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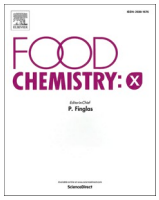

Eco-Friendly Extraction: A green approach to maximizing bioactive extraction from pumpkin (*Curcubita moschata* L.)

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ABSTRACT

The research focused on optimizing the accelerated solvent extraction (ASE) of carotenoids and polyphenols from pumpkin powder. The study optimized accelerated solvent extraction (ASE) of carotenoids and polyphenols from pumpkin powder. Using a mix of standard score (SS) and artificial neural network (ANN) methods, the extraction process was fine-tuned. The ANN model assessed extraction parameters' significance, achieving high predictability for total carotenoid content (TCC), total phenolic content (TPC), and free radical scavenging capacity (DPPH and ABTS methods). The analysis highlighted the most effective extraction at 50 % concentration, 120 °C temperature, 5 min duration, and 2 cycles, yielding high carotenoid and phenolic content (TCC 571.49 μg/g, TPC 7.85 mg GAE/g). HPLC-DAD profiles of the optimized ASE extract confirmed major carotenoids and phenolic compounds. Strong correlations were found between bioactive compounds and antioxidant activity, emphasizing potential health benefits.

1. Introduction

The food industry is devoting considerable resources to innovate and create new functional food products. These functional foods go beyond the basic provision of essential nutrition; they are tailored to have a positive impact on specific body functions, contributing to overall health and well-being. This heightened focus on functional foods reflects the increasing awareness of the link between diet and health, and the demand that food offers more than just sustenance. As a result of this significant potential, there has been a notable rise in consumer interest and demand for functional foods enriched with natural bioactive compounds (McClements et al., 2015). Extracting and utilizing these bioactives as functional ingredients in value-added products have become a key area of interest (Vrgović et al., 2022).

Pumpkin (*Cucurbita moschata*), a member of the plant family *Cucurbitaceae*, is abundant in bioactive compounds, namely carotenoids, which are known for their numerous positive effects on human health mostly because of their antioxidant abilities. Besides being powerful antioxidants, these natural colorants can improve the aesthetic appeal of food products and at the same time replace the artificial coloring compounds in the food industry (Sharma & Bhat, 2021). The most dominant carotenoids in pumpkin are α-Carotene, β-carotene, and lutein, however, the carotenoids profile and their concentrations depend on the pumpkin species (Bergantin et al., 2018; Stupar et al., 2021). Additionally to the carotenoids, the most common polyphenols in pumpkin are chlorogenic acid, quercetin, caffeic acid, gallic acid, *p*-coumaric acid, and ferulic acid (Babbar, Oberoi, & Sandhu, 2015).

In 2021, the Food and Agriculture Organization (FAO) reported that a pumpkin harvest exceeded 22.9 million tons (Huang et al., 2023), making the pumpkin one of the major vegetables in agricultural regions around the world (Maran, Mekala, & Manikandan, 2013). As a result of its high content of bioactive ingredients (carotenoids and polyphenols), and consequently their health benefits (antibacterial, antitumor, anti-inflammatory, antihypertensive, etc.), pumpkin holds a distinguished status in the realm of functional foods with potential medicinal value (Rošul et al., 2022). In recent applications, pumpkin has been utilized as a functional food through the utilization of extracts and dried powders, either independently or as an ingredient in bakery products (cakes, biscuits, bread) and other food products (juice, porridge, soup) (Hussain et al., 2022). However, the complexity that arises from the diverse

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arrays of carotenoids found in various sources, each with different levels of polarity, makes their extraction and separation a challenging task. Additionally, their hydrophobic character and sensitivity to light, heat, and oxygen, make it difficult not only to be extracted in high quantities but also to be effectively incorporated into food products (Norshazila et al., 2017; Stupar et al., 2021).

Bioactives found in pumpkin, such as carotenoids and phenolic compounds have been linked to various health benefits, including antioxidant and anti-inflammatory effects, immune system support, and potential disease prevention. However solely consumption of foods rich in carotenoids as a source of these bioactives, especially β-carotene, mostly results in low bioavailability of carotenoids from plant sources (10–65 %), due to resistance of carotene-protein complexes, fibers, and plant cell walls to digestion and degradation (Rošul et al., 2022). In order to improve bioavailability, carotenoids from plant sources can be supplemented in the form of plant extracts.

Traditionally, the extraction of bioactive compounds, such as carotenoids, has relied on volatile solvents, which come with significant drawbacks. These solvents are toxic to human health and the environment and contribute to high energy consumption (Yara-Varón et al., 2016). Conventional extraction processes face challenges like low extract recovery, prolonged duration, and high energy consumption due to intensive heating and mixing. Furthermore, conventional methods, reliant on petroleum solvents, face challenges in meeting quality criteria due to toxicity (Chemat et al., 2019). Moreover, the presence of residual traces of organic solvents in the extract can potentially compromise its bioactive properties (Vieira, Rebocho, Craveiro, Paiva, & Duarte, 2022). To address these issues, researchers have directed their focus toward more environmentally friendly solvents (e.g. ethanol, supercritical CO_2) compared to commonly used solvents such as hexane, diethyl ether, dichloromethane, and chloroform for carotenoid extraction (Alfonsi et al., 2008; Norshazila et al., 2017). For example, Capello, Fischer and Hungerbühler (2007) different alcohol-water mixtures (ethanol–water) showed to be more environmentally suitable compared to pure alcohol or propanol-water mixtures.

In the pursuit of sustainability, eco-friendly extraction methods, known as “green methods,” are crucial for addressing environmental and health concerns. Green extraction of natural products aims to develop processes that minimize energy usage and eliminate reliance on organic solvents, while guaranteeing the safety and quality of extracts. Innovative techniques, including microwave, ultrasound, and pressurized liquid systems, aim to enhance efficiency and reduce environmental impact. These “green” extractions offer increased yield and lower solvent consumption, meeting requirements for simplicity, speed, and cost-effectiveness, prioritizing both economic viability and environmental responsibility. Embracing these approaches ensures efficient extractions while minimizing adverse effects on both human well-being and the ecosystem (Tsiaka, Sinanoglou, & Zoumpoulakis, 2017, Cvetanović Kljakić et al., 2023). According to Sarkarat, Mohamadnia, and Tavakoli (2023), accelerated solvent extraction (ASE) also known as pressurized liquid extraction (PLE) offers several advantages over traditional extraction methods (Saini & Keum, 2018). These advantages include shorter extraction times, efficient mass transfer, high extraction yields, lower solvent consumption, selectivity, and the potential for automation. As accelerated solvent extraction, involves heating a mixture of extraction solvents under constant high pressure, it leads to improved cell wall rupture, and enhanced penetration of the extracting solvent. Consequently, it optimizes the mass transfer of bioactive compounds, making it a suitable choice for carotenoid extraction or other bioactive compounds as phenolic one. However, it is important to note that ASE does have some drawbacks, such as the need for a cleanup step and the initial high investment cost.

Accordingly, the primary objective of this research is to optimize the ASE to obtain an extract with a high content of bioactive compounds, with a focus on carotenoids, and high antioxidant potential. To accomplish this objective, the study employs a modeling approach, specifically ANN to enhance the quality of the model fitting, analyze the influential factors impacting the extraction process, and enhance the accuracy of the extraction optimization. Consequently, further incorporation of obtained pumpkin extracts with enhanced health-promoting properties into food products holds significant promise for growing the functional food market.

2. Materials and methods

2.1. Chemicals

Folin-Ciocalteau reagent and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and gallic acid were purchased from Sigma-Aldrich GmbH (Taufkirchen, Germany). Ethanol (96 % v/v) and methanol (95 % v/v) were obtained from Zorka Pharma (Šabac, Srbija). Diatomaceous earth was bought from Dionex Corporation (Sunnyvale, CA, USA), and sodium carbonate from Carl Roth GmbH (Karlsruhe, Germany). Distilled water was produced using a water purification system Crystal EX purchased from Animalab (Poznan, Poland).

2.2. Plant material

Commercial pumpkin powder – flour (100 % pumpkin pulp) from Jakovov producer (Ševarice, Serbia) was used in experiments.

2.3. Extraction procedure

2.3.1. Solid-liquid extraction (SLE)

Conventional solid–liquid extractions were conducted using water and ethanol in different water–ethanol mixtures (30 %, 50 %, 70 %, 96 % v/v ethanol) as solvents. The concentration range of extracts was 50–100 mg/ml. The extractions were carried out at room temperature for 24 h shaking at 150 rpm, in the dark. Following the extraction process, the extracts were immediately filtered using a vacuum filter. Subsequently, the filtered extracts were collected in glass vials and stored at 4 °C prior to analysis.

2.3.2. Accelerated solvent extraction (ASE)

ASE was performed in ASE 350 system Dionex Corporation (Sunnyvale, CA, USA) equipped with stainless steel extraction cells (22 mL volume) and collection vials. One gram of pumpkin powder mixed with diatomaceous earth was placed in the extraction cell. The ethanol–water mixture was used as a solvent, varying ethanol concentration, extraction temperature, and the number of extraction cycles defined in the experimental design (Table 1). After the extraction, each cell was rinsed with fresh solvent and purged with a flow of nitrogen. The extracts were filtered before further analysis.

2.4. Spectrophotometric analysis

2.4.1. Total carotenoid content

The content of total carotenoids in the obtained extracts was determined by the spectrophotometric method of Nagata & Yamashita (1992). β-carotene was used for the construction of the calibration curve, and the total carotenoid content was expressed as equivalent β-carotene equivalents (μg β-car/g of pumpkin powder).

2.4.2. Total phenolic content

The total phenolic content (TPC) was determined using the spectrophotometric method described by Platzer, Kiese, Herfellner, Schweiggert-Weisz, & Eisner (2021) with some modifications. The absorbance was measured at 750 nm using a spectrophotometer (Specord M40, Carl Zeiss, Jena, Germany). Gallic acid was used as the standard for the construction of the calibration curve, and the TPC was

Table 1
Central composite design of the three-levels and four-variables with observed responses under different experimental conditions.

RunNo.	Ethanol concentration (%)	Temperature (°C)	Time (min)	No of cycles	TCC(μg/g sample)	TPC (mg GAE/gsample)	DPPH (μmol TE/gsample)	ABTS (mmol TE/gsample)
1	50	120	15	2	349.51	5.32	7.07	0.26
2	80	90	10	3	233.61	2.99	3.05	1.13
3	70	120	5	4	218.16	3.70	3.55	1.27
4	60	90	10	1	296.02	4.56	6.17	0.66
5	40	90	10	3	219.98	3.74	3.76	0.46
6	60	90	10	3	302.22	3.15	3.92	0.14
7	50	60	5	2	260.40	4.23	4.14	0.20
8	60	90	10	5	113.11	2.50	3.55	0.30
9	60	90	10	3	301.41	3.97	3.96	0.14
10	70	120	15	4	411.56	8.26	7.40	1.30
11	60	90	20	3	540.48	4.48	8.55	0.68
12	50	60	15	4	205.10	1.41	3.10	0.12
13	60	90	10	3	303.22	3.59	3.98	0.14
14	70	120	5	2	397.80	2.02	3.18	0.22
15	50	120	5	2	563.34	5.85	7.20	1.00
16	60	90	10	3	291.66	3.87	3.91	0.14
17	70	60	15	4	382.60	2.93	3.71	0.59
18	70	60	5	2	282.12	3.36	3.19	0.18
19	50	60	5	4	235.19	3.14	4.05	1.19
20	60	150	10	3	301.68	3.15	4.30	0.09
21	70	60	15	2	613.91	3.39	4.14	0.14
22	60	90	10	3	294.54	3.23	3.97	0.14
23	70	120	15	2	286.24	3.50	4.20	0.13
24	60	30	10	3	249.01	3.09	3.17	0.17
25	70	60	5	4	322.50	2.91	2.57	0.15
26	50	120	15	4	425.96	8.42	7.87	0.31
27	60	90	0	3	396.91	3.14	3.40	0.19
28	50	120	5	4	326.03	3.49	4.55	0.19
29	50	60	15	2	470.46	1.37	3.51	0.24
30	60	90	10	3	296.48	2.16	2.58	0.22

expressed as gallic acid equivalents (GAE) (mg GAE/g of pumpkin powder).

2.4.3. The free radical scavenging capacity

The free radical scavenging capacity of the examined extracts was analyzed by the ABTS method, according to the method reported by Tumbas Šaponjac et al. (2014). Potential capacity was indicated by observing ABTS•+ radicals' color change at 414 nm. A calibration curve was constructed with Trolox, and the results were then expressed as mmol Trolox equivalents (TE) (mmol TE/g of pumpkin powder).

The free radical scavenging capacity of the extracts was also assessed using the DPPH method, where the reduction of the intense purple DPPH• radical to the yellow DPPH-H form indicates antioxidant presence. The results were expressed as μmol TE/g of pumpkin powder.

2.5. Chromatographic analysis

2.5.1. Phenolic profile determination by HPLC

The phenolic compounds in the extract acquired under optimal ASE conditions were analyzed using high-performance liquid chromatography (HPLC) employing an Agilent 1200 series liquid chromatography system. The HPLC system was equipped with an Agilent Eclipse XDB-C18 column measuring 4.6 × 50 mm and 1.8 μm, along with a diode array detector (DAD), in accordance with the methodology established by Mišan et al. (2011). The chromatographic separation was done using a solvent linear gradient program comprising solvent A (methanol) and solvent B (1 % formic acid in water) as follows: initial 85 % B; 0 to 6.2 min, 85 % B; 6.2 to 8 min, 85 % to 75 % B; 8 to 13 min, 75 % to 61 % B; 13 to 15 min, 61 % B; 15 to 20 min, 61 % to 40 % B; 20 to 25 min, 40 % to 0 % B. The flow rate was maintained at 1.000 mL/min, and the column temperature was set to 30 °C. Spectra were recorded within the range of 190–400 nm, and chromatograms were plotted at 280 nm, 330 nm, and 350 nm. The HPLC-DAD method was fully validated on standard solutions in terms of linearity range, limit of detection (LOD), limit of quantitation (LOQ), precision and accuracy. Quantification was performed using the external standard method. Identification of phenolic compounds was achieved by comparing their retention times and spectral characteristics with those of established standards. In instances where a standard was unavailable, the detected compound content was expressed as an equivalent of the corresponding phenolic compound.

2.5.2. Carotenoids profile determination by HPLC

The carotenoid composition and quantification in the extract acquired under optimal ASE conditions were analyzed following the method described by Kevrešan, Mastilović, Mandić, & Torbica (2013). An Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a DAD detector and a Zorbax SB C18 column (3.0 × 250 mm i.d., particle size = 5 μm). The separation of pigments was conducted at an ambient temperature of 24 ± 1 °C, with a flow rate of 1.5 mL/min. Two eluents, (A) acetone/water (75:25, v/v) and (B) acetone/methanol (75:25, v/v), were utilized with a gradient profile as follows: from 0 to 25 % B in 10 min, from 25 to 100 % B in 35 min, and 100 % B for 10 min. HPLC-DAD methods was fully validated using new column on standard solutions in terms of linearity range, limit of detection, limit of quantitation, precision and accuracy. Peaks were identified by comparing their retention time and spectra with literature data and calculated as β-carotene equivalents.

2.6. Experimental design and statistical analysis

The central-composite experimental design was applied in order to investigate the impact of the ASE parameters on target responses and to optimize the extraction process of carotenoids and polyphenols. Three extraction parameters were arranged at three levels, with five replicates at the central point (30 runs). In the ASE experimental setup, extraction parameters that influence the extraction efficiency of the target component are extraction temperature (30–150 °C), extraction cycles (1–5), and ethanol concentrations (40–80 % v/v) were optimized at five levels. The parameters and levels were chosen based on preliminary

experiments and adjusted according to the experimental design.

2.6.2. Standard scores

Ranking of 30 samples was done by comparing their raw data to extreme values, following the method by Brlek et al. (2013). Criteria for ranking included parameters like TCC, TPC, DPPH, and ABTS tests, favoring higher values.

2.6.3. Artificial neural network (ANN) model)

The data for ANN modeling (30 samples as mentioned in the previous sections) was split into training (60 %), cross-validation (20 %), and testing (20 %) sets. To improve accuracy, input and output standardization was applied using min–max normalization. The proposed multilayer perceptron model (MLP) included a three-layer, with a feedforward architecture and backpropagation training (Kavuncuoglu et al., 2017; Pavlić et al., 2020; Bajić et al., 2020). The hidden layer contained 5 to 10 neurons, and various activation functions (tangent, sigmoidal, exponential, identity) were tested. The BFGS algorithm was used to build the ANN model and iteratively adjusted weights and biases using 100,000 different configurations. The objective was to minimize square error until both learning and cross-validation curves approached zero. Computation was done with StatSoft Statistica (ver. 10.0, Palo Alto, CA, USA).

The model's accuracy was evaluated through various standard computational tests, including the coefficient of determination (r^2), reduced chi-square (χ^2), mean bias error (MBE), root mean square error (RMSE), mean percentage error (MPE), sum of squared errors (SSE) and average absolute relative deviation (AARD). Yoon's interpretation method was used to determine the relative influence of the solution type, temperature, extraction time, and number of cycles on TCC, TPC, DPPH, and ABTS for the ANN model.

3. Results and disscussion

3.1. ASE extraction

Extraction is the first step in the isolation of pumpkins' valuable bioactive, and the choice of solvents and extraction methods play an important role in successful isolation and further use. In the process of solvent screening, preference was given to environmentally friendly options, primarily focusing on water and ethanol mixtures. The maceration technique highlighted 70 % v/v ethanol as the most effective solvent, yielding the highest quantities of carotenoids (27.6 μg β-car/g of pumpkin powder) and phenols (0.30 mg GAE/g of pumpkin powder). However, for the ASE various ethanol concentrations were applied (40 %, 50 %, 60 %, 70 %, 80 % v/v), since solvent properties can be significantly altered (e.g. dielectric constant) at elevated pressure and temperatures that are often applied during ASE (Cvetanović Kljakić at al., 2023, Maravić et al., 2022). This approach aimed to ensure a comprehensive extraction of bioactive compounds, including more polar carotenoids, by employing solvent mixtures with differing polarities.

Huang et al. (2022) also optimized an efficient ASE method for rapid carotenoid extraction from paprika. The carotenoid content exhibited a significant increase when employing ASE in contrast to maceration extraction (e.g. 432 μg/g of β-carotene compared to 293 μg/g β-carotene).

To address the limitations of traditional approaches like the "one-variable-at-a-time" method, this study employs the efficient and cost-effective ANN modeling approach. Demonstrated in recent research, ANN has proven effective in predicting extraction parameters and achieving desired outcomes, even with limited datasets (Bajić et al., 2020; Pavlić et al., 2020; Stupar et al., 2021). Therefore, ASE of pumpkin powder was performed within a face-centered central-composite design investigating the influence of ethanol concentration (40–80 % v/v), extraction temperature (30–150 °C), extraction cycles (1–5), and extraction time (0–20 min) on target responses. By considering these variables, the study aimed to refine the extraction process ensuring the production of extracts with high bioactive potential while upholding principles of sustainability and eco-consciousness.

3.2. Effects of extraction parameters

Experimental results of the accelerated solvent extraction are presented in the Table 1 with the outcomes of various key parameters. Specifically, the total carotenoid content (TCC), the total phenolic content (TPC), and the free radical scavenging capacity evaluated by DPPH and ABTS assays were determined. According to the obtained results, the TCC exhibited a notable range, varying from 113.11 to 613.91 μg/g, while TPC was in the range of 1.37 to 8.42 mg GAE/g sample. Additionally, the free radical scavenging capacity, assessed with DPPH test, displayed values ranging from 2.57 to 8.55 μmol TE/g sample. Similarly, the results obtained via the ABTS test demonstrated values spanning from 9.73 to 130.58 mmol TE/100 g sample, highlighting the wide spectrum of antioxidant effectiveness across the studied parameters.

As a target bioactive compound, the highest carotenoid content was obtained using 70 % ethanol, same as in SLE, followed by the 50 % ethanol as a solvent. However, extraction time was much shorter and the content of bioactive compounds was up to 20 time higher. Notably, a 50 % ethanol–water mixture in ASE exhibited the most favorable results for the isolation of phenolic compounds, which is about 25 fold more than in classical SLE, maceration. Several studies were also pressurized solvent extraction was applied, have highlighted the efficacy of a hydro-alcoholic mixture, specifically ethanol and water in a 50:50 % v/v ratio, as the preferred solvent for polyphenolic compounds (Fernández-Ponce et al., 2015; Machado,Pasquel-Reátegui,Barbero,& Martínez, 2015; Tumbas Šaponjac et al., 2021; del Pilar Garcia-Mendoza et al., 2017). Furthermore, in the research conducted by Tumbas Šaponjac et al. (2021), the 50 % ethanol mixture also demonstrated favorable results for carotenoid ASE from carrots.

The influence of input variables on TCC, TPC, DPPH, and ABTS was studied and presented in Fig. 1. According to Fig. 1, extraction time was the most positively influential parameter on TCC with an approximately relative importance of + 57.60 %, Fig. 1a. On the other hand, solution concentration, temperature, extraction time, and 2- and 4-cycle extraction were negatively affecting parameters for TPC (-5.13 %, −4.29 %, −21.52 %, −2.34 %, and −30.61 %, respectively), Fig. 1b. Extraction time was the most positively influential parameter for the free radical scavenging capacity measured by DPPH test with an approximately relative importance of + 46.94 %, Fig. 1c. The most negative influence on ABTS was recorded by solution concentration, reaching the approximate value of −29.04 %, while the most positive influence on ABTS radical scavenging activity was obtained by temperature (+25.94 %), Fig. 1d.

Even though increased temperature contributes by increasing the diffusion rate which enhances penetration of desired compounds into the matrix, and by increasing the solubility of those compounds in the solvent, shorter time in combination with the higher temperature gave better results. The time required for extraction depends on factors like the chosen temperature and the characteristics of the matrix and target compounds. Extending the extraction time can lead to increased energy and operational costs, and prolonged heating during extraction may result in the degradation of compounds, as highlighted by Tomšik et al. (2017). Therefore, the key aim is to strike a balance between the advantages of high temperatures and longer durations, which can enhance extraction yield, and the benefits of lower temperatures and shorter durations, which help prevent the thermal degradation of bioactive compounds. A lower temperature and shorter extraction time helps avoid oxidative degradation, isomerization of carotenoids, and the generation of free radicals, which can be initiated by high temperatures in combination with other extraction parameters (Stupar et al., 2021). However, it was shown more than once that high temperatures do not

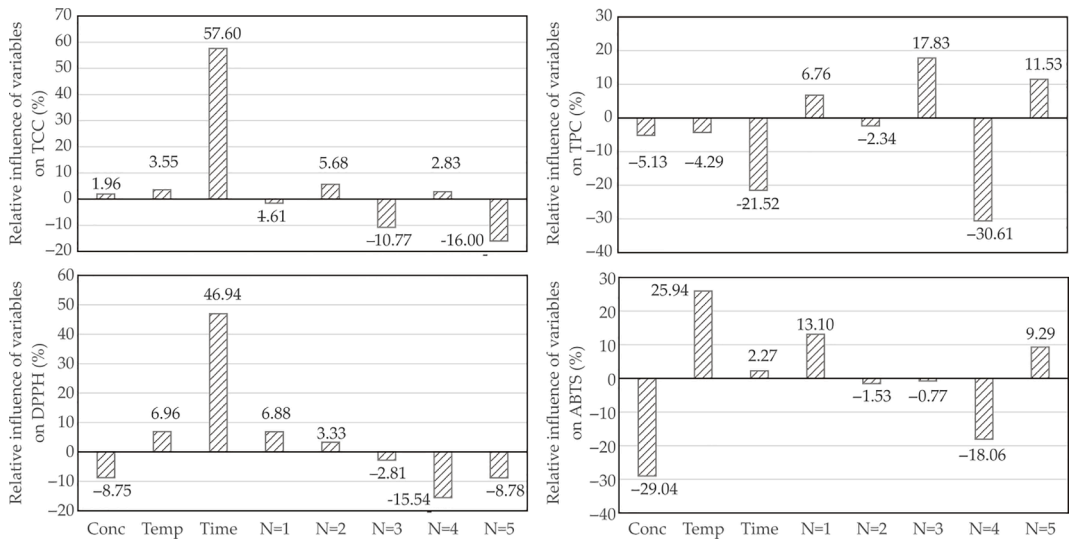

Fig. 1. The relative importance of the solution type, temperature, extraction time, and number of cycles on: (a) TCC, (b) TPC, (c) DPPH, and (d) ABTS.

always harm generally unstable natural compounds associated with antioxidant activity. In solvents mixed with water, increasing the temperature decreases the polarity of the water and increases the solubility of the targeted components, and thus their extraction (Herrero, Castro-Puyana, Mendiola, & Ibañez, 2013; Herrero, del Pilar Sánchez-Camargo, Cifuentes, & Ibáñez, 2015). Regarding the solvent concentration, it is fundamental to choose the appropriate ratio in a solvent mixture, as it affects the selectivity and therefore the chemical composition and functional characteristics of the final extract (Raspe, da Silva, C., & da Costa, 2023).

According to correlation analysis, strong positive relations between DPPH and TPC, with correlation coefficients reaching r = 0.818 (statistically significant at $p \leq 0.001$). Furthermore, DPPH exhibited a positive correlation with TCC as well, with a correlation coefficient of r = 0.521 ($p \leq 0.01$). Similarly, Rodríguez et al. (2016) reported a strong correlation between polyphenols, carotenoids, and other bioactive components and the observed free radical scavenging capacity. Their findings demonstrated that elevating temperature to 60 °C resulted in increased content of bioactive components of maqui berries extracts, followed by an increase in the free radical scavenging capacity. This phenomenon can be attributed to the Maillard reactions that occur at higher temperatures, leading to an elevation in the final polyphenol content. Usually formed phenols during Maillard reaction are vanillic acid, p-coumaric acid, ferulic acid, catechin, quercetin, kaempferol, etc. As indicated by Machado, Pasquel-Reátegui, Barbero, & Martínez (2015), the presence of polyphenols and other phytochemicals, along with their interactions, contributes significantly to the enhancement of the free radical scavenging capacity, particularly in terms of antiradical scavenging activity (DPPH), at elevated temperatures. Notably, both temperature and the type of solvent exhibited a notable influence on the free radical scavenging capacity of the blackberry ASE extracts. The research highlighted that the optimal extract was obtained using a solvent mixture comprising ethanol and water (50:50 % v/v) at 100 °C.

A positive correlation between ABTS and TPC was noticed (r = 0.378, $p \leq 0.05$). A similar correlation was observed in research (Kulczyński et al., 2020), a positive correlation between and ABTS tests (r = 0.41; $p < 0.01$) was detected for aqueous extract, while the analysis of the results for the aqueous–methanol extracts revealed a positive correlation between the total polyphenolic content and the free radical scavenging capacity assayed in the ABTS test (r = 0.37; $p < 0.05$). ABTS activity may be influenced by other bioactives such as carotenoids. Existing literature on *in vitro* antioxidant activity of β-carotene and related carotenoids has yielded disparate findings, likely attributed to the utilization of different test systems. According to Mueller & Boehm (2011) when they used the ABTS test, the degradation of β-carotene to β-apo-8′-carotenal and its carotenoic acid ester led to a reduction in conjugated double bonds and, consequently, a decrease in free radical scavenging capacity, but the (all-E)-BC and its (Z)-isomers exhibited significantly higher ABTS●+ bleaching activity compared to α-tocopherol, indicating their potent antioxidant properties. Their findings provide valuable insights into the complex relationship between the structure of carotenoids and their antioxidant activity, highlighting the significance of considering molecular interactions in such assessments (Mueller & Boehm, 2011).

3.3. Model validation via artificial neural network and process optimization

In order to obtain more accurate predictions of ANN modeling, the standard score was obtained by summing the normalized scores for each variable (TCC, TPC, DPPH, and ABTS), which are then multiplied by their respective weights (0.4, 0.25, 0.25, and 0.1, respectively) (Fig. 2). Maximizing the SS function indicates optimal processing parameters and values for TCC, TPC, DPPH, and ABTS, with a higher SS value approaching 1 indicating a stronger likelihood of optimal parameters.

The artificial neural network model's structure and results heavily rely on the initial assumptions for matrix parameters (biases and weights). These assumptions are critical for fitting the model to the actual experimental data. The performance of the model is also influenced by the number of neurons in the hidden layer. To address this, 100,000 runs with randomized topologies eliminated random correlations from initial assumptions and weight initialization. The model achieved the highest r^2 value with nine hidden neurons (referring to Fig. 3a). Each ANN model underwent training for 100 epochs, and the training results, namely the training accuracy and error (loss), are presented in Fig. 3b. The training accuracy increased with each training cycle until reaching a nearly constant value around the 50th to 60th epoch. Training for more than 60 epochs could potentially lead to significant overfitting, while 60 epochs proved sufficient for achieving high model accuracy without the risk of overfitting (see Fig. 3b).

The optimized neural network models demonstrated strong generalization for the experimental data, accurately predicting output based on input parameters. The ANN model used 8 neurons (network MLP 8–8-4) to achieve high r^2 values (0.975, 0.965, and 0.970 for training, testing, and validation). Matrix W1 and vector B1 (bias row) are detailed in Table 2, while Table 3 provides elements of matrix W2 and vector B2 (bias) for the hidden layer. The artificial neural network models exhibited good accuracy in predicting the experimental variables across

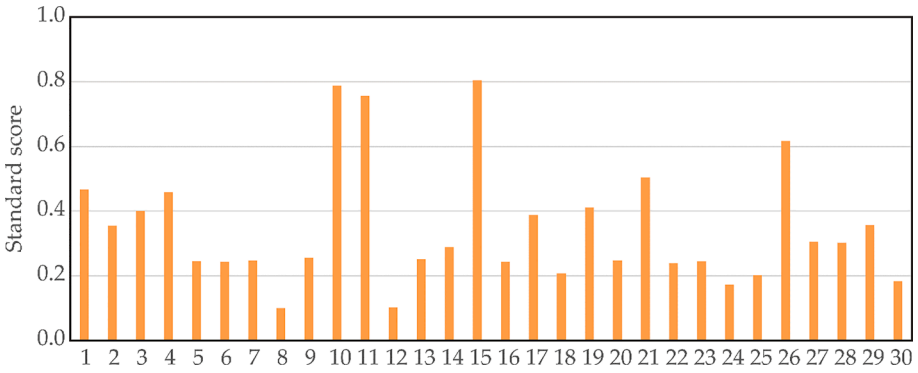

Fig. 2. Standard scores for 30 samples during extraction.

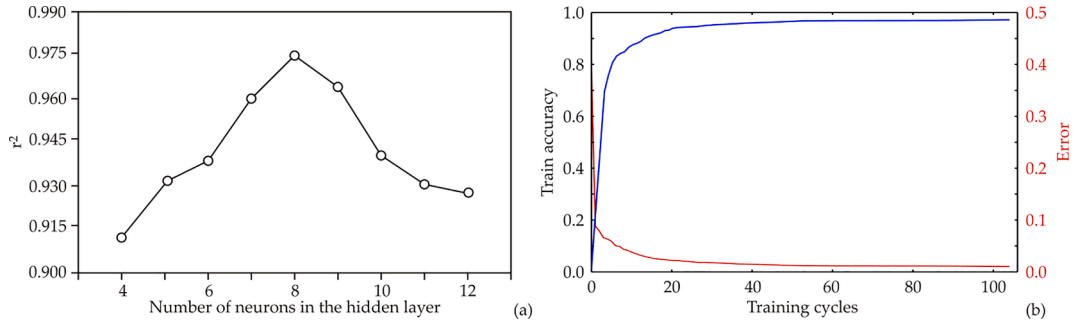

Fig. 3. ANN calculation: (a) The dependence of the r2 value of the number of neurons in the hidden layer in the ANN model, (b) Training results per epoch.

Table 2
The weight coefficients and biases W1 and B1 for the ANN model.

	1	2	3	4	5	6	7	8
Conc	1.761	0.162	0.515	−0.253	−1.574	−1.124	−4.129	0.162
Temp	−0.919	0.089	0.923	2.489	−0.825	3.001	5.743	3.359
Time	−0.412	−1.645	−7.277	8.227	0.447	−1.530	0.977	−0.927
N = 1	−0.576	−1.108	−1.163	0.889	−1.065	1.211	2.063	0.095
N = 2	0.247	0.011	−0.884	0.574	1.726	−2.139	−0.141	−0.351
N = 3	−0.845	2.080	3.088	−4.321	2.874	1.079	−3.640	−1.538
N = 4	0.817	0.160	−0.264	1.179	−3.238	−0.607	1.874	1.466
N = 5	−0.278	−1.181	0.658	−1.285	−0.603	1.310	−0.678	−1.300
Bias	−0.616	−0.155	1.409	−2.910	−0.403	0.917	−0.607	−1.679

Table 3
The weight coefficients and biases W2 and B2 for ANN model.

	1	2	3	4	5	6	7	8	Bias
TCC	−0.001	4.321	−2.183	3.091	0.327	0.591	−0.856	−0.187	−0.169
TPC	0.470	−0.877	−0.909	−0.954	0.769	0.749	−0.241	0.928	0.576
DPPH	0.226	0.153	−1.277	−0.254	0.652	0.740	−0.232	0.719	0.334
ABTS	4.165	−0.536	−0.872	−0.562	2.196	2.715	1.757	−1.214	0.242

a diverse range of process variables. The visual aspect of the model accuracy is presented in Fig. 4, where the graph illustrates the close agreement between the experimentally measured values and the values predicted by the ANN model.

The model feature fit was examined and presented in Table 4. The results show that the ANN models had a minor lack of fit tests, which implies that the models satisfactorily predicted the values of the analyzed parameters. The obtained r^2 for TCC, TPC, DPPH, and ABTS prediction (0.980, 0.957, 0.966, and 0.999) suggests that the variation was accurately evaluated and that the data fit adequately to the suggested model.

The developed ANN was evaluated using mean relative percent error (ranging from 2.204 to 6.095), root mean square error (RMSE) values between 0.294 and 16.080, mean percent error (MPE) within the 2.204 to 6.095 range, and average absolute relative deviation (AARD) also spanning 2.204 to 6.095. These assessments, outlined in Table 4, demonstrate the statistical significance of the ANN model and its alignment with experimental outcomes. Furthermore, the residual data analysis was performed on the model developed, as an additional model test. The skewness and kurtosis values in Table 4 offer crucial insights into the data distribution, influencing preprocessing steps and model selection for predictive analysis. The negative skewness values and relatively lower kurtosis indicate a left-skewed distribution, suggesting less pronounced. These metrics play a key role in describing data shape, providing valuable insights into symmetry, tails, and the presence of outliers or extreme values (Taylor, 2006).

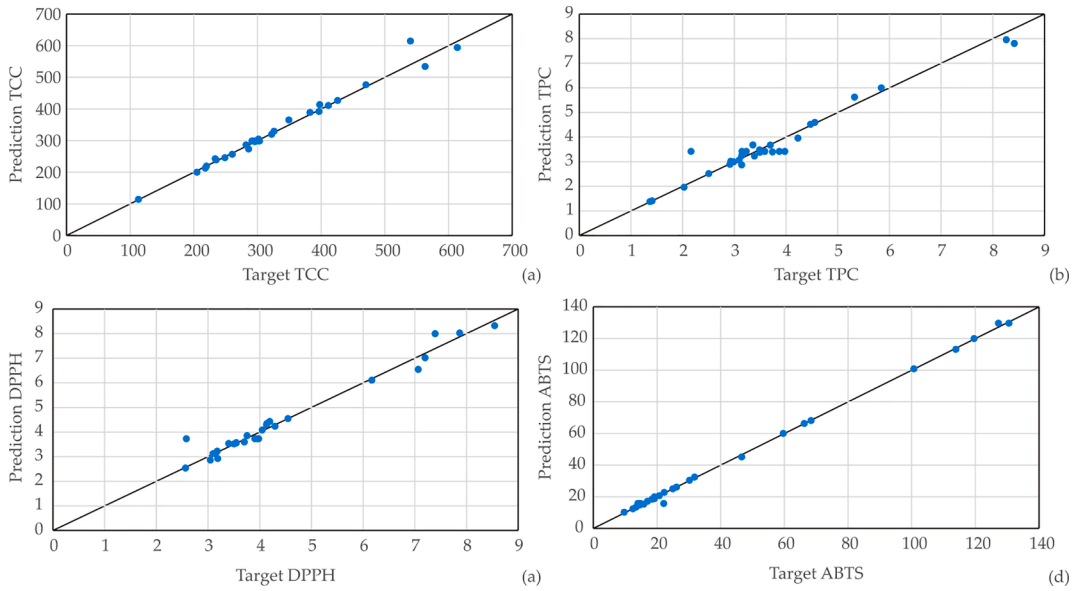

Fig. 4. Experimental and predicted values obtained for (a) TCC, (b) TPC, (c) DPPH, and (d) ABTS.

Table 4
The "goodness of fit" tests for the developed ANN model.

	χ^2	RMSE	MBE	MPE	SSE	AARD	r^2	Skew	Kurt	Mean	StDev	Var
TCC	267.496	16.080	−1.547	2.204	7757.385	2.204	0.980	−2.836	13.588	−1.547	16.279	265.021
TPC	0.113	0.330	0.020	6.095	3.274	6.095	0.957	−1.600	6.590	0.020	0.335	0.112
DPPH	0.089	0.294	−0.003	4.725	2.585	4.725	0.966	−2.044	6.971	−0.003	0.299	0.089
ABTS	2.157	1.444	−0.044	3.465	62.560	3.465	0.999	3.033	13.844	−0.044	1.468	2.155

The optimization of the outputs was performed by utilizing the experimental data presented in Table 1, which were applied to the developed ANN model. The optimal predicted result introducing the maximum values for TCC, TPC, DPPH, and ABTS were determined to be 571.49 μg/g; 7.85 GAE/g sample; 8.08 umol TE/g sample, and 130.22 mM TE/100 g sample, respectively. These optimal outputs were achieved by employing the process parameters: 67.73 % solution, 120 °C, 9.66 min, and 4 extraction cycles.

The optimal result obtained using the standard score, maximizing TCC, TPC, DPPH, and ABTS were 563.34 μg/g, 5.84 GAE/g sample, 7.20 umol TE/g sample, and 100.75 mM TE/100 g sample, respectively. The optimal sample was obtained by employing the process parameters: 50 % solution, 120 °C, 5 min of extraction, and 2 extraction cycles. Fig. 4 provides an overview of these standard scores. AS ANN predicted multiple response values (TPC, TCC, ABTS, and DPPH), only the ANN optimum was experimentally validated as a result.

According to aforementioned influence of extraction parameters, process optimization is of high importance. To ensure the highest yield and the optimal processing conditions, optimization of the ASE was performed using the developed ANN model. In order to identify the processing variables (solution type, temperature, extraction time, and number of cycles) that yield optimal values for TCC, TPC, DPPH, and ABTS, based on the experimentally obtained data, standard scores were calculated. The superiority of ASE over SLE results, in our case maceration, and the justified optimization of ASE using ANN underscore the significance of the adopted approach.

3.4. Chomatographic profile of optimized ASE extract

Significant variations in carotenoid content are commonly observed within the same species and variety, attributed to factors such as cultivar type, environmental conditions (temperature, sunlight intensity, nutrient availability, soil quality), and post-harvesting processes (Atencio et al., 2022; Kulczyński et al., 2019). Consequently, concentration ranges reported in the literature are notably wide, reflecting the diversity of these influencing factors. Typically, one to four carotenoids are predominant in the pumpkin species, with several other compounds detected in low concentrations or traces. In our study, the HPLC-DAD profiles clearly evidenced the presence of two main peaks. The first peak was characterized according to data available in the literature; its identity was plausibly attributed to the α-carotene. The second detected peak was established as

Carotene based on the correspondence between its retention time and that of the reference standard. These two carotenoids collectively constituted over 70 % of the total chromatographic area, while several minor peaks were present in trace amounts, making their identification challenging. The concentrations of the dominant carotenoids detected in the optimal ASE extract were as follows: α-carotene at 3.53 ± 0.24 mg β-car equivalent/100 g DW and β-carotene at 3.88 ± 0.19 mg/100 g DW (Table 5). Several authors have reported a trend comparable to our findings. According to Pinna et al. (2022), the carotenoid composition in

Table 5
Detected phenolic and carotenoid compounds by HPLC methods.

Phenolic compounds	
detected compounds	concentration (mg/100 g DW)
Gallic acid	12.78 ± 0.71
Catechin	29.57 ± 0.56
Ferulic acid	1.26 ± 0.21
Sinapic Acid	6.57 ± 0.19
Quercetin	1.34 ± 0.23
Carotenoids	
detected compounds	concentration (mg/100 g DW)
β-carotene	3.53 ± 0.24
α-Carotene*	3.88 ± 0.19
* (expressed as β-carotene equivalent)	

raw *C.moschata* varieties presented a higher content of α-carotene (1.26 mg/100 g DW) and β-carotene (1.95 mg/100 g DW) compared to lutein content. Kulczyński & Gramza-Michałowska (2019) have investigated various pumpkins, including the *C.moschata* where depending on the cultivar, the content of β-carotene was in the range from 1.29 to 5.26 mg/100 g DW, while retinol equivalent was in the range 0.52 to 2.12 mg/100 g DW. In this research lutein and zeaxantin were detected in some varieties.

Despite the association of pumpkins with carotenoids, pumpkins are also recognized for their polyphenolic content. The specific polyphenolic composition of pumpkins can also vary widely depending on factors like pumpkin variety, growing conditions, and processing methods. The analysis of polyphenolics in the optimal ASE extract is presented in Table 5. Gallic acid (12.87 mg GAE/100 g DW) was found to be the most abundant phenolic acid, followed by sinapic acid (6.57 mg GAE/100 g DW). The content of gallic acid was similar to the one measured in the 'Buttercup' pumpkin cultivar analyzed by Kulczyński and Gramza-Michałowska (2019) (14.22 mg GAE/100 g DW), however, their SLE involved hexane and ethyl acetate as solvents, with prior saponification for SLE. In contrast, our extraction technique is more environmentally friendly, requiring less time and solvent usage. Pinna et al.(2022) have employed contemporary extraction techniques as are ultrasound-assisted extraction (UAE) and microwave-assisted extraction techniques MAE for carotenoids extraction from *C. moschata* obtaining similar result as in our study (12.33 mg GAE/100 g DW). Nevertheless, their extractions lack in "green" solvent as they used hexane:isopropanol, 60:40 v/v or hexane: acetone: ethanol 50:25:25 v/v/v as a solvents. In terms of flavonols, catechin (29.57 mg GAE/100 g DW) and quercetin (1.34 mg GAE/100 g DW) were identified as the most prevalent compounds. Quercetin was not detected in most cultivars in a study by Kulczyński & Gramza-Michałowska (2019), except in one *C.moschata* cultivar (1.92 mg GAE/100 g DW), which is in accordance with our results.

3.5. Comparative overview of methods for efficient bioactives extraction from pumpkin

A critical comparison of various extraction methods is essential for determining optimal approaches based on extraction yields, solvent consumption, and energy efficiency. This section evaluates and compares proposed methods with published works, emphasizing "green" solvents for environmental sustainability and reduced energy consumption, with a focus on pumpkin's dominant compounds, carotenoids.

Extraction of carotenes from dried vegetables typically involves labor-intensive processes, toxic solvents, and overnight saponification, which, although yielding high extraction efficiencies, is less effective for individual carotenoid analysis due to degradation and isomerization (Adadi et al., 2018). Carotenoid extraction faces challenges due to strong associations with proteins and fatty acids, hindering mass transfer; thus, initial extraction steps employ physical, chemical, enzymatic, or biological means to disrupt these barriers (Saini et al., 2022). To over come this initial step, extractions as ultrasound assisted extraction (UAE), microvawe ectraction (MAE), sperercritical extraction (SFE) annd accelerated solvent extracton may be applied. While ultrasound-assisted extraction (UAE) and microwave extraction (MAE) prove effective in disrupting cell material and accelerating phenolic extraction(Cvetanović Kljakić et al., 2023; Pavlić et al., 2023; Zengin et al., 2020), their drawbacks include thermal degradation and reliance on organic solvents for carotenoid extraction, diminishing bioavailability and health benefits. For instance, intermittent microwave radiation combined with MAE was employed to extract carotenoids and β-carotene from carrot peels. By utilizing $\alpha = 1/4$ along with appropriate microwave powers and solvent-to-sample ratios (180 W/75 mL:2 g and 300 W/150 mL:2 g), larger amounts of extractable β-carotene and total carotenoids (289.2 ± 5.4 mg/100 g d.b) were obtained compared to continuous MAE (132.7 ± 5.4 mg/100 g d.b). However, the applied solvent consisted of 50 % (v/v) hexane, 25 % (v/v) acetone, and 25 % (v/v) ethanol. (Hiranvarachat & Devahastin, 2014). Several studies investigated the potentiality of using vegetable oils as a green solvent/co-solvent along with other innovative technologies for the extraction of carotenoids. Sebdani et al. (2023) utilized sunflower oil with ultrasound-assisted extraction, achieving optimal conditions at 0.08 g/mL solid/solvent, 30 °C, and 55 min, yielding carotenoid content of 13.93 mg/100 g and β-carotene content of 13.30 mg/kg. Despite the considered green nature of the extraction technique and solvent, this approach yielded approximately four times lower carotenoid content than our results. Our applied accelerated solvent extraction (ASE) conditions, with automatic filtration and cleaning, offer advantages in terms of extraction efficiency, reduced extraction time, and material preservation.

Another example of an extraction method using a "green" solvent, which can be considered and compared to ASE, is supercritical CO2 extraction, successfully applied in carotenoid extraction (Adadi et al., 2018; Pavlić et al., 2023). Durante and coauthors addressed the results of extracting carotenoid-rich oil from pumpkin using SFE-CO2, comparing it with classical solvent extraction (CSE). They observed that SFE-CO2 exhibited much higher efficiency in terms of solid–liquid ratio, temperature, extraction time, and yield compared to classical solvent extraction. However, it is essential to consider that during SFE-CO2, other compounds, such as oil, can be co-extracted, impacting the extract's lipophilic character. Additionally, the capital investment and complex operating system associated with SFE-CO2 are higher than in ASE.

Accordingly, Accelerated Solvent Extraction (ASE) can be identified as a promising and efficient method and recommended for carotenoid extraction.

4. Conclusions

This study underscored the challenge of low bioavailability of carotenoids from plant sources, emphasizing the need for efficient extraction methods to maximize the yield of these valuable compounds. Artificial neural network (ANN) modeling was employed to optimize the extraction process, taking into account various parameters such as solvent type, temperature, extraction time, and the number of cycles during accelerated solvent extraction, with an emphasis on the careful use of environmentally friendly options. This accelerated solvent extraction has proven to be successful, and the obtained extract can be further incorporated into functional products. In line with the emphasis on eco-consciousness and sustainability, future work should continue to investigate green and environmentally friendly extraction techniques, ensuring minimal ecological impact. Additionally, there is a growing market for nutraceuticals, and the development of novel pumpkin-based products with enhanced bioactive content could be a promising trend.

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CRediT authorship contribution statement

Milana Matić: Writing – original draft, Formal analysis, Data curation. **Alena Stupar:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Data curation, Conceptualization. **Lato Pezo:** Writing – original draft, Visualization, Validation,

Methodology, Data curation. **Nataša Đerić Ilić:** Writing – original draft, Visualization, Formal analysis. **Aleksandra Mišan:** Writing – review & editing, Supervision, Formal analysis. **Nemanja Teslić:** Writing – original draft, Formal analysis. **Milica Pojić:** Writing – original draft, Validation, Data curation. **Anamarija Mandić:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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