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STABILIZATION OF OXIDATIVE PROCESSES IN COOKED SAUSAGES BY OPTIMIZATION OF INCORPORATED BIOLOGICALLY ACTIVE SUBSTANCES

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ABSTRACT

Nowadays, many substances of plant origin are used for their antioxidant properties. The aim of the study was to stabilize oxidative processes in cooked sausages by optimization of the incorporated biologically active substances (BAS). For this purpose, a full factorial design with three factors at two levels was used. Sodium L-ascorbate (x1); dihydroquercetin from *Larix sibirica* Ledeb (x2) and lyophilized (30% w/v) ethanol extract from dry distilled rose (*Rosa damascena* Mill) petals (DDRPE) (x3) were used as BAS. The antioxidant activity (DPPH and FRAP assays), the stabilizing effect on the oxidative processes in the lipid and protein fraction, and the color stability of the cross-cut surface were investigated. The optimization was performed according to the target functions as follows: Minimum TBA values and protein carbonyls as well as maximum color lightness (L*) and redness (a*). Cooked sausages with optimized BAS composition were characterized by: TBA value = 0.80 mg MDA / kg; PC = 0.118 nmol DNPH / mg protein; L* = 56.69 and a* = 18.73. The mathematically defined combined optimum according to the four target functions was determined at: 0.10 g x1/ kg; 0.09 g x2/ kg and 0.10 g x3/ kg.

1.Introduction

The ongoing oxidative changes in cooked meat products lead to a decrease in nutritional value (Dominguez *et al.,* 2019) and an increased risk to human health (García-Lomillo *et al.,* 2017; Estévez, 2021). The malondialdehyde (MDA) and protein carbonyls (PC) as end products of the lipid and protein oxidation are used as indicators for the quality of cooked meat products (Estévez *et al.,* 2005).

The substances capable of electron donation or antioxidants are those who inhibit the oxidative processes. Their origin can be either synthetic or natural and all of them comes with their pros and cons (Aminzare *et al.,* 2019). Synthetic antioxidants such as butylated hydroxytoluene (BHT) were used for years to improve the food quality and extend the shelf life (Hashemi Gahruie *et al.,* 2017), but there are evidences linking them to an increased health risks (Carocho *et al.,* 2015). Recently, the so-called "clean label" products gained interest (Oswell *et al.,* 2018; Estévez, 2021). In this regard, more and more extracts of natural origin (Aminzare *et al.,* 2019; Rather *et al.,* 2016) have established themselves as possible additives in the production of meat products. Found in fruits and herbs, bioactive substances such as polyphenols, carotenoids, and tocopherols with strong antioxidant properties (Oswell *et al.,* 2018) can slow down the development of lipid oxidation (García-Lomillo *et al.,* 2017). Ascorbic acid and its salts like sodium L-

ascorbate are widely used in meat processing and under the regulation of the EC (Cenci-Goga *et al.,* 2020). Dihydroquercetin isolated from Siberian larch (*Larix sibirica* Ledeb) has been used to suppress oxidative processes in meat products (Ivanov *et al.,* 2009; Balev *et al.,* 2017). Kobyalko *et al.,* (2009) suggest the combination of dihydroquercetin and L-ascorbic acid as a possible option. Vlahova-Vangelova *et al.,* (2014) suggest the use dry distilled rose (*Rosa damascena* Mill.) petals extract in combination with dihydroquercetin. Therefore, the quality of cooked sausages can be improved due to the rich content of biologically active substances (BAS) with well-defined antioxidant and antimicrobial properties (Baydar and Baydar, 2013; Dragoev *et al.,* 2021).

Our hypothesis is that the triple optimized BAS blend (Sodium L-ascorbate - x1, Dihydroquercetin - x2 and Dry distilled rose petals extract - x3) can increase the oxidative stability of cooked sausages. Therefore, the aim of the study is to inhibit lipid and protein oxidation expressed by the TBA value and the formation of protein carbonyls (PC) as well as to limit the discoloration (L*, a*) of the cross-cut surface of the model system of cooked sausages by optimized incorporation of BAS.

2. Materials and methods

2.1. Materials

The chilled deboned beef shoulder and pork bacon were delivered from the slaughterhouse of Unitemp Ltd., Voyvodinovo village, Bulgaria (48 h *post mortem*). The experiment was carried out in the Department of Meat and Fish Technology at the University of Food Technologies, Plovdiv, Bulgaria. The beef and pork (1:1 w/w), chilled to -1°C are ground in a mincer with mesh diameter of 3 mm and then divided into 9 portions. Each part of minced meat is mixed with the appropriate pre-prepared amounts of BAS (Table 1) and mixed for 10 min using a mixer. Mixed filling masses are separately stuffed in polyamide coatings with diameter of 50 mm. An industrial steam boiler (ALLROUND – SYSTEM "RONDAIR", Rauch and Wärmetechnik GmbH&Co.KG, West Germany) was used for the cooking process. The cooking is considered as finished upon reaching 72°C in the diametrical center. The cooked sausages are water cooled for 20 min and stored at 0+4°C for 7 days.

Table 1. Full factorial design of the experiment

Design points	Coded values of added antioxidant compounds			Sodium L-ascorbate, g/kg	Dihydroquercetin isolate, g/kg	Lyophilized DDRPE, g/kg
	x1	x2	x3	x1	x2	x3
1	-	-	-	0.00	0.00	0.00
2	+	-	-	0.10	0.00	0.00
3	-	+	-	0.00	0.10	0.00
4	+	+	-	0.10	0.10	0.00
5	-	-	+	0.00	0.00	0.10
6	+	-	+	0.10	0.00	0.10
7	-	+	+	0.00	0.10	0.10
8	+	+	+	0.10	0.10	0.10
9	C	C	C	0.05	0.05	0.05

Triple BAS blend: sodium L-ascorbate (x1) which is commonly used, antioxidant with defined synergistic properties (Staykov *et al.,* 2016) is food grade and bough form certified

local distributor; Dihydroquercetin isolate of *Larix sibirica* Ledeb (x2) was bought from Flavit Ltd. (Pushtino, Russia) (dihydroquercetin (96%), dihydrokaempferol (3%) and naringenin (approx. 1%) (Ivanov *et al.,* 2009); the lyophilized (30% w/v) ethanol extract of dry distilled rose *Rosa damascena* Mill petals (x3) was prepared in the Department of Food Preservation and Refrigeration Technology at the University of Food Technologies. The dried distilled rose petals are a by-product of the rose oil industry, containing more than 30 polyphenolic compounds with antioxidant properties (Dragoev *et al.,* 2021).

All other reagents and standards are an analytical grade. Trichloroacetic acid, 2-Thiobarbituric acid, Malondialdehyde, 2,4-Dinitrophenylhydrazine, Hydrochloric acid (37%, puriss.), 2,4,6-Tris(2-pyridyl)-s-triazine, Trolox, 2,2-Diphenyl-1-picrylhydrazyl are purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Guanidine Hydrochloride from Fisher Scientific (New Jersey, USA). The Iron (III) chloride is from Reidel-de Haen (Germany). Sodium Chloride, Ethanol (99%), Ethyl acetate, Methyl alcohol, Sodium acetate and Acetic acid (99%) are from Fillab Ltd. (Plovdiv, Bulgaria).

2.2. Methods

A homogenized mean sample was prepared according the recommendations of Esbensen & Wagner, (2014) and immediately tested.

2.2.1. Antioxidant activity analysis

For the methanol extraction, mixture of 10 g homogenized sample and 100 cm^3 methyl alcohol (99.9%) were left for 12 hours in a refrigerator (4 + 8° C). The solution was filtered through folded filter paper (Filtrax, Grade 391). The resulting extract is stored in a refrigerator.

Radical scavenging activity was determined by DPPH analysis, based on the method of Brand-Williams, Cuvelier and Berset (1995), with modifications by Dinkova et. al (2014). 250 μL extract directly mixed in a UV-macro cuvette with 2250 μL of methanolic DPPH solution (2,2-Diphenyl-1-picrylhydrazyl) (6×10^{-5} M). The absorbance is measured at 515 nm after 15 min in dark. The results are presented as μmol TE (Trolox equivalent)/100g sample.

The iron reducing potential was measured by FRAP test according to Benzie and Strain (1996) with some modifications by Dinkova et al. (2014). The FRAP reagent was prepared by mixing 2.5 mL of a solution of TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) (10 mmol / L) in hydrochloric acid (40 mmol / L), 2,5 mL aqueous $FeCl_3$ solution (20 mmol / L) and 25 mL acetate buffer (0.3 mol / L, pH 3.6). 250 μL extract mixed with 2250 μL of FRAP reagent in a UV-macro cuvette. After 4 minutes in dark at room temperature the absorbance is measured at 593 nm. The results are presented as μmol TE (Trolox equivalent)/100g sample.

2.3. Color characteristics

A Konica Minolta colorimeter CR-410 (Konica Minolta Holding, New Jersey, USA) was used to assess the lightness (L*), redness (a*) and yellowness (b*) on the cross-cut surface of the cooked sausages (Hunt *et al.,* 2012).

2.4. Determination of lipid and protein oxidation's products

The 2-thiobarbituric acid (TBA) test was determined by the method proposed by Botsoglou *et al.,* (1994). Ten grams homogenized sample are mixed with 50 cm^3 0.9% NaCl and 50 cm^3 10% trichloroacetic acid, stirred, left for 10 min and filtrated. A 4 cm^3 extract is mixed with fresh 1% solution of 2-Thiobarbituric acid and heated at 70°C for 30 min. Absorbance is measured at 532 nm using a dual-beam UV-VIS spectrophotometer Camspec, model M 550 (Camspec Ltd., Sawston, UK). The results are presented as mg MDA/kg sample.

Protein oxidation products, expressed by the concentration of carbonyl groups, were determined by the method of Mercier et al., (2004). Briefly, two parallel 0.5 cm^3 aliquot homogenate for each sample were mixed with 10% trichloroacetic acid and centrifuged at 5500 rpm for 10 min. To the first one a 1 cm^3 of 2 N HCl is added and to the second a same volume of 0.2% (w/v) 2,4-dinitrophenylhydrazine

(DNPH) in 2 N HCl. After 1h incubation at room temperature and occasional shaking, bout sample were precipitated with 10% trichloroacetic acid and centrifuged at 5500 rpm for 10 min. The precipitate is washed twice ethanol: ethyl acetate (1:1v/v), and the left proteins are dissolved in 2 cm^3 6M guanidine HCl. Last centrifuging is done at 5500 rpm for 10 min to eliminate insoluble impurities. A dual-beam UV-VIS spectrophotometer Camspec, model M 550 (Camspec Ltd., Sawston, UK) was used to measure the absorbance at 280 nm for HCl controls and at 370 nm for the DNPH treated samples. The results are presented as nmol DNPH/ mg protein.

2.6. Data analysis

For the antioxidant activity analysis, a two-way ANOVA was used. The BAS mixture and time of storage were used as the two factors of the statistical analyze at $\alpha = 0.05$.

A full factorial design was determined by three factors (x1, x2 and x3) at two levels (0.00 and 0.10g/kg) following the methodology suggested by Severino et al., (2012) (Table 1). The optimization was performed according to the target functions as follows: minimum values of the lipid oxidation by-products (TBA value) and protein oxidation products (PC) as well as maximum values of lightness (L*) and redness (a*) of the color on the cross-cut surface. Target functions are assessed after 7 days of refrigerated storage at 0 - 4°C.

3.Results and discussions

3.1. Antioxidant activity of the cooked sausages

The free radical scavenging activity of the cooked sausages evaluated by the DPPH• radical showed that the triple BAS blend in concentrations of 0.10 g/kg led to a 95.6% ($p<0.05$) increased radical scavenging activity compared to control (Table 2).

Table 2. Antioxidant activity expressed by DPPH and FRAP assay

Design points	DPPH•, µmol TE/100 g	DPPH•, µmol TE/100 g	FRAP, µmol TE/100 g	FRAP, µmol TE/100 g
	1st day	7th day	1st day	7th day
1	$16.33^{a,x}\pm0.61$	$12.67^{a,y}\pm0.56$	$151.73^{a,x}\pm0.88$	$141.89^{b,y}\pm0.54$
2	$18.67^{b,x}\pm0.61$	$14.67^{b,y}\pm0.37$	$161.40^{b,x}\pm1.21$	$135.96^{a,y}\pm0.63$
3	$80.50^{c,x}\pm0.94$	$60.00^{c,y}\pm1.94$	$266.42^{d,x}\pm1.48$	$230.76^{d,y}\pm1.61$
4	$216.33^{f,x}\pm1.35$	$206.67^{g,y}\pm0.64$	$420.47^{g,x}\pm0.85$	$390.95^{h,y}\pm1.40$
5	$200.33^{e,x}\pm1.78$	$170.00^{e,y}\pm2.04$	$201.40^{c,x}\pm1.22$	$182.20^{c,y}\pm0.84$
6	$135.67^{d,x}\pm1.15$	$119.67^{d,y}\pm1.43$	$283.89^{e,x}\pm0.56$	$274.08^{e,y}\pm0.90$
7	$239.33^{h,x}\pm1.99$	$180.33^{f,y}\pm1.46$	$445.41^{h,x}\pm1.37$	$387.43^{g,y}\pm0.89$
8	$369.00^{i,x}\pm1.19$	$355.00^{h,y}\pm0.49$	$561.07^{i,x}\pm1.68$	$539.63^{i,y}\pm1.29$
9	$227.67^{g,x}\pm0.98$	$168.33^{e,y}\pm0.72$	$340.19^{f,x}\pm1.15$	$297.10^{f,y}\pm0.65$

*Results are presented as Means±SEM

a,b,c,d,e,f,g,h,i indexes indicating significant differences ($p<0.05$) between Means by columns

x,y indexes indicating significant differences ($p<0.05$) between Means by columns for one each parameter separately

The results from the ferric reduction activity potential (FRAP) assay showed similar to the DPPH• values, correlation between the combinations of the BAS. Both at 1-st and 7-th day of the storage (0+4°C), highest ($p<0.05$) FRAP values were measured in design point 8 (DP 8) (Table 2). A well-defined trend in both DPPH• and FRAP values was observed.

Lowest DPPH•/FRAP values – control < Single BAS< Combination of two BAS < Triple BAS blend - Highest DPPH•/FRAP values.

This trend confirmed our hypothesis that the combination of two or three BAS can increased the overall antioxidant activity and by that to increase the oxidative stability of the cooked sausages.

A decrease in the DPPH• and FRAP values on the seventh day of the experiment was evaluated. This could be explained by the ongoing oxidative processes, which leads to the consumption of substances with antioxidant properties.

3.2. Oxidative stability of the cooked sausages

Four response surfaces (Fig. 1) and four second-order polynomial equations (equation 1-4) were generated showing the correlation between the values of the target functions and either combination or concentrations of added BAS.

$$TBA = 0.925 + 0.853 * x1 + 0.483 * x2 + 0.173 * x3 - 6.225 * x1^2 - 12.500 * x1 * x2 + 3.500 * x1 * x3 - 3.225 * x2^2 - 6.500 * x2 * x3 - 4.225 * x3^2, mg\ MDA/kg \quad (1)$$

$$PC = 0.145 + 0.170 * x1 + 1.160 * x2 - 1.590 * x3 + 3.803 * x1^2 - 1.000 * x1 * x2 - 12.000 * x1 * x3 - 6.197 * x2^2 - 5.000 * x2 * x3 + 19.803 * x3^2, nmol\ DNPH\ /\ mg\ protein \quad (2)$$

$$L^* = 56.074 - 9.379 * x1 + 13.991 * x2 - 5.019 * x3 + 17.690 * x1^2 + 44.000 * x1 * x2 + 133.000 * x1 * x3 - 78.310 * x2^2 - 76.000 * x2 * x3 + 21.690 * x3^2 \quad (3)$$

$$a^* = 19.427 + 4.166 * x1 + 0.506 * x2 - 35.484 * x3 - 41.859 * x1^2 + 7.500 * x1 * x2 + 70.500 * x1 * x3 - 39.859 * x2^2 + 66.500 * x2 * x3 + 176.141 * x3^2 \quad (4)$$

Each of three design points 2, 3 and 5 showed an inhibitory effect on MDA formation (Fig. 2 a), Equation 1). However, the combination of the triple BAS blend (DP 8) was evaluated with the lowest ($p<0.05$) accumulation of MDA (Fig. 1 a). At the 7-th day of the cold storage (0+4°C), cooked sausages prepared with the triple BAS blend in concentration of 0.10 g/kg (DP 8) are characterized by the least amount of MDA (0.76±0.04 mg MDA/kg, $p<0.05$), which is 26.2% lower than the control (DP 1) – 1.03±0.04 mg MDA/kg ($p<0.05$). The formation of secondary products of lipid oxidation well correlates with the established antioxidant activity (DPPH and FRAP values) of the cooked sausages (Table 2).

The results for protein oxidation were similar to the obtained for lipid oxidation. Significant reduction on the formation of protein carbonyls was found at concentrations 3×0.05 g/kg of the triple BAS blend (DP 9 – 0.11±0.01 nmol DNPH/mg protein, $p<0.05$) (Fig. 1 b). The most pronounced was in DP 8 at 3×0.10 g/kg of the triple BAS blend (Fig. 2 b), Equation 2). The protein oxidation in the DP 8 was 33.3% (0.10±0.02 nmol DNPH/mg protein, $p<0.05$) lower than the control (0.15±0.01 nmol DNPH/mg protein, $p<0.05$). One possible reason for those results are the highest DDPH and FRAP values in DP 8 and DP 9.

Near to control (56.01±0.03) was L* value in sausages prepared with triple BAS blend (DP 9 – 56.30±0.05). The lightness of the color in DP 8 (56.75±0.03) after 7 days of cold storage (0+4°C) is comparable and a little bit higher (by 1.30%, $p\leq0.05$) to the control. In the design points prepared with single use of BAS or combination of two is observed a darkening of the color (lower L* values) of cross cut surface (Fig. 1 c) and 2 c), Equation 3).

The results evaluated in the a* values show opposite to L* values trend (Fig 1 d). The triple BAS blend in concentrations of 0.10 g/kg (DP 8) showed a slight decrease in a* values compared

to the control (Fig. 2 d), Equation 4). At the end of storage period the control was characterized by the highest redness of the color (19.36±0.07) which was 3.25% higher ($p \leq 0.05$) than the evaluated in DP 8 (18.73±0.07).

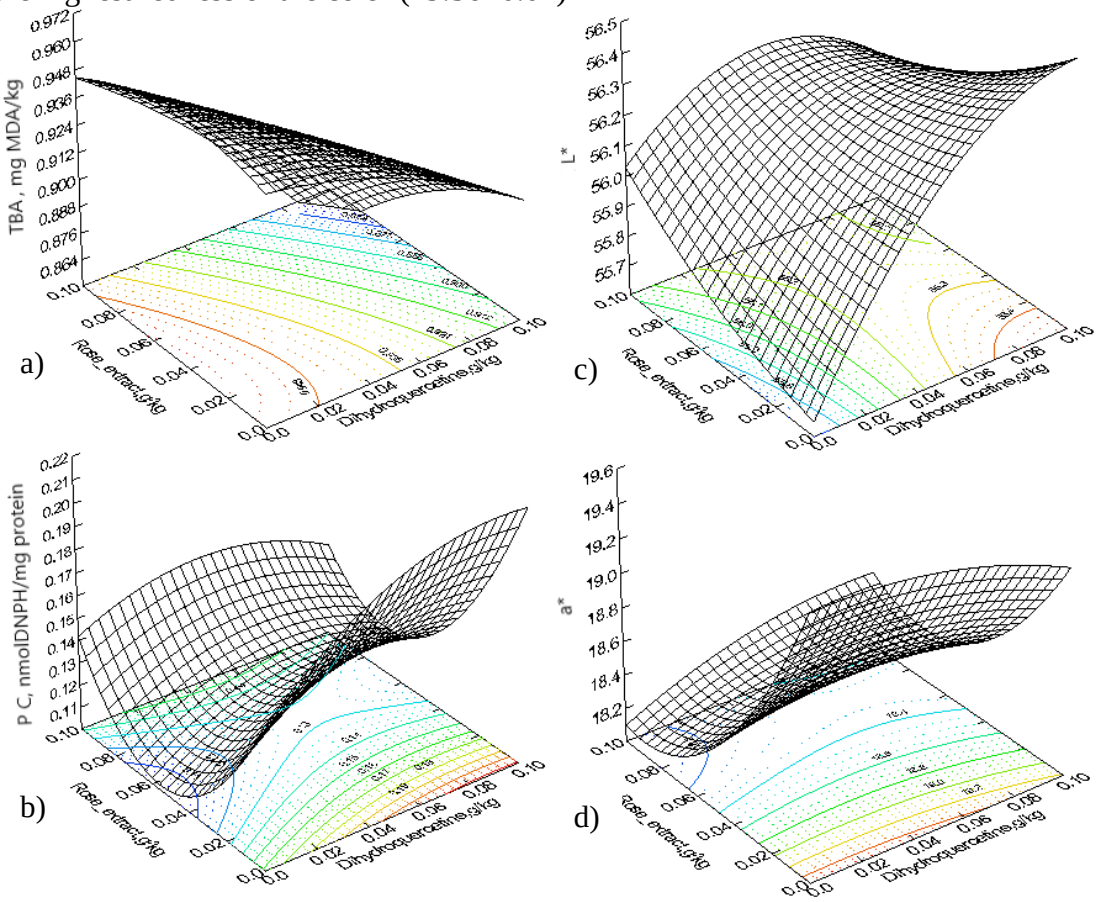

Figure 1. Response surfaces of the four target functions: a) TBA value = f(x2, x3), x1= const; b) PC = f(x2, x3), x1= const; L*= f(x2, x3), x1= const; a*= f(x2, x3), x1= const

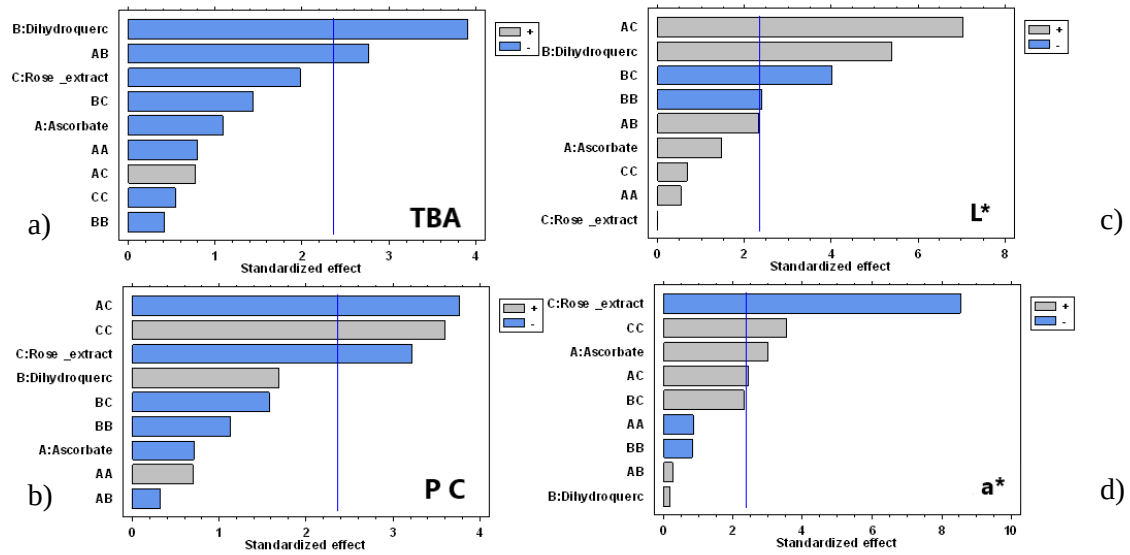

Figure 2. Pareto chart of standardized effects on the target functions of the experiment

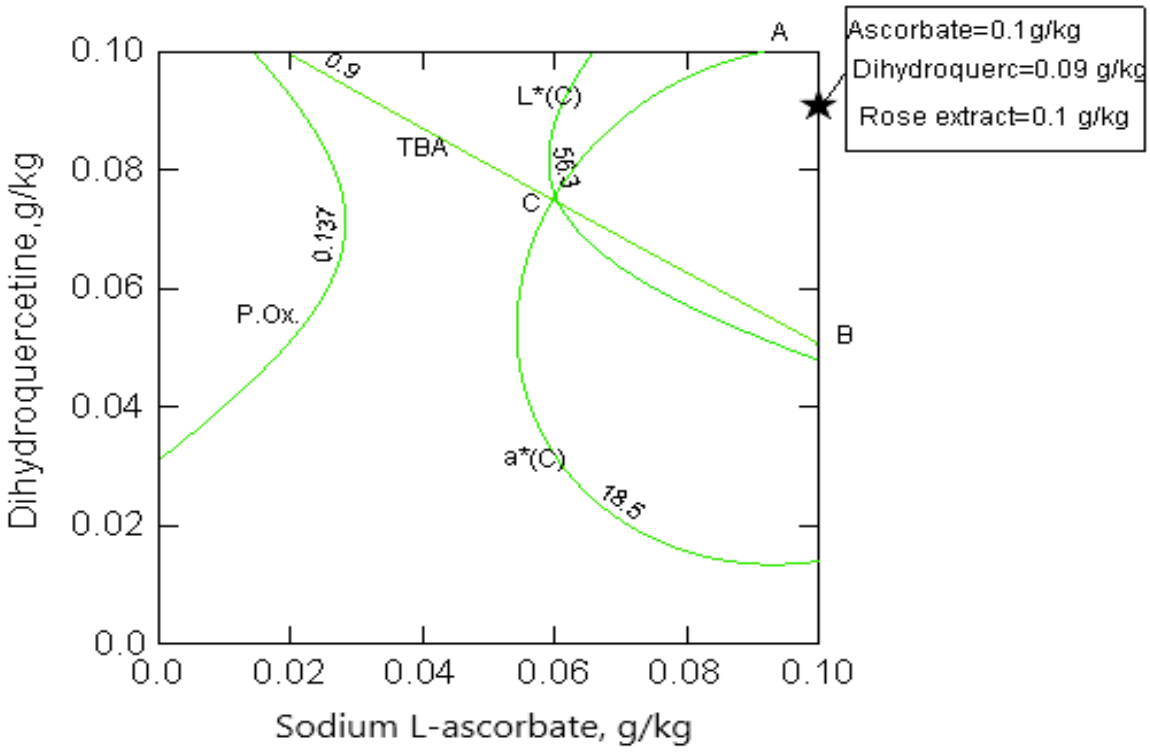

Figure 3. Graphical optimization of the added biologically active substances

The mathematically defined combined optimum (Fig. 3) according to the four target functions was determined at: 0.10 g sodium L-ascorbate (x1)/kg, 0.09 g dihydroquercetin (x2)/kg and 0.10 g DDRPE (x3)/kg.

The potential synergistic effect between the three used BAS may be the reason for the studied results. The use of triple BAS blend resulted in lower levels of MDA (inhibition of lipid oxidation), protein carbonyls (suppression of protein degradation) as well as prevention of the discoloration (inhibition of color pigments' oxidation) of the cooked sausages. In combination with a strong antioxidant (dihydroquercetin) the sodium L-ascorbate act as a synergist. Such synergism in three-component mixture of antioxidants (10 g/L dihydroquercetin, 5 g/L rosemary extract and 1 g/L L-ascorbic acid) was reported by Staykov et al., (2016). Minimal changes in color characteristics were found in DP 8 (Table 1). This suggests that antioxidant properties of used dihydroquercetin and DDREP could suspend the oxidation of the meat pigments (Ivanov et al., 2009; Vlahova-Vangelova et al., 2014). Kaempferol and quercetin glycosides found in DDREP (Baydar and Baydar, 2013; Dragoev et al., 2021) demonstrate free radical scavenging activity and ferric ion reducing antioxidant power (Table 2). The synergistic effects between dihydroquercetin and ascorbic acid, observed by Kobyalko et al., (2009) correspond to the inhibition of lipid and protein oxidative processes found by us. The protein oxidation could be inhibited when DDRPE was added to a matrix of cooked functional sausages with reduced nitrite content (Balev et al., 2019). Vlahova-Vangelova *et al.*, (2020) also reported the possibility of reducing the addition of nitrites in cooked sausages produced with the addition of dihydroquercetin or pork obtained from pigs fed with feed to which dry distilled rose petals had been added.

4. Conclusions

The increase of the antioxidant activity, the inhibition of the formation and accumulation of malondialdehyde, the decreased amounts of

protein carbonyls, and preservation of the cross-cut surface's lightness may be a consequence of synergism between the three biologically active substances.

The combination of sodium L-ascorbate, dihydroquercetin and lyophilized dried distilled rose petals extract in concentrations of 0.10 g/kg each, can be used as antioxidant mixture to inhibit both lipid and protein oxidation and to suspend the discoloration of the cross-cut surface of cooked sausages.

The optimized triple BAS blend can potentially be used in processing of cooked sausages with reduced nitrite addition or extended shelf life.

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