



Food Science and Applied Biotechnology

e-ISSN: 2603-3380

Journal home page: www.ijfsab.com
<https://doi.org/10.30721/fsab2025.v8.i1>



Research Article

Optimization of bioactive compound mixtures: Polyphenol content, antioxidant activity, and synergistic effect

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Abstract

The rapid expansion of the food industry has significantly increased the generation of by-products rich in bioactive compounds with untapped potential. This study aims to optimise the mixtures of bioactive compound by evaluation of the polyphenol content, antioxidant and synergistic effects. The Simplex-Centroid design and response surface methodology (RSM) combined with correlation analysis were used for this purpose. Each of the three investigated bioactive substances Sodium L-ascorbate (SLA); Dihydroquercetin isolate from *Larix sibirica* Ledeb (DHQ), and freeze-dried (30% w/v) ethanol extract from distilled rose (*Rosa damascena* Mill.) petals (FDRPE) contribute uniquely to redox reactions. Although FDRPE exhibited the lowest total polyphenol content (TPC) and weakest antioxidant activity, its combinations with SLA and DHQ demonstrated enhanced radical scavenging and metal-chelating activities. Response surface methodology indicated that dihydroquercetin (DHQ) alone could achieve the strongest antioxidant activity but requires higher concentrations. The results suggest that the combined use of SLA, DHQ, and FDRPE positively impacts radical scavenging activity, as well as transition metal-chelating activity (FRAP and CUPRAC). The optimum is achieved by combining a 6.11962×10^{-7} SLA + 88.5131 DHQ + 11.4869 FDRPE mg.L⁻¹. The optimized mixture have the potential to be incorporated into various food matrices at lower concentrations compared to their individual use, enhancing the practical application in the food industry.

Keywords

Simplex-Centroid design, sodium L-ascorbate, dihydroquercetin; freeze-dried spent rose petals extract

Abbreviations

HAT – hydrogen atom transfer; SET – single electron transfer; RMS – response surface methodology; SLA – sodium L-ascorbate; DHQ – dihydroquercetin; FDRPE – freeze-dried spent rose petals extract.

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Article history:

Received 16 September 2024

Reviewed 07 January 2025

Accepted 11 February 2025

Available on-line 18 March 2025

<https://doi.org/10.30721/fsab2025.v8.i1.466>
2025, UFT Academic publishing house, Plovdiv

Introduction

Polyphenols are secondary metabolic products from the development of plants and fruits (Efenberger-Szmechtyk et al. 2021), playing a crucial role in human health and regulation of oxidative stress (Zahra et al. 2024). The molecular structure of polyphenols includes an aromatic ring with one or more hydroxyl groups (-OH), and their antioxidant activity is directly related to the location and number of -OH groups (Birsa and Sarbu 2024). The antioxidant activity is also influenced by other functional groups associated with the aromatic ring (Gulcin 2020). Flavonoids and phenolic acids exhibit their radical-scavenging activity through a hydrogen atom transfer (HAT), single electron donation/transfer (SET) mechanism. They also bind the ions of transition metals (Fe^{3+} and Cu^{2+}) in the chelation process (Perron et al. 2010; Zahra et al. 2024). The metal-chelation inhibits the Fenton and Haber–Weiss reactions mainly responsible for the formation of reactive species such as hydroxyl ($\text{OH}\cdot$) radicals (Gulcin and Alwasel 2023).

Almost every processing of raw materials into intermediate or final products generates by-products, often referred to as "waste" products (Constantin et al. 2024). The expansion of food industry in last few decades lead to enormous increase of by-products generation. Failure to utilize these by-products leads to environmental pollution (Ebrahimi and Lante 2022; Şahin and Kurtulbaş 2024). By-products from the food industry are often rich in biologically active substances, such as phenolic acids, flavanols, anthocyanins, stilbenes, tannins, and L-ascorbic acid (Constantin et al. 2024; Dinkova et al. 2022; Dragoev et al. 2021).

The *Rosaceae* family includes about 2000 species, including rhizomatous, thorny, low-stem shrubs, or trees. The most important for the food industry are the dog rose (*Rosa canina* L.), white oil rose (*Rosa alba* L.), and Damask rose (*Rosa damascena* Mill.) (Petkova et al. 2020). *Rosa damascena* Mill. oil is an extremely expensive product, resulting from the enormous quantities of petals needed for its production, as its content is only 0.03 – 0.045% of the total mass of the petals (Slavov et al. 2017). According to reports from the Ministry of Agriculture, Food and Forestry, Bulgaria produces 10,000 to 15,000 tons of rose petals for oil-bearing roses annually (MAFF 2020; 2021; 2022; 2023).

The large quantities of by-products are a direct result of the low yield, and their processing and utilization would lead to significant economic and environmental benefits. According to Slavov et al. (2020) the extract from by-products of the white oil rose (*Rosa alba* L.) contains significant quantities of some phenolic acids, including rosmarinic acid, as well as substantial amounts of quercetin, quercetin-3- β -glucoside, kaempferol, and catechin. In a study of extracts from dried distilled petals of *Rosa damascena* Mill. Dragoev et al. (2021) report a similar quantitative ratio of quercetin, its glucosides, and kaempferol.

Various methods are reported for obtaining extracts from both fresh and spent petals, using different solvents: water, n-hexane, methanol, or ethanol (Liu et al. 2020; Petkova et al. 2020; Önder 2023). Baydar and Baydar (2013) investigate different methanol extracts from spent rose (*Rosa damascena* Mill.) petals, determining the total phenolic content using the Folin–Ciocalteu method, radical-scavenging ability (DPPH $\cdot$), and ferric reducing antioxidant power (FRAP). In addition of being a perfumery component, the extracts from the spent petals of *Rosa damascena* Mill. exhibit antioxidant activity against free radicals like DPPH $\cdot$ and ABTS $\cdot^+$, also iron-reducing activity potential (FRAP) and cupric reducing antioxidant capacity – CUPRAC, similar to that of α -tocopherol (Önder 2023). First reports date back in 2005, when Schieber et al. (2005) successfully extracted flavanol glycosides from distilled petals of *Rosa damascena* Mill. Later on, Shikov et al. (2008; 2012) and Dinkova et al. (2022) reported the presence of flavanol glycosides, mainly quercetin and kaempferol in a 30% aqueous-ethanol (v/v) extract. The extracts from spent petals of *Rosa damascena* Mill were used in various food matrixes like, canned strawberries (Shikov et al. 2012), cooked sausages (Kolev et al. 2022), aromatized Wine-Type Beverages (Slavov et al. 2023).

Dihydroquercetin (3,5,7,3,4-pentahydroxy flavanone) is a representative of the flavonoid group, produced from the bark of Siberian larch (*Larix sibirica* Ledeb) (Schauss et al. 2015). Dihydroquercetin inhibits the formation of free radicals and increases the oxidative stability of lipids in cell membranes (Rokaityte et al. 2019). It has been reported that dihydroquercetin exhibits antiradical activity in vivo even at low concen-

trations (0.0001 - 0.00001%). Studies on its antioxidant activity evaluated by the Oxygen Radical Absorbance Capacity (ORAC) at 95% purity of the extract have shown approximately 9.5 times stronger activity than vitamin C (Fomichev et al. 2016). Another study reports a 50 times greater antioxidant activity of the 99.6% pure Dihydroquercetin compared to vitamin C (Vilimanovich and Jevremovic 2019). As a representative of the flavonoid group, it also exhibits strong iron-reducing potential (Zahra et al. 2024). Gulcin (2020) describes the mechanism of forming a complex with free metal ions, and Topal et al. (2016) confirm that one molecule of dihydroquercetin can bind up to three iron ions. Some researches aim to increase the quality of the final product by increased shelf-life (Rokaitytė et al. 2017), while other successfully enhanced the sensory profile of the products (Staykov et al. 2016).

Mixtures/blends of biologically active substances exhibit significantly stronger antioxidant activity, even at lower concentrations, compared to their separate use (Bayram and Decker 2023). The observed phenomenon, expressed as an increase in inhibitory action when using a combination of two or more substances, is known as synergism (Efenberger-Szmechtyk et al. 2021). It's important that the occurrence of synergism can be influenced by multiple factors, such as the pH value, type of matrix, antioxidant type/concentration/ratio, also possible interactions with other food components, last but not least the processing conditions (Bayram and Decker 2023). Therefore, it's almost impossible to evaluate the occurrence of synergism with a single analytical method. In the meantime, some substances, usually considered "true" synergists, do not possess antioxidant activity themselves but enhance the effect of phenolic antioxidants when added to them (Rokaitytė et al. 2017; Kolev et al. 2022). These include some organic and inorganic acids - phosphoric, citric, L-ascorbic, etc. Instead of L-ascorbic acid (E 300) or in combination with it, its salts - sodium L-ascorbate (E 301) and sodium erythorbate (E 316) are also used (Wang and Chang 2015). Both L-ascorbic acid and sodium L-ascorbate possess radical-scavenging abilities against superoxide ($O_2^{\bullet-}$) and hydroxyl ($OH^{\bullet-}$) radicals (Bobolaki et al. 2018). The combination of dihydroquercetin with L-ascorbic acid not only inhibits lipid peroxidation and the formation of

malondialdehyde in vivo (Kobyalko et al. 2009) but has also been used in various model systems of meat products. A synergistic effect has been reported by Staykov et al. (2016), who studied beef sprayed with a three-component antioxidant mixture (10 g.L⁻¹ dihydroquercetin, 5 g.L⁻¹ rosemary extract *Rosmarinus officinalis*, and 1 g.L⁻¹ L-ascorbic acid) beef cuts. Rokaityte et al. (2019) reported inhibitory effect of dihydroquercetin on lipolysis and oxidation in dry-fermented sausages. Meanwhile Abilmazhinova et al. (2020) report a synergistic effect against oxidative process between dihydroquercetin with L-ascorbic in the production of minced meat. Most recently Kolev et al. (2022) studied the stabilization of oxidative processes in cooked sausages by optimization the incorporated blend of biologically active substances (Sodium L-ascorbate 0.10 g.kg⁻¹; dihydroquercetin 0.09 g.kg⁻¹ and lyophilised extract from dry distilled rose *Rosa damascena* Mill. petals). In modern food industry, antioxidant blends represent several appropriately selected antioxidants and synergists (Staykov et al. 2016). For example, substances that interrupt chain-radical processes in combination with neutralizers of the formed hydroperoxides (Gulcin 2020). This study aims to optimize bioactive compound mixtures by assessing polyphenol content, antioxidant potential, and synergistic interactions, with the goal of identifying sustainable, practical antioxidant solutions for the food industry.

Materials and Methods

Materials. For the purpose of the study were used: Sodium L-ascorbate (SLA) – 99% food grade (E301), bought from PI LTD (Bulgaria); Dihydroquercetin isolate from *Larix sibirica* Ledeb (DHQ), bought from Flavit Ltd. (Pushtino, Russia) containing - 96% dihydroquercetin, 3% dihydrokaempferol and approx. 1% naringenin and freeze-dried (30% w/v) ethanol extract from distilled rose (*Rosa damascena* Mill.) petals (FDRPE). The aliquot extract was concentrated under a vacuum at 60-70°C, later on, freeze-dried at -45 and 0.400 Mbar for 36 h using laboratory freeze dryer CRYODOS—50 (Telstar Industrial, Spain). For DHQ and FDPRE solutions was used 0.5 mL 96% ethanol to redissolve the substance before mixing with the distilled water.

Plan of the experiment and sample preparation.

Nine aliquot solutions of the biologically active substances (BAS) were prepared following the Simplex-Centroid design (Fig. 1). Contour plots of the response were used to determine the optimal ratio of the components and obtain a mixture with the strongest antioxidant activity. The selected model type is quadratic and the design fitted a model with both first and second-order terms (1).

$$Y = \alpha_1 * x_1 + \alpha_2 * x_2 + \alpha_3 * x_3 - \alpha_{12} * x_1 * x_2 + \alpha_{13} * x_1 * x_3 + \alpha_{23} * x_2 * x_3 \quad (1)$$

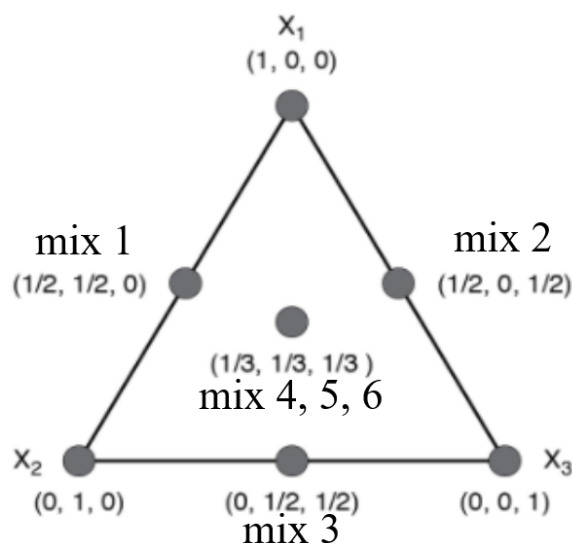


Figure 1. Diagram of simplex-centroid design: 1 – 100 mg.L⁻¹ SLA (X₁); 2 – 100 mg.L⁻¹ DHQ (X₂); 3 – 100 mg.L⁻¹ FDPRE (X₃); mix 1 – X₁+X₂/ 50+50 mg.L⁻¹; mix 2 – X₁+X₃/ 50+50 mg.L⁻¹; mix 3 – X₂+X₃/ 50+50 mg.L⁻¹; mix 4,5,6 – X₁+X₂+X₃/ 33,3+33,3+33,3 mg.L⁻¹

Total phenolic content (TPC). The method is based on the reaction between phenolic compounds and the Folin–Ciocalteu reagent and the absorbance was measured at $\lambda = 760$ nm, described by Vardakas et al. (2024). The standard curve was prepared using a gallic acid and results are presented as gallic acid equivalent.L⁻¹ (GAE.L⁻¹).

Content of phenolic compounds, phenolic acids and flavonoids. The Glories method was used as described in detail by Shopska et al. (2022), where the absorbance was measured at $\lambda = 280, 320$ and 360 nm. The results are presented as gallic acid equivalent.L⁻¹(GAE.L⁻¹) for phenolic compounds, caffeic acid equivalent.L⁻¹ (CAE.L⁻¹) for phenolic

acids, and quercetin equivalent.L⁻¹ (QE.L⁻¹) for flavonoids.

Determination of radical scavenging capacity.

For this purpose, the 1,1-diphenyl-2-picrylhydrazil radical (DPPH•) was used according to the method of Brand-Williams et al. (1995), with modifications from Dinkova et al. (2014). The absorbance of the decolorized solution was measured at $\lambda = 517$ nm. Also, the radical scavenging capacity against the 2,2-azinobis 3-ethylbenzthiazoline-6-sulfonic acid radical (ABTS•⁺) was evaluated at $\lambda = 734$ nm, following the methodology described by Shopska et al. (2022). Both radical scavenging capacities are presented as Trolox equivalent.L⁻¹ (TE.L⁻¹).

Determination of transition metals-chelating activity.

The ferric (Fe³⁺) reduction ability of plasma - FRAP assay was assessed at $\lambda = 593$ nm, according to the method of Benzie and Strain (1996) with modifications from Dinkova et al. (2014). At the same time, the cupric (Cu²⁺) reducing antioxidant capacity - CUPRAC test was established at $\lambda = 450$ nm, following the method described in detail by Shopska et al. (2022).

All above mentioned measurements were executed using a Helios Omega UV–Vis spectrophotometer coupled by VISION lite software (Thermo Fisher Scientific, Madison, USA).

Statistical analysis. The analysis was processed through the Statgraphics Centurion XVI® software. Results are presented as Estimated by the Quadratic Model Fitting values and Actual (measured) values are means $\pm$ standard deviation (n=10). Data was processed by the ANOVA ($p < 0.01$) analysis, the means were examined by the Tukey test ($\alpha = 0.01$). All studied dependencies establish inadequacy for ANOVA lack of fit, therefore the models are adequate. Pearson's correlations were used for factor analyses. Desirability analysis was conducted for 4 of the evaluated parameters (TPC, TF, DPPH and FRAP) to calculate the optimal concentration of BAS required to asses maximum phenol content, antioxidant activity and synergism. All measurements were performed in triplicates.

Results and Discussion

Phenolic content. Total phenolic content (TPC) is directly influenced by the used biologically active substance and its concentration. Solely use of DHQ exerted the highest TPC, followed by the SLA and

FDRPE (Fig. 2 - 3a). The all examined combinations of BAS followed a foreseen trend of the total phenolic content. The established effect is displayed by the equation of the fitted quadric model (2).

The optimized response for TPC is established in mixture composed by: 0 mg.L⁻¹ SLA; 100 mg.L⁻¹ – DHQ and 0 mg.L⁻¹ – FDRPE, with optimum value – 328.1 mg GAE.L⁻¹.

$$\text{TPC} = 292.191 * x_1 + 328.1 * x_2 + 70.8653 * x_3 - 23.6777 * x_1 * x_2 + 31.7706 * x_1 * x_3 + 41.651 * x_2 * x_3, \quad (2)$$

mg GAE.L⁻¹

The content of phenolic compounds (PC) assessed by the Glories method is significantly lower compared to the TPC by FC method, yet the samples followed a similar trend (Fig. 2 - 3b). Such a difference was also reported by others, when comparing phenolic content via both methods (Aleixandre-Tudo et al. 2017; Shopska et al. 2022)

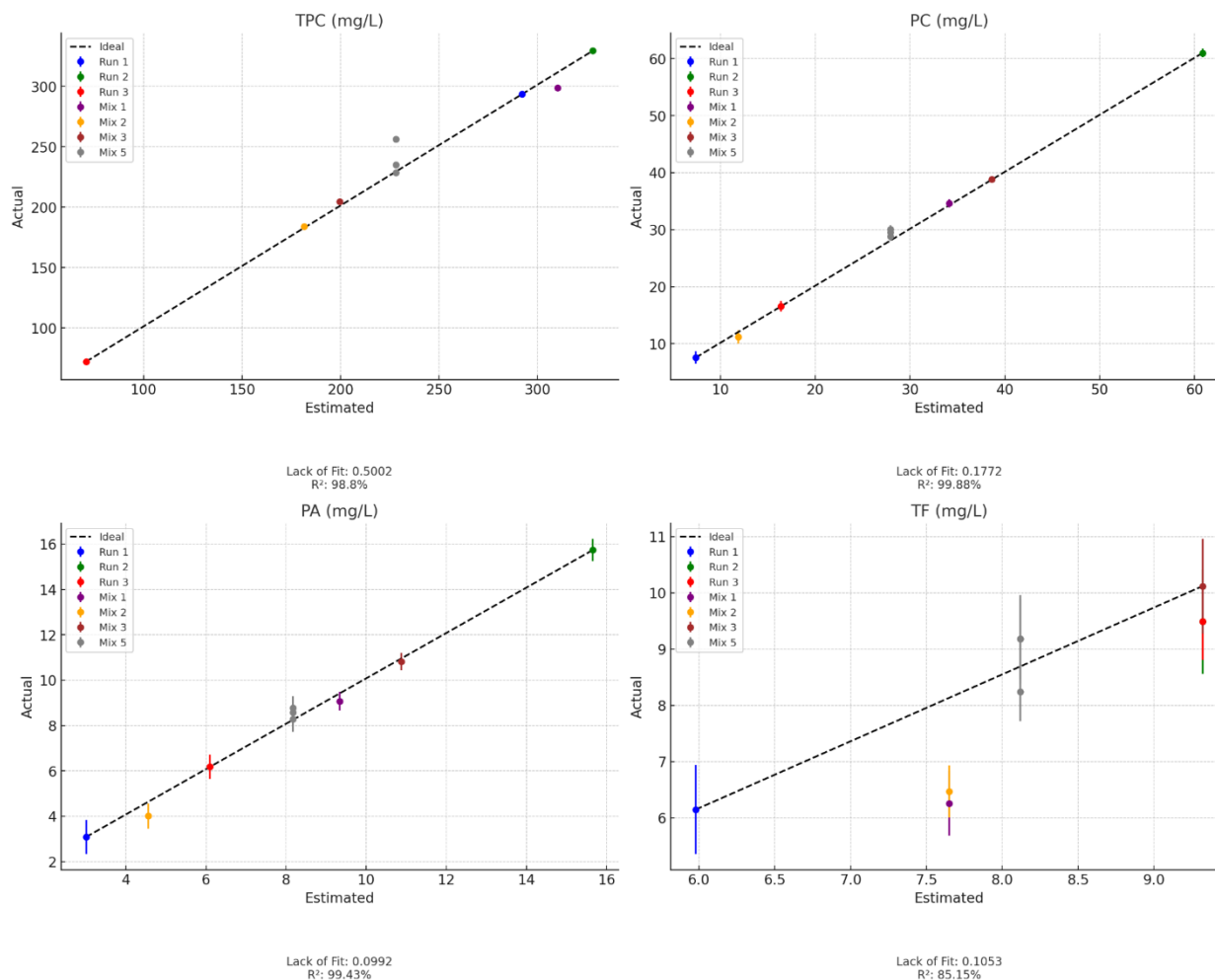


Figure 2. Scatter plots of the experimental runs for the total phenolic content (TPC), phenolic compounds (PC), phenolic acids (PA) and total flavonoids (TF)

As Aleixandre-Tudo et al. (2017) stated the main drawback of the FC method is that the reaction used for quantification of the presence of phenolic compounds is SET-based. Therefore, each substance possessing electron donation properties even not a phenolic compound will be quantified as such. The electron donation properties of the SLA

are well-known (Ramanathan et al. 2021) and they are confirmed by the presented results (Fig. 3a-b). As to the optimized response for PC, it's identical as for TPC - 0 mg.L⁻¹ SLA; 100 mg.L⁻¹ – DHQ and 0 mg.L⁻¹ – FDRPE, with optimum value – 60.84 mg GAE.L⁻¹.

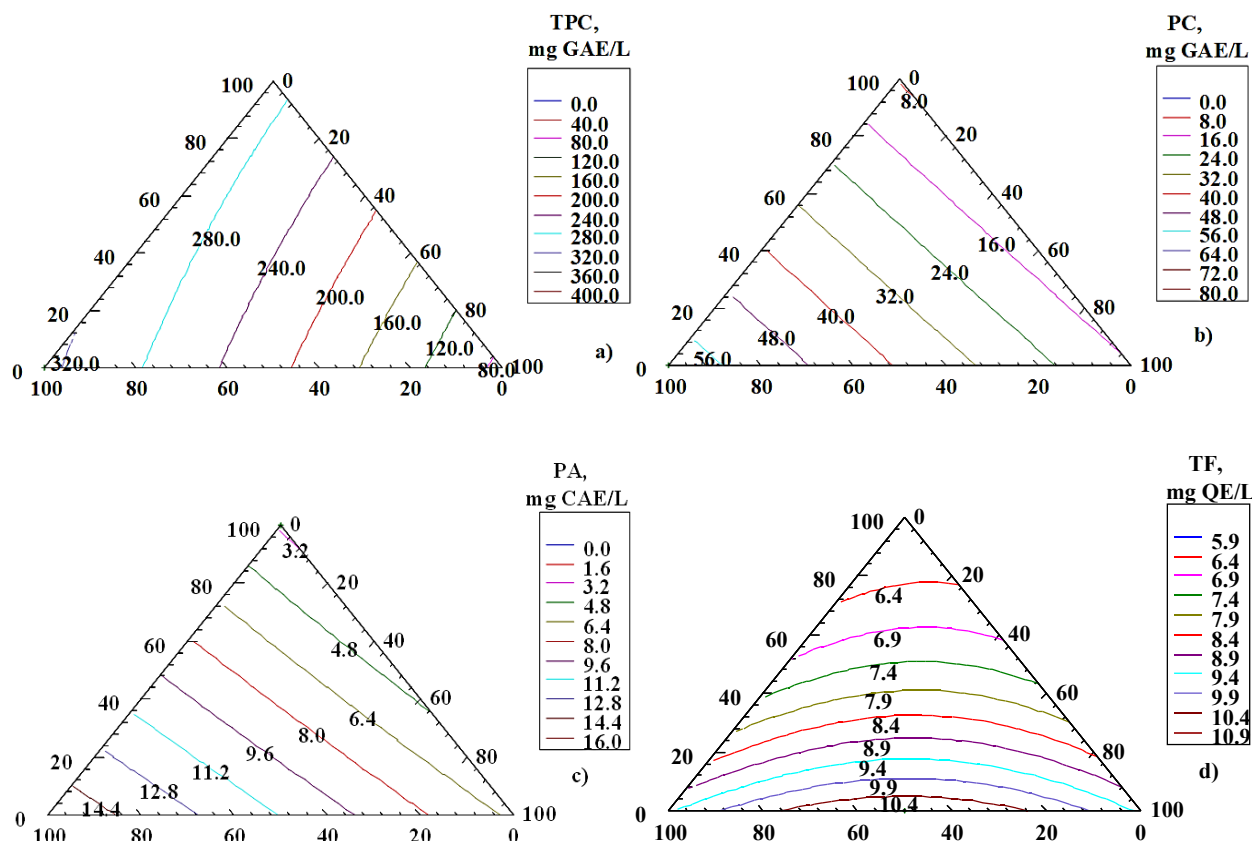


Figure 3. Contours plots for: a) Total phenolic content (TPC); b) Phenolic compounds (PC); c) Phenolic acids (PA) and d) Total flavonoids (TF)

The content of phenolic acids (PA) and total flavonoids (TF) in samples with solely use of DQH is the highest, followed by the FDRPE and SLA (Fig. 2 - 3c, d). The double and triple combination significantly influence the content of PC, PA and TF. These three effect are described by the following fitted quadric model equations (3-5).

The contour plots used to determine the optimum for achieving maximal PA and TF content showed optimum value for PA is 15.66 CAE.L⁻¹ and can be achieved at 0 mg.L⁻¹ – SLA; 100 mg.L⁻¹ – DHQ and 0 mg.L⁻¹ – FDRPE. For the TF the optimum value

is 10.81 QE.L⁻¹, established in mixture containing - 0 mg.L⁻¹ – SLA; 50 mg.L⁻¹ – DHQ and 50 mg.L⁻¹ – FDRPE.

It can be concluded that the DHQ has the most significant influence on the TPC, PC, PA and TF content. According to Zahra et al. (2024) the role and significance of flavonoids in human diet is connected with their function as signalling molecules, regulating the cell growth, trigger apoptosis, and decrease the production of reactive oxygen species (ROS).

$$PC = 7.42149*x_1 + 60.8414*x_2 + 16.3781*x_3 + 4.47292*x_1*x_2 - 0.431909*x_1*x_3 + 3.1934*x_2*x_3, \quad (3)$$

mgGAE.L⁻¹

$$PA = 3.01018*x_1 + 15.66*x_2 + 6.10236*x_3 + 0.234273*x_1*x_2 - 0.890154*x_1*x_3 + 1.07759*x_2*x_3, \quad (4)$$

mgCAE.L⁻¹

$$TF = 5.98063*x_1 + 9.31866*x_2 + 9.31866*x_3 - 2.78167*x_1*x_2 - 1.94717*x_1*x_3 + 5.98065*x_2*x_3, \quad (5)$$

mg QE.L⁻¹

Radical scavenging capacity. The known property of SLA to act as an electron donor explains its strong radical scavenging activity against the DPPH• (Ramanathan et al. 2021). The solely use of DHQ had the highest values (Fig. 4 and Fig. 5a) and the mechanism of the reduction from DPPH• to DPPH-H is described in detail by Gulcin and Alwasel (2023). Even though that solely use of

FDRPE had the weakest radical scavenging activity, its combination with SLA and DHQ exerted a greater activity against DPPH• compared to the estimated values. This can be explained by a possible synergistic effect between the components of the mixtures. The fitted quadric model equation (6) has the coefficient of determination $R^2=86.48\%$.

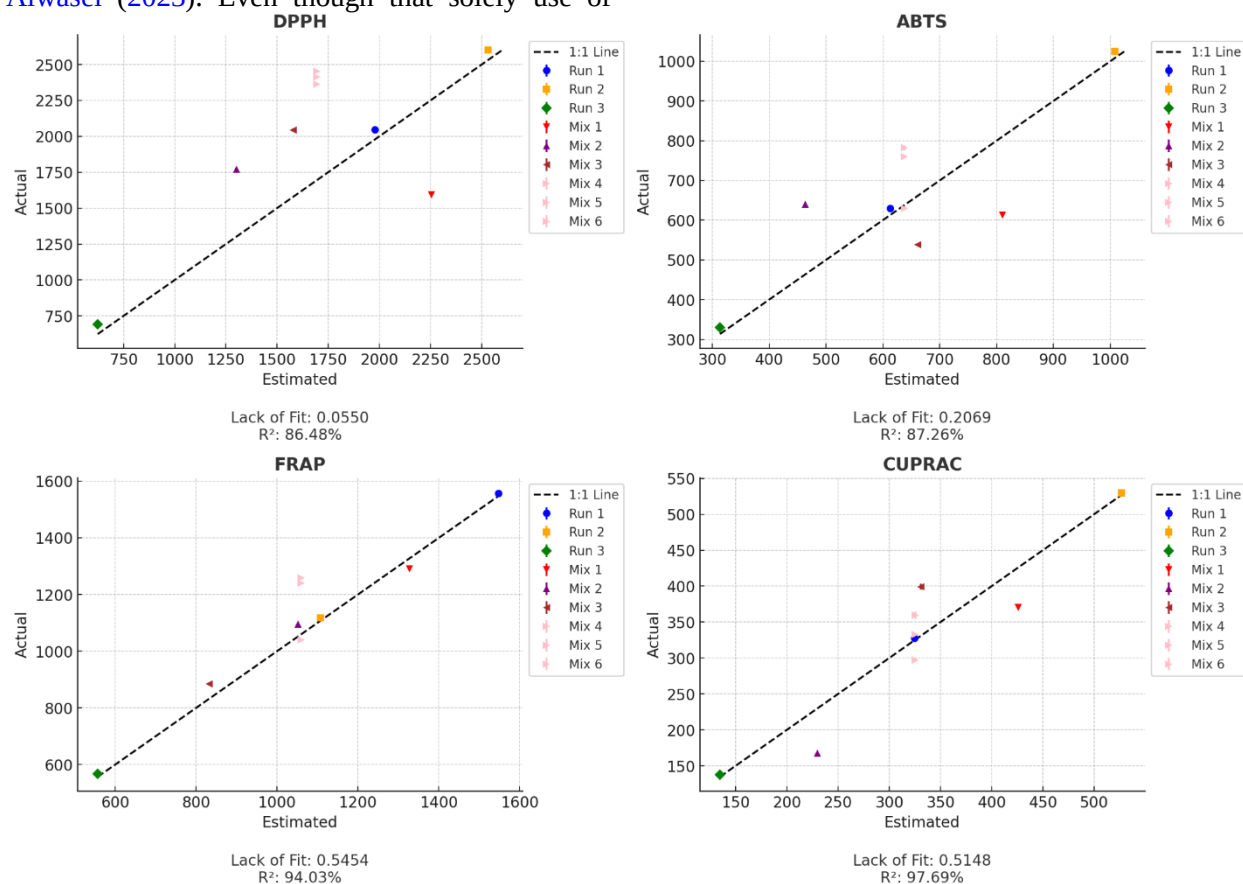


Figure 4. Scatter plots of the experimental runs for radical scavenging activity against DPPH and ABTS radicals and transition metals-chelating activity against Ferric (Fe^{3+}) ions (FRAP) Cupric (Cu^{2+}) ions (CUPRAC)

$$\text{DPPH} = 1978.12 \cdot x_1 + 2531.89 \cdot x_2 + 623.675 \cdot x_3 - 1539.97 \cdot x_1 \cdot x_2 + 2988.08 \cdot x_1 \cdot x_3 + 2971.46 \cdot x_2 \cdot x_3, \text{ mmol TE.L}^{-1} \quad (6)$$

The mixture of the three BAS has also similar radical scavenging activity against the DPPH• as the pure DHQ even though that they are only 1/3 of their initial concentrations. The triple mixture has a more 30% stronger antioxidant activity compared the estimated value (Fig. 4). This phenomenon can be explained by the manifestation of a synergistic effect and mutual reinforcement of the radical-scavenging activity of the BAS (Bobolaki et al. 2018). A synergistic effect between dihydroquercetin and L-ascorbic acid (Abilmazhinova et al.

2020), and synergism in three-component mixture of antioxidants containing 10 g.L⁻¹ dihydroquercetin, 5 g.L⁻¹ rosemary extract and 1 g.L⁻¹ L-ascorbic acid were reported (Staykov et al. 2016). By the use of contour plots was determined that 2627.00 mmol TE.L⁻¹ is an optimal maximum value for the radical scavenging activity against the DPPH• in mixture containing - 4.81745E⁻⁸ mg.L⁻¹ – SLA; 82.1092 mg.L⁻¹ – DHQ and 17.8908 mg.L⁻¹ – FDRPE. dihydroquercetin and L-ascorbic acid (Abilmazhinova et al. 2020), and synergism in

three-component mixture of antioxidants containing 10 g.L⁻¹ dihydroquercetin, 5 g.L⁻¹ rosemary extract and 1 g.L⁻¹ L-ascorbic acid were reported (Staykov et al. 2016). By the use of contour plots was determined that 2627.00 mmol TE.L⁻¹ is an optimal maximum value for the radical scavenging activity against the DPPH• in mixture containing - 4.81745E-8 mg.L⁻¹ – SLA; 82.1092 mg.L⁻¹ – DHQ and 17.8908 mg.L⁻¹ – FDRPE.

The further evaluation of the radical scavenging activity against the ABTS•⁺ radical, followed the all-ready established trend for the solely use of the BAS (Fig. 4). This time the values were from 2 to 3.5 times lower compared to the radical scavenging activity against the DPPH•. The evaluated relations between the established results and concentration of BAS are described by equation (7).

The main difference found in the radical scavenging activity against the ABTS•⁺ radical are the values for the double combinations of BAS. The mix 3 (DHQ + FDRPE/ 50 + 50 mg.L⁻¹) have the weakest

antioxidant activity against the ABTS•⁺ radical (Fig 3b) that differs from the evaluated strongest against the DPPH•. Yet the measured antioxidant activity against the ABTS•⁺ radical of mixture 3 is significantly stronger that the estimated value (Fig. 4) In the meantime, the mixtures 4 and 6 composed by SLA+DHQ+FDRPE/ 33.3+33.3+33.3 mg.L⁻¹ exerted a significantly higher values compared to the estimated ones. This, as previously stated, could due to e synergism of the components. As a results of the optimization of the radical scavenging activity against the ABTS•⁺ radical can be concluded that the maximal optimum value is 1008.12 mmol TE.L⁻¹, achievable at solely use of 100 mg.L⁻¹ DHQ.

Both assays are based on reduction by hydrogen atom transfer (HAT) and single electron transfer (SET). Gulcin and Alwasel (2023) describe the limitation of both methods and note that the DPPH radical is reduced much faster by electron transfer than by hydrogen atom transfer.

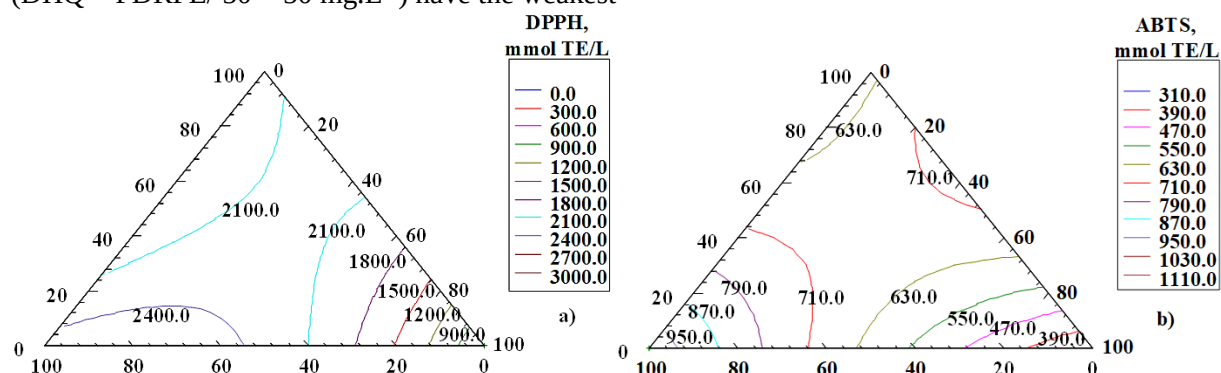


Figure 5. Contours for the radical scavenging activity against: a) DPPH• radical; b) ABTS•⁺ radical

$$ABTS = 613.102 \cdot x_1 + 1008.12 \cdot x_2 + 313.455 \cdot x_3 - 514.147 \cdot x_1 \cdot x_2 + 979.729 \cdot x_1 \cdot x_3 - 215.647 \cdot x_2 \cdot x_3, \quad (7)$$

mmol TE.L⁻¹

It has been reported that the electron transfer is quite fast, but still slower than that in the reactions of the ABTS•⁺ radical, due to the difficult accessibility of the active centre of the DPPH molecule. It is believed that more complex molecules interfere with each other, possibly blocking access to DPPH• radicals, while simpler molecules, such as that of SLA, do not. Additionally, the measurement at different wavelengths, could cause or eliminate colour interference from the sample (Alexandre-Tudo et al. 2017). Our results confirm previous statements that assessing antioxidant activity by

only one methodology sometimes can be misleading (Gulcin and Alwasel 2023; Önder 2023).

No matter of the established difference in both radical scavenging activities their connection with the above presented results for the TPC, PC, PA and TF is almost linear. The solutions with high phenolic content exerted a stronger antioxidant activity against the free radicals. Meanwhile their combination manifested a synergistic effect and increased radical scavenging activity more than expected with the decrease of the concentration.

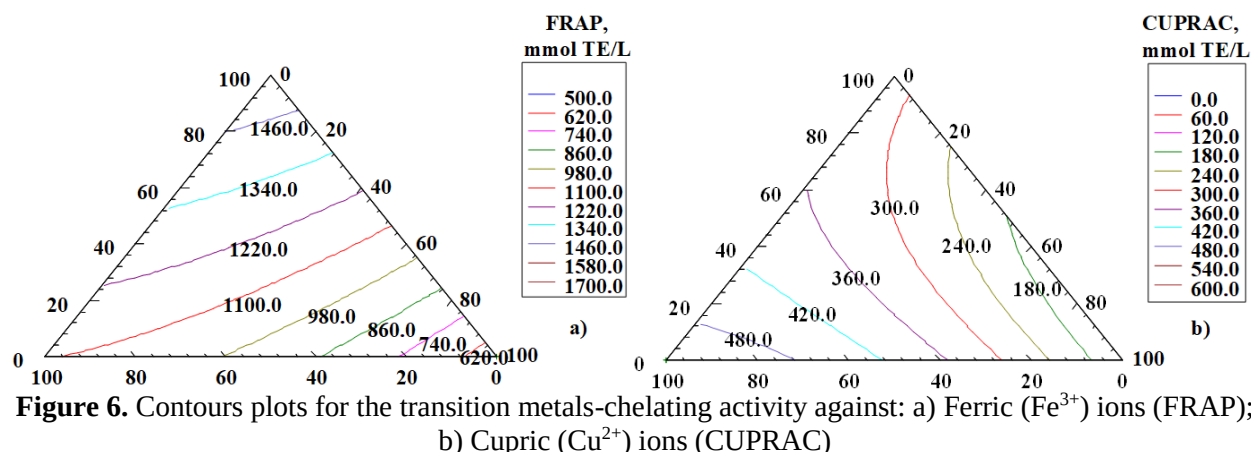
Transition metals-chelating activity. Compared to DHQ, the SLA and FDRPE have 2 to 3 times lower iron-reducing potential (Fig. 4 - 6a). The highest FRAP values for the mixtures were evaluated in mix 1 – SLA+DHQ/ 50+50 mg.L⁻¹ (Fig. 4a), but they are not higher than the estimated value. A synergism between DHQ and SLA is reported for *in vivo* systems (Kobyalko et al. 2009). While the mixture 2 and 3 containing FDRPE exhibited a stronger iron chelating activity compared to the estimated values (Fig. 4). The presence of synergism is also seen in the mixtures 4, 5, and 6, containing all three BAS - 33,33 mg.L⁻¹ each. It is well-known that the dihydroquercetin molecule, in addition to scavenging free radicals, also has the ability to bind up to three iron ions in stable complexes (Gulcin 2020). The metal-chelating ability of DHQ isn't only oriented to iron but to other transition and some heavy metals (Kobyalko et al. 2009; Fomichev et al. 2016; Bobolaki et al. 2018) and could explain the evaluated results. Above stated is confirmed by the structural relationship whereby DHQ with its three metal binding sites can probably chelate three iron ions (Fe³⁺) through its hydroxyl and carbonyl groups (Topal et al. 2016). Similar levels of radical-scavenging activity and iron-reducing potential of FDRPE have been reported by other authors (Shikov et al. 2008, Baydar and Baydar 2013). Stronger antioxidant activity of FDRPE is reported by Slavov et al. (2017), Dragoev et al. (2021) and Dinkova et al. (2022). The kaempferol has one less hydroxyl group than dihydroquercetin, which also accounts for the weaker iron-reducing potential (Gulcin 2020). Also, the polyphenols with gallol groups, such as DHQ can have faster iron oxidation rates than their catechol analogues (Perron et al. 2010). As the goal is to achieve maximum iron chelating activity, the optimum value of 1547.14 mmol TE.L⁻¹ determined by the contour plot can be achieved by the solution containing only 100 mg.L⁻¹ SLA.

The evaluation of the cupric (Cu²⁺) reducing antioxidant capacity – CUPRAC showed that DHQ binds cupric ion better compared to the other two BAS (Fig. 4, Fig. 6b). In the meantime, only mix 3 from the double mixtures demonstrated a synergistic effect and increased cupric (Cu²⁺) reducing antioxidant capacity further than expected with the decrease of the concentration. The triple

combination of the BAS in concentration of 33,33 mg.L⁻¹ each led to increase of cupric reducing antioxidant capacity, compared to the estimated value. Using the contour plots is evaluated that the maximal optimum value is 527.04 mmol TE.L⁻¹ and can be achieved by solely use of 100 mg.L⁻¹ DHQ.

The broad spectrum of polyphenols in the FDRPE, such as 4 kaempferol glycosides (Kaempferol disaccharide; Kaempferol 3-glucoside; Kaempferolpentoside and kaempferol aglycone) and 9 quercetin glycosides (quercetin 3-O-rutinoside, quercetin 3-O-galactoside, quercetin 3-O-glucoside, quercetin 3-O-xyloside and kaempferol 3-O-glucoside) identified by Dinkova et al. (2022), could possibly manifest a synergism in the evaluated mixtures. Our results confirm the reported by Petkova et al. (2020), no significant correlation between the total phenolic content and FRAP and CUPRAC results. This may be explained not only by the phenolic content or composition, but by the conditions of the analysis. FRAP assay is performed at pH of 3.6 while in CUPRAC test, a buffer with pH of 7.0 is used (Dinkova et al. 2014; Shopska et al. 2022). Well-known fact is that most of the polyphenols, such as kaempferol and quercetin glycoside exhibit stronger antioxidant activity in acid solutions such as fruit beverages (Shikov et al. 2012). Both transition metal-chelating activities FRAP and CUPRAC are described by the following fitted quadric model equations (8-9).

The Pearson's correlations analysis showed that variables such as PC and PA, TPC and CUPRAC, and ABTS and DPPH have strong correlations, implying strong interdependence. Also, there is a strong correlation between the TCP and radical scavenging activity, as well as transition metals-chelation activity (Table 1). Both radical scavenging activity against DPPH and ABTS radicals have a strong correlation with $r = 0.84$. In the meantime, TF show weak or negative correlations with ABTS and FRAP, indicating different behaviour or measurement scales. The moderate negative correlation, between TF and FRAP, indicating that as TF increases, FRAP tends to decrease is an interesting finding. It's in contrast with the DHQ as a typical flavonoid with the ability to chelate iron (Fe³⁺) ions (Topal et al. 2016; Zahra et al. 2024). These correlations can help predict the outcomes in further analysis.



$$\text{FRAP} = 1547.14 \cdot x_1 + 1107.19 \cdot x_2 + 556.638 \cdot x_3 + 10.3246 \cdot x_1 \cdot x_2 + 332.02 \cdot x_1 \cdot x_3 + 371.75 \cdot x_2 \cdot x_3, \quad (8)$$

mmol TE.L⁻¹

$$\text{CUPRAC} = 324.796 \cdot x_1 + 527.043 \cdot x_2 + 134.633 \cdot x_3 - 178.818 \cdot x_1 \cdot x_2 - 204.904 \cdot x_1 \cdot x_3 + 317.734 \cdot x_2 \cdot x_3, \quad (9)$$

mmol TE.L⁻¹

Table 1. Correlation analysis for total phenolic content, phenolic acids, total flavonoids, radical scavenging activity and transition metal-chelating activity

	TPC	PC	PA	TF	DPPH	ABTS	FRAP	CUPRAC
TPC	1							
PC	0.48071	1						
PA	0.40484	0.99512	1					
TF	-0.29084	0.53556	0.60973	1				
DPPH	0.72722	0.48731	0.45369	0.145	1			
ABTS	0.77258	0.62881	0.58716	0.03023	0.84028	1		
FRAP	0.80857	-0.09474	-0.173	-0.59742	0.56203	0.45038	1	
CUPRAC	0.82360	0.82704	0.79031	0.25933	0.70923	0.71901	0.40429	1

*The Pearson correlation coefficient (r), $1 \geq r > 0$ - positive correlation: when one variable changes, the other variable changes in the same direction; $r = 0$ - no correlation; $0 \geq r > -1$ - negative correlation: when one variable changes, the other variable changes in the opposite direction.

The optimized desirability of the response for maximum of total phenolic content, total flavonoids, radical scavenging activity against DPPH• and iron-reducing potential is achieved at: 6.11962×10^{-7} SLA + 88.5131 DHQ + 11.4869 FDRPE mg.L⁻¹.

Conclusions

Each of the three studied biologically active substances plays its role in the redox reactions. Even though that the FDRPE had the lowest TPC and weakest antioxidant activity of all three, its combinations with SLA and DHQ exhibited stronger that estimated radical scavenging and metal-chelating activities. The applied Simplex Centroid design revealed that in most cases the

solely use of dihydroquercetin is optimal for achieving the strongest antioxidant activity, but in greater concentrations. All results led to a conclusion that combination of SLA, DHQ and FDRPE have a positive impact on the radical scavenging activity radicals and transition metals-chelating activity. The evaluated mixtures have the potential to be incorporated in different food matrixes and most important in decreased concentrations compared to their solely use.

Acknowledgements

This research is supported by the Bulgarian Ministry of Education and Science under the National program “Young Scientists and Postdoctoral Students - 2”.

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