%\Delta A_{515}$) 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})
Ag / AA	98.01±1.81	85.85 / 104.15
St / AA	64.41±3.12	105 / 105
Mf / AA	84.71±4.46	105 / 105

In all mixtures examined, the extract was the most powerful antioxidant and therefore the antagonism observed was rather a consequence of regeneration of AA 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. 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 AA by the polyphenols resulting in antagonism could occur if the polyphenol-containing extract had, in total, lower oxidation potential than AA.

A strong background to support such a theory are studies carried out with cyclic voltametry, where it has been demonstrated that polyphenols such as catechin possess lower oxidation potential than AA and that catechin exhibited significantly lower antioxidant activity in the presence of AA (Abou Samra *et al*., 2011). This is particularly important, because detailed analysis of the extracts used, in a previous work (Karvela *et al.*, 2009), indicated that the predominant polyphenolic compounds are catechins and some proanthocyanidins, which are catechin oligomers. Thus it would be reasonable to presume that the antiradical activity of extracts in the presence of AA behaves in a similar manner to that seen in catechin / AA mixtures.

Although the antagonistic phenomena recorded could also be revealed by using the equations extracted from the linear regressions (Table 2), 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 DPPH• and FRAP assays (Hidalgo *et al*., 2010), combinations of α-tocopherol and AA with various flavonols (Hiramoto *et al.*, 2002), quercetin with α-tocopherol and astaxanthin (Becker *et al*., 2007), and combinations of α-tocopherol and myricetin (Marinova *et al.*, 2008).

The outcome of the factorial design approach indicated that the TP and AA concentration had always statistically significant contribution in the expression of antiradical activity in all mixtures tested (Table 7). Although for St / AA and Mf / AA mixtures the optimal theoretical responses were obtained using solutions with equal AA and TP concentrations (Table 8), in the case of Ag / AA mixtures, it was shown that the ratio deviates from this analogy. Thus by using equal amounts of AA and TP, the antioxidant responses recorded might be misleading with respect to the magnitude of antagonism, as maximal effects could greatly depend on the relative amounts of two antioxidants interacting.

Conclusion

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 $\%\Delta A_{515}$ 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 AA over a wider range of concentrations. The response surface methodology used disclosed that polyphenol-containing extracts from a rich source, such as grape seeds, exhibit antagonism when combined with AA, a fact ascribed to the regenerating ability of polyphenols towards AA. It was also emphasised that maximal efficiency in the mixtures tested is a result of an ideal ratio of concentrations of TP and AA. 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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