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ORIGINAL PAPER

Phenolic composition and antioxidant properties of *Pleurotus ostreatus* and *Pleurotus eryngii* enriched with selenium and zinc

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Abstract The aim of the study was to investigate the antioxidant properties, phenolic and flavonoid contents and composition and content of ascorbic acid in *Pleurotus ostreatus* and *Pleurotus eryngii* enriched simultaneously with selenium (Se) and zinc (Zn). Non-enriched mushrooms contained Se and Zn at the level as in the most popular mushrooms. The total phenolic content (TPC) for non-enriched *P. ostreatus* and *P. eryngii* was 9.64 ± 0.33 and 7.91 ± 1.02 mg/g of extract, the total flavonoid content was 2.11 ± 0.19 and 1.26 ± 0.17 mg/g of extract, and ascorbic acid content ranged from 10.28 ± 0.39 to 16.64 ± 0.47 mg/100 g DW, respectively. Methanolic extracts contained 4-hydroxybenzoic, ferulic, *p*-coumaric, protocatechuic, *t*-cinnamic and vanillic acids and naringenin. In methanolic extract of *P. eryngii*, 2,5-dihydroxybenzoic acid was also quantified. The correlation between the TPC and antioxidant activity in mushroom was confirmed. Additionally, the correlations between Zn and Se concentration in fruiting bodies and EC_{50} value and phenolic compounds were confirmed. Our results with simultaneous supplementation with Zn and Se provide the opportunity to increase the content of the elements in fruiting bodies and to improve antioxidant properties and antioxidant contents in enriched mushrooms. Additionally, the obtained results demonstrated that simultaneous enrichment with micronutrients with a contrary effect on antioxidant properties can activate synthesis of phenolic compounds and ascorbic acid. The investigation is the first study evaluating the effect of addition of two elements to the substrate at the same time on antioxidant properties of mushrooms.

Keywords Phenolic and flavonoid compounds · Selenium · Zinc · Ascorbic acid · Antioxidant properties · *Pleurotus*

Introduction

Mushrooms are a valuable component of the human diet due to their nutritional and medicinal value. They are a source of compounds responsible for antimicrobial, antioxidant, antitumor and antiinflammatory properties [1–8]. The consumption of food rich in antioxidants plays a protective role for human health, because of the reduction in oxidative damage resulting in enhanced generation of free radicals [9, 10]. Free radicals are unstable and highly reactive, due to having unpaired electrons; they are responsible for oxidative stress, and in consequence, they cause DNA damage, carcinogenesis, oxidation of biomolecules and cellular degradation related to aging, etc. [11–14].

Phenolic compounds are mushroom antioxidants which are strong radical scavengers and free radical inhibitors and phytonutrients [15, 16]. The common feature of this very diverse group of metabolites including flavonoids, phenolic acids, stilbenes, lignin, tannins is possessing in the structure one or more aromatic rings with hydroxyl groups [15, 17].

Ascorbic acid is the enolic form of an α-ketolactone (2,3-didehydr L-threo-hexano-1,4-lactone) and is the

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functional form of vitamin C [18], which has a protective role in human health and acts as an electron donor for human enzymes (reducing agent or antioxidant) [19].

Selenium (Se) and zinc (Zn) are trace elements important for human health [20]. Se is a component of antioxidant enzymes via amino acid (selenocysteine, selenomethionine) and proteins [21–23]. Zn is a component of enzymes participating in the synthesis and degradation of different biomolecules including lipids, proteins, carbohydrates and nucleic acids as well as in the metabolism of other micronutrients [19]. The antioxidant properties of Zn in detoxification of reactive oxygen species (ROS) such as $OH^{\cdot}$, $O_2^{\cdot-}$ and H_2O_2 were confirmed in Zn-metallothionein and in the enzyme Cu–Zn superoxide dismutase [12, 24].

The deficiency of micronutrient is associated with health disturbances, and thus, different cultivation practices have been successfully implemented to increase the nutritional value of mushrooms [21, 25, 26]. It was also documented that enrichment with micronutrients had an impact on antioxidant activities and phenolic content [26–28]. In publications mentioned, the impact of a single element on antioxidants was determined. The present study is a continuation of our experiment on mushroom enrichment [28].

The main objective of the study was to determine phenolic, flavonoid and ascorbic acid contents and antioxidant activity of methanolic extracts from *P. ostreatus* and *P. eryngii* enriched with Se and Zn. Additionally, the individual profile of phenolics in extracts of the mushrooms was determined. To our knowledge, it is the first study on the impact of simultaneous enrichment of substrates with different micronutrients on antioxidants in edible mushrooms.

Materials and methods

Mushroom material

Experiments were designed following Gąsecka et al. [28] with some modifications. Wheat straw cut into chaff 4–5 cm long was placed in polypropylene bags with 60 % moisture content. After pasteurization at 60 °C for 24 h, it was used as substrate for the *P. ostreatus* experiments. Sodium selenite [Na_2SeO_3 (IV)], sodium selenate [Na_2SeO_4 (VI)] and zinc nitrate hexahydrate [$Zn(NO_3)_2 \times 6H_2O$] were dissolved in an amount of sterile water sufficient to obtain their appropriate concentration in the substrate. After addition of the salt solution, the substrate reached the moisture content of 70 %. The final concentration of the salts was 1.5 mM (each in five replicates). The substrate was mixed with 3 % of spawn (wheat grain) using a POLYMIX PX-SR 90 D stirrer (Kinematica AG, Littau-Luzern, Switzerland). Then, 1 kg of the substrate was placed in bags of perforated foil and incubated at 25 °C and 85–90 % air relative humidity (RH). Once the bags were totally colonized, they were placed in the cultivation chamber (15–16 °C and 85–90 % RH) and illuminated with fluorescent light of 500 lux intensity 10 h a day. The growth facility was aerated to maintain CO_2 concentration below 1000 ppm.

The substrate for *P. eryngii* was prepared as a mixture of beech sawdust and flax shives (3:1 vol.) supplemented with wheat bran in the amount of 20 %, corn flour 5 % and gypsum 1 % in relation to the substrate dry matter. Substrate moisture was adjusted to 45 % using distilled water, and then, it was bagged in polypropylene bags and sterilized at 121 °C for 1 h and finally cooled down to 25 °C. Se and Zn salt solutions were prepared as described above and were added to the substrate to obtain the appropriate concentration (1.5 mM each in five replicates) in the substrate with 60 % moisture. Then, the substrate with Se and Zn addition was mixed with spawn (wheat grain) of the mushroom (5 % of substrate weight). A total of 350 g of the substrate was placed in polypropylene bottles of 1 dm^3 volume and closed with a cover with a filter. The incubation was conducted at the temperature of 25 °C and 80–85 % RH until the substrate became completely colonized with mycelium. Next, the bottles without covers were placed in the cultivation chamber (85–90 % RH and 14 ± 1 °C) and lit with fluorescent light of 500 lx intensity 12 h a day. The growth facility was aerated in such a way as to maintain CO_2 concentration below 1000 ppm.

Fruiting bodies of *P. ostreatus* and *P. eryngii* were collected after maturation, dried in an electric oven (SLW 53 STD, Pol-Eko, Wodzisław Śląski, Poland) at 50 ± 2 °C for 48 h and ground for 0.5 min in a Cutting Boll Mill 200 (Retsch GmbH, Haan, Germany). For the extraction procedure, five representative powdered samples were used.

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), Na_2SeO_3, Na_2SeO_4, $Zn(NO_3)_2 \times 6H_2O$, $NaNO_2$, $AlCl_3$, Na_2CO_3, NaOH, HNO_3, H_2O_2, Folin–Ciocalteu phenol reagent, formic, gallic, protocatechuic, benzoic, 2,5-dihydroxybenzoic, 4-hydroxybenzoic, caffeic, chlorogenic, vanillic, salicylic, syringic, *p*-coumaric, ferulic, sinapic and *t*-cinnamic acids, rutin, catechin, kaempferol, quercetin, vitexin, luteolin, naringenin, apigenin and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Se standard solution was purchased from Merck (Darmstadt, Germany).

Se determination

Mineralization of dry fruiting bodies of the mushrooms (1.0000 ± 0.0001 g) was performed using 8 mL of 65 % HNO_3 and 1 mL of 30 % H_2O_2 with a CEM Mars 5 Xpress microwave mineralization system (CEM Corp., Matthews,

NC, USA) according to the following microwave three-stage program: first stage—power 400 W, time 2 min, temperature 100 °C; second stage—power 600 W, time 5 min, temperature 160 °C; the third stage—power 1000 W, time 10 min, temperature 200 °C. Solutions after mineralization were filtered through 45-mm filters (Qualitative Filter Papers, Whatman, Grade 595: 4–7 μm, UK). Then, the whole contents were made up to a final volume of 50.0 mL with deionized water (Milli-Q Advantage A10 Water Purification Systems, Merck Millipore, Darmstadt, Germany). Total Se concentration in samples was determined by electrothermal atomic absorption spectrometry (ETAAS) using an Agilent Technologies AA Duo—AA280FS/AA280Z spectrometer (Agilent Technologies, Mulgrave, Victoria, Australia). Pyrolytic graphite tubes and a Se hollow cathode lamp (wavelength 196.0 nm, slit 1.0 nm, lamp current 10 mA) were used. The optimized temperature program was used: drying step at 85–120 °C for 55 s; ashing step at 1000 C for 8 s; atomization at 2600 °C. As a chemical modifier, palladium solution was used (10 μL of 500 mg/L for 20 μL of sample). For the preparation of the calibration curve, the commercial Se standard in 1 g/L concentration was used as the appropriate diluted standard. The linearity of the calibration curve was 0.9996; the detection limit for liquid samples was 0.0007 mg/L. The range of the calibration curve was from the detection limit to 0.080 mg/L. Precision measured as relative standard deviation was at the level of 3–5 %. The uncertainty of the whole analytical procedure (sample preparation and analysis) did not exceed 20 %. Due to the lack of reference material, the traceability was determined in a standard addition procedure. The obtained recovery values were in the range 96.7–104.1 %. The determination level for the solid samples was 0.1 mg/kg [29, 30].

For Zn determination, flame (air-acetylene) atomic absorption spectrometry was used. The following determination conditions were used: a stoichiometric flame (2.0 L/min acetylene and 13.5 L/min air), using hollow cathode lamps (HCL) and background correction with a deuterium lamp. The spectral conditions were: wavelength 213.9 nm and slit 1.0 nm. A Zn hollow cathode lamp (HCL) was used with the current 5 mA. The determination limit was 1.0 mg/kg. The uncertainty (understood as a parameter characterizing the dispersion of the values attributed to a measured parameter; the main component of uncertainty was precision) of the entire analytical process (sample preparation and spectrometric measurements) did not exceed 15 %. Due to the lack of reference material, the traceability was determined in a standard addition procedure. The obtained recovery values were in the range 94.3–103.8 %.

Extraction

Ten grams of the powdered mushroom samples or substrates was mixed with 100 mL of 80 % methanol. Samples were sonicated, shaken in an Ika KS 260 shaker (IKA-Werke GmbH & Co. Kg, Staufen, Germany) for 8 h, centrifuged at 3000 rpm with a Universal 320 R centrifuge (Hettich, Tuttlingen, Germany) and then filtered through Whatman No. 4 paper (UK). The extraction was repeated twice, and both supernatants were mixed and evaporated at 40 °C to dryness using Büchi Rotavapor R-205 (Flawil, Switzerland). The obtained residues were weighed and stored at −12 °C until the analyses. For further analysis, the extract was redissolved in 1 mL of 80 % of methanol [28].

Total phenolic and ascorbic acid contents

The total phenolic content (TPC) was determined with the Folin–Ciocalteu reagent [31]. To improve the specificity for TPC determination with the Folin–Ciocalteu method, the modification based on simultaneous quantification of ascorbic acid content (AAC) was introduced according to Sánchez-Rangel et al. [32] and Isabelle et al. [33]. Methanolic extract was mixed with diluted Folin–Ciocalteu phenol reagent (1:1 with water, v:v), and after 3 min, the absorbance at $\lambda = 765$ nm was measured with a Varian Cary 300 Bio UV–Visible scanning spectrophotometer. Afterward, 20 % Na_2CO_2 was added and the samples were kept in the dark for 2 h at room temperature. The absorbance at $\lambda = 765$ nm was measured. The corrected TPC was obtained by subtracting AA reducing activity from the TPC absorbance values. The results of TPC were expressed in mg of chlorogenic acid (CHA) equivalents per g of dried extract (mg CHA/g). The results of AAC were expressed as mg per 100 g of dried weight of mushroom. The results of AA reducing activity were expressed as AAC multiplied by 1.43 [32].

Total flavonoid content

Total flavonoid content (TFC) was measured according to Choi et al. [34], and also Lin and Tang [35] with some modifications. A total of 250 μL of methanolic extract, 1.25 mL of distilled water and 75 μL of 5 % $NaNO_2$ were mixed together. After 6 min, 150 μL of 10 % $AlCl_3$ was added. After the next 6 min, 4 mL of 4 % NaOH and 2.5 mL of deionized water were added. The absorbance was measured at 510 nm, and TFC was expressed as mg rutin equivalents per g of dried extract.

Chromatographic analysis

Chromatographic analysis was performed with a Waters ACQUITY UPLC H-Class System (Waters Corp., Milford, MA, USA), consisting of a quaternary pump solvent management system, an online degasser and an autosampler. An a Acquity UPLC HSS T3 C_{18} column (150 mm × 2.1 mm, particle size 1.8 μm) (Waters, Ireland) was applied for all analyses. The raw data were acquired and processed with Empower software. Before injection, the extracts were filtered through a 0.22 mm syringe filter. The mobile phase was composed of A (water, containing 0.10 % formic acid) and B (acetonitrile, containing 0.10 % formic acid) with a gradient program as follows: flow 0.4 mL/min—5 % B (2 min), 5–16 % B (5 min), 16 % B (3 min), 16–20 % B (7 min), 20–28 % B (11 min) flow 0.45 mL/min—28 % (1 min), 28–60 % B (3 min) flow 5.0 mL/min—60–95 % B (1 min), 65 % B (1 min), 95–5 % B (0.1 min) flow 0.4 mL/min 5 % B (1.9 min). The detection was carried out in a photodiode array detector (PDA) eλ (Waters Corporation, Milford, MA, USA), and measurements of phenolic compound concentrations were performed using an external standard at wavelengths $\lambda = 320$ nm (chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, salicylic acid, sinapic acid, vitexin, rutin, quercetin, luteolin, apigenin, kaempferol), $\lambda = 280$ nm (gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, vanillin, syringic acid, vanillic acid, *t*-cinnamic acid, naringenin) and $\lambda = 230$ nm (catechin, benzoic acid). Compounds were identified based on a comparison of retention times of the examined peak with that of the standard and by adding a specific amount of the standard to the tested sample and repeated analyses. The limit of detection was 1 mg/kg [36].

DPPH radical scavenging assay

The radical scavenging assay was prepared as previously described [28]. One milliliter of methanolic extracts at a concentration between 2 and 20 mg/mL was mixed with 2.7 mL of 6 μmol/L methanolic solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. The mixture was shaken and kept in the dark at room temperature for 60 min. The reduction of the DPPH radical was measured by monitoring the decrease in absorbance at 517 nm. The DPPH radical scavenging activity was calculated according to the formula [37]:

$$\text{Inhibition}\,(\%) = (A - A_C)/A \times 100.$$

where A absorbance of control (DPPH solution without extract), A_C absorbance of methanolic extract of mushroom.

The scavenging activity expressed as EC_{50} represented the concentration of a sample having 50 % of the DPPH radical scavenging effect and was estimated graphically.

Statistical analysis

All analyses were prepared in five replicates, and the results were expressed as mean value ± SD. The data were processed using Microsoft Excel 2010. Statistical analysis was done using STATISTICA 10 (StatSoft, USA) statistical software with one-way ANOVA followed by post hoc Tukey's test (the results marked with identical letters in rows exhibit no differences at the significance level $\alpha = 0.05$). The Pearson correlation coefficients for selected pairs of parameters were also estimated.

Results and discussion

Se and Zn enrichment and their content

The contents of Se and Zn in substrates and in fruiting bodies of *Pleurotus* species are shown in Table 1. The Se and Zn concentration in substrates significantly increased after supplementation. The Se concentration in non-enriched *P. ostreatus* (control) was 2.73 ± 0.26 mg/

Table 1 Se and Zn concentration in substrates and in *P. ostreatus* and *P. eryngii* fruiting bodies (mg/kg DW)

	SPoc	SPoSeZn	Poc	PoSeZn
Se	5.12 ± 0.76^{b}	54.87 ± 0.76^{a}	2.73 ± 0.26^{b}	109.74 ± 11.06^{a}
Zn	14.72 ± 0.51^{b}	$45.67^{a} \pm 1.07$	26.00 ± 0.82^{b}	32.93 ± 0.99^{a}
	SPec	SPeSeZn	Pec	PeSeZn
Se	7.32 ± 0.93^{b}	59.98 ± 1.33^{a}	2.07 ± 0.68^{b}	54.39 ± 0.79^{a}
Zn	22.45 ± 1.03^{b}	56.23 ± 0.47^{a}	22.98 ± 0.84^{b}	85.95 ± 2.22^{a}

Mean values ($n = 5$) ± SDs; identical superscripts in row denote no significant ($\alpha < 0.05$) difference between mean values according to Tukey's HSD test (ANOVA)

DW, dry weight; SPoc, substrate of non-enriched *P. ostreatus* (control); SPoSeZn, substrate of enriched *P. ostreatus*; Poc, non-enriched *P. ostreatus* (control); PoSeZn, *P. ostreatus* enriched with Se and Zn; SPe, substrate of non-enriched *P. eryngii* (control); SPeSeZn, substrate of enriched *P. eryngii*; Pec, non-enriched *P. eryngii* (control); PeSeZn, *P. eryngii* enriched with Se and Zn

kg DW, and it was comparable as in our earlier experiments [28, 30]. The concentration of Zn in the control of *P. ostreatus* was 26.00 ± 0.82 mg/kg DW. The enrichment of the substrate with Se + Zn resulted in a significant increase in the elements in fruiting bodies up to 109.74 ± 11.06 mg/kg DW and 32.926 ± 0.987 mg/kg DW Se and Zn, respectively. The Se concentration in the control of *P. eryngii* was 2.07 ± 0.68 mg/kg DW, and a significant rise up to 54.39 ± 0.79 mg/kg DW was observed for Se + Zn-enriched mushrooms. The concentration of Zn in control and Se + Zn-enriched *P. eryngii* was as follows: 22.98 ± 0.84 and 85.95 ± 2.22 mg/kg DW, respectively. Our experiment confirmed that non-enriched *P. ostreatus* and *P. eryngii* contained Se and Zn at the level as in the most popular mushrooms, which range from <1 to 20 µg/g DW for Se and from ~5 to more than 100 µg/g DW for Zn [21, 25, 28, 38–44]. Because mushrooms have great ability to accumulate selected elements [44], supplementation of substrate with different micronutrients was successfully used to enhance their content in fruiting bodies [21, 25, 26, 29]. In our experiment, after Se and Zn enrichment substrates possessed higher mineral availability for fruiting bodies and it influenced the micronutrient accumulation in mushrooms. The concentration of Se after enrichment was ~40 and ~27 times higher for *P. ostreatus* and *P. eryngii*. The results were consistent with our earlier experiment [28, 30], because the Se concentration in enriched fruiting bodies was significantly higher than in the control. Bhatia et al. [25] revealed that Se-enriched *P. florida* was able to accumulate even 800 times higher concentration of the micronutrient in comparison with the control. The increase in the content of Zn in enriched mushroom was slight in *P. ostreatus*, while it was large (nearly 4 times) for *P. eryngii*. However, Vieira et al. [27] reported that enrichment with Zn did not lead to changes of its concentration in the fruiting bodies. Our results for *P. eryngii* are opposite; the increase in Zn content in our experiment could be due to an interaction between Zn and Se which favors increased Zn content in mushrooms.

Furthermore, the mushroom enrichment is dependent on species, pH, composition of substrate, concentration of the added element and other minerals [45].

Yield, total phenolic, flavonoid and ascorbic acid contents

The yield of extract ranged from 25.89 ± 1.88 to 26.49 ± 1.23 g per 100 g DW and from 28.15 ± 0.27 to 28.85 ± 2.85 g per 100 g DW for *P. ostreatus* and *P. eryngii* (respectively), and there were no significant differences ($\alpha = 0.05$) between control and enriched mushrooms (Table 2). The results were higher than those obtained by Yang et al. [46], but similar to Oke and Aslim [47]. Other studies demonstrated that the yield mainly depends on polarity of the solvent [6, 47, 48]. The Folin–Ciocalteu method is a colorimetric method commonly used to determine the total phenolic content [7, 47]. The Folin–Ciocalteu assay is sensitive to other metabolites, which causes overestimation of the results due to interference of the metabolites with the reagent, and therefore, the method is not specific for the determination of total phenolic content [5]. To improve the specificity for total phenolic content determination in our study, modifications suggested by Sánchez-Rangel et al. [32] involving the simultaneous quantification of total ascorbic acid by the Folin–Ciocalteu assay were applied. The total phenolic content in non-enriched

Table 2 Extraction yield, total phenolic, flavonoid and ascorbic acid contents and EC_{50} value in *P. ostreatus* and *P. eryngii* fruiting bodies

	Poc	PoSeZn
Yield (g/100 g DW)	25.89 ± 1.88[a]	26.49 ± 1.23[a]
TPC (mg/g of extract)	9.64 ± 0.33[b]	13.38 ± 0.58[a]
TFC (mg/g of extract)	2.11 ± 0.19[b]	2.72 ± 0.09[a]
AAC (mg/100 g DW)	10.28 ± 0.39[b]	15.76 ± 0.57[a]
EC_{50} (mg/mL) (DPPH)	4.42 ± 0.08[a]	3.84 ± 0.09[b]
	Pec	PeSeZn
Yield (g/100 g DW)	28.85 ± 2.85[a]	28.15 ± 0.27[a]
TPC (mg/g of extract)	7.91 ± 1.02[b]	10.86 ± 1.48[a]
TFC (mg/g of extract)	1.26 ± 0.17[b]	1.89 ± 0.09[a]
AAC (mg/100 g DW)	16.64 ± 0.47[b]	34.74 ± 1.99[a]
EC_{50} (mg/mL) (DPPH)	7.34 ± 0.11[a]	3.35 ± 0.11[b]

Mean values ($n = 5$) ± SDs; identical superscripts in row denote no significant ($\alpha < 0.05$) difference between mean values according to Tukey's HSD test (ANOVA)

DW, dry weight; TPC, total phenolic content; TFC, total flavonoid content; AAC, ascorbic acid content; Poc, non-enriched *P. ostreatus* (control); PoSeZn, *P. ostreatus* enriched with Se and Zn; Pec, non-enriched *P. eryngii* (control); PeSeZn, *P. eryngii* enriched with Se and Zn

mushrooms for *P. ostreatus* and *P. eryngii* was 9.64 ± 0.33 and 7.91 ± 1.02 mg/g of extract, respectively. The total flavonoid content was 2.11 ± 0.19 and 1.26 ± 0.17 mg/g of extract, respectively. It was documented that major components of mushroom extract are phenolics and *Pleurotus* species exhibited different contents of total phenolic ranging from ~2 to >30 mg/g of extract [27, 37, 49]. In comparison with our earlier study [28], the results obtained for non-enriched mushrooms were slightly higher. The reason should be sought in the composition of substrates. It has been reported that mushrooms contain different compounds of phenolics. Some authors have stated that mushrooms contain mainly phenolics acids [7, 50], while others reported a high amount of flavonoids in extracts [5, 37, 51]. The total flavonoid content in different species of *Pleurotus* was found at the level from 1.2 to 2.9 μg/g of extract [49], even to 7.79 mg/g of extract [37]. However, Vieira et al. [27] did not detect flavonoids in *P. ostreatus*. It was found that AAC was dependent on species and ranged from <5 up to as high as 50 mg/100 g DW [40, 52, 53]. In our experiment, the ascorbic acid level in non-enriched *Pleurotus* species ranged from 10.28 ± 0.39 to 16.64 ± 0.47 mg/100 g DW. The enrichment of substrates with Se and Zn resulted in significant increases in TPC, TFC and AAC. It was documented that Se treatments enhanced the phenolic content in plants and mushrooms [26, 54, 55]. However, no changes of phenolic content in zinc-enriched *P. ostreatus* were detected by Vieira [27]. In the case of Se, it was suggested that the element enhances accumulation of some sugars [54] or inhibits enzymatic polyphenol oxidation [26], while Zn is involved in the regulation of sugar metabolism [56, 57]. Thus, the mechanism is probably related to changes in concentration of glucose, which is an important substrate in many metabolic pathways, and in that way enhanced the phenolic content. Additionally, Zn was able to induce oxidative stress [58] and in consequence stimulate synthesis of antioxidants such as ascorbic acid and phenolics. The higher content of the metabolites in enriched mushrooms could be the result of detoxification mechanisms caused by elevated levels of the elements.

Chromatographic profile

In the study, we used 22 standards for identification of phenolics, among which the phenolic acids and flavonoids were detected in substrates and *Pleurotus* species. In substrates, caffeic, chlorogenic, ferulic, syringic, *p*-coumaric, vanillic and *t*-cinnamic acids and naringenin were detected (Table 3). We quantified derivatives of benzoic (4-hydroxybenzoic, 2,5-dihydroxybenzoic, protocatechuic and vanillic acids) and *t*-cinnamic acids (*p*-coumaric, ferulic and *t*-cinnamic acids) and flavanones (naringenin) in fruiting bodies (Table 4). Six phenolic acids were detected in the extract from *P. ostreatus:* 4-hydroxybenzoic, ferulic, *p*-coumaric, protocatechuic, *t*-cinnamic and vanillic acids. Additionally, naringenin was also quantified. The extract from *P. eryngii* additionally contained 2,5-dihydroxybenzoic acid. Among the compounds, ferulic acid was dominant in both analyzed *Pleurotus* species. The monitoring of chromatograms showed that mushroom contains different phenolic compounds [5, 7, 47, 50]. In our previous experiment [28], we detected only 4-hydroxybenzoic, *p*-coumaric, ferulic acids and myricetin. The differences could be due to the impact of Zn on phenolic composition. Additionally, substrates could have influenced phenolics in fruiting bodies because of decomposition of lignin by mycelium. This polymer contains in the structure some phenolic acids or their derivatives. Woldegiorgis et al. [7] detected in *P. ostreatus* caffeic, gallic and *p*-hydroxybenzoic acids and myricetin. Kim et al. [59] additionally quantified homogentisic, protocatechuic, chlorogenic acids, naringin and myricetin. The study of *P. ostreatus* by Palacios et al. [5] confirmed *p*-coumaric, ferulic, gallic, gentisic, *p*-hydroxybenzoic, homogentisic and protocatechuic acids and myricetin in fruiting bodies. Kim

Table 3 Phenolic acid and flavonoid composition of substrates (μg/g DW)

Compounds	SPoc	SPoSeZn	SPec	SPeSeZn
Chlorogenic acid	2.50 ± 0.09[a]	2.70 ± 0.11[a]	1.27 ± 0.18[a]	1.49 ± 0.40[a]
Syringic acid	2.30 ± 0.19[b]	3.70 ± 0.11[a]	2.22 ± 0.22[b]	3.87 ± 0.22[a]
Ferulic acid	3.00 ± 0.33[b]	4.46 ± 0.24[a]	4.87 ± 0.33[b]	6.29 ± 0.47[a]
p-Coumaric acid	7.17 ± 0.23[b]	9.12 ± 0.12[a]	6.49 ± 0.22[b]	8.00 ± 0.18[a]
Caffeic acid	1.21 ± 0.08[b]	2.52 ± 0.09[a]	1.23 ± 0.21[b]	3.81 ± 0.17[a]
t-Cinnamic acid	2.35 ± 0.15[b]	5.97 ± 0.59[a]	2.79 ± 0.18[a]	2.69 ± 0.11[a]
Vanillic acid	1.34 ± 0.06[b]	3.87 ± 0.52[a]	1.59 ± 0.22[b]	2.85 ± 0.18[a]
Naringenin	0.15 ± 0.03[b]	0.53 ± 0.17[a]	0.42 ± 0.04[b]	0.63 ± 0.09[a]

Mean values ($n = 5$) ± SDs; identical superscripts in rows for each mushroom species denote no significant ($p < 0.05$) difference between mean values according to Tukey's HSD test (ANOVA)

SPoc, substrate of non-enriched *P. ostreatus* (control); SPoSeZn, substrate of enriched *P. ostreatus*; SPec, non-enriched *P. ostreatus* (control); SPeSeZn, substrate of enriched *P. eryngii*

Table 4 Phenolic acid and flavonoid composition of *P. ostreatus* and *P. eryngii* (μg/g DW)

Compounds	Poc	PoSeZn	Pec	PeSeZn
2,5-Dihydroxybenzoic acid	nd	nd	1.37 ± 0.30^{b}	5.49 ± 0.41^{a}
4-Hydroxybenzoic acid	5.30 ± 0.20^{b}	6.70 ± 0.19^{a}	2.32 ± 0.32^{b}	3.59 ± 0.20^{a}
Ferulic acid	30.00 ± 1.00^{b}	34.46 ± 2.94^{a}	29.00 ± 1.00^{b}	36.29 ± 1.47^{a}
p-Coumaric acid	10.54 ± 0.70^{b}	15.82 ± 1.02^{a}	13.49 ± 2.22^{b}	20.00 ± 0.88^{a}
Protocatechuic acid	0.21 ± 0.09^{b}	0.52 ± 0.07^{a}	1.43 ± 0.24^{b}	7.81 ± 1.67^{a}
t-Cinnamic acid	0.35 ± 0.05^{b}	0.97 ± 0.05^{a}	0.79 ± 0.15^{b}	1.69 ± 0.01^{a}
Vanillic acid	0.34 ± 0.03^{b}	0.87 ± 0.12^{a}	0.59 ± 0.04^{b}	0.85 ± 0.18^{a}
Naringenin	0.18 ± 0.05^{b}	0.73 ± 0.18^{a}	0.18 ± 0.04^{b}	0.43 ± 0.09^{a}

Mean values ($n = 5$) ± SDs; identical superscripts in rows for each mushroom species denote no significant ($p < 0.05$) difference between mean values according to Tukey's HSD test (ANOVA)

Poc, non-enriched *P. ostreatus* (control); PoSeZn, *P. ostreatus* enriched with Se and Zn; Pec, non-enriched *P. eryngii* (control); PeSeZn, *P. eryngii* enriched with Se and Zn

et al. [59] examined *P. eryngii* and documented gallic and protocatechuic acids and naringin in the mushroom. According to Reis et al. [50] in *P. ostreatus* and *P. eryngii*, the presence of protocatechuic, *p*-hydroxybenzoic, *p*-coumaric and cinnamic acids was found. Oke and Aslim [47] quantified gallic, *p*-hydroxybenzoic, caffeic, syringic, *p*-coumaric, ferulic and cinnamic acids and catechin in *P. eryngii*. In other species of mushroom, additionally α-resorcylic and syringic acids were detected [6, 7, 59]. Flavonoids or their aglycones (myricetin, quercetin, naringenin, kaempferol and hesperetin) were also detected in different species of edible mushroom [5, 7, 59] (although neither fungi nor animals, but only plants have the ability to biosynthesize flavonoids [60]).

Activation of synthesis of free phenolic acids was also observed in our previous study [28] as well as in *L. sativus* [61]. As mentioned above, it could be the result of the elements on metabolism of sugars and in consequence on metabolism of other biomolecules.

Table 2 presents DPPH scavenging ability expressed as EC_{50} values. Better scavenging ability for non-enriched mushroom was obtained for *P. ostreatus* (4.42 mg/mL). The drop in EC_{50} values observed for enriched mushroom confirmed improvement of the antioxidant properties. Additionally, both enriched species of *Pleurotus* had similar EC_{50} values. Scavenging effects on DPPH of extract from non-enriched mushroom increased with concentrations (Fig. 1). For *P.* ostreatus, it ranged from 19.7 to 76.7 %, and for *P. eryngii*, it was between 17.9 and 62.1 %. The Se and Zn addition resulted in increases in the scavenging effects by 35.8–87.8 and 40.3–91.4 %, respectively. In other experiments EC_{50} values for *P. ostreatus* were 8.4 mg/mL [7] and 6.54 mg/mL [50], while EC_{50} value for *P. eryngii* was 8.67 mg/mL [50]. Se-rich mushroom showed a larger DPPH scavenging effect than non-enriched mushroom [26, 28]. In the study, we demonstrated that simultaneous supplementation of micronutrients significantly affected the antioxidant properties.

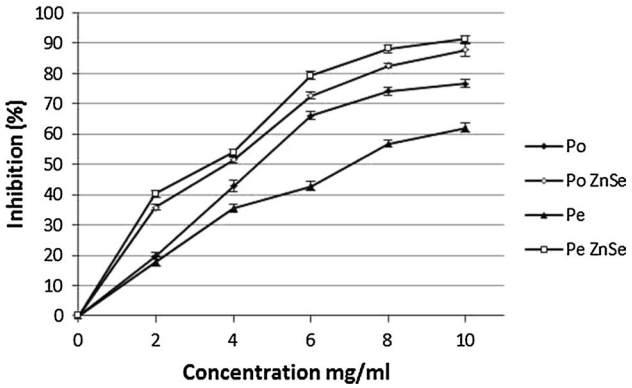

Fig. 1 Scavenging ability of methanolic extracts from *P. ostreatus* and *P. eryngii* on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. Each value is expressed as mean ± SDs ($n = 5$). Po, non-enriched *P. ostreatus*; PoZnSe, enriched *P. ostreatus*; Pe, non-enriched *P. eryngii*; PeZnSe, non-enriched *P. eryngii*

The correlation between the total phenolic content and antioxidant activity in mushroom was confirmed [62]. In our previous study for enriched *P. ostreatus*, a correlation between inhibition of the radicals and the total phenolic and flavonoid content was found, whereas Vieira et al. [27] found no correlations for iron-, zinc- and lithium-enriched *P. ostreatus.* In the present study for *P. ostreatus*, we found a correlation between EC_{50} value and phenolic compounds (except naringin) and Zn and Se concentration in fruiting bodies and between micronutrient concentrations and phenolic compounds (except ferulic acids) (Table 5). For *P. eryngii*, strong correlations were found between EC_{50} value and phenolic compounds (except vanillic acid) and Zn and Se concentrations in fruiting bodies and between micronutrient concentrations and phenolic compounds (except vanillic acid) (Table 6). Ions of some metals including Zn can form a complex with polyphenols [63]. Moreover, phenolics complexed by metals have reduced antioxidant

Table 5 Pearson correlation coefficients (*r*) between Se and Zn concentrations in fruiting bodies and EC_{50}, phenolic and flavonoid components and between EC_{50} and phenolic and flavonoid components for *P. ostreatus*

Compounds	EC_{50}	Se	Zn
4-Hydroxybenzoic acid	−0.922	0.967	0.918
Protocatechuic acid	−0.822	0.950	0.859
Vanillic acid	−0.822	0.988	0.925
p-Coumaric acid	−0.935	0.949	0.969
Ferulic acid	−0.910	ns	ns
t-Cinnamic acid	−0.901	0.995	0.948
Naringin	ns	0.955	0.906
TPC	−0.950	0.988	0.969
TFC	−0.999	0.987	0.938
AAC	−0.999	0.989	0.973
EC_{50}		−0.893	−0.914
Se	−0.893		0.967
Zn	−0.914	0.967	

TPC, total phenolic content; TFC, total flavonoid content; AAC, ascorbic acid content

Table 6 Pearson correlation coefficients (*r*) between Se and Zn concentration in the fruiting bodies and EC_{50}, phenolic and flavonoid components and between EC_{50} and phenolic and flavonoid components for *P. eryngii*

Compounds	EC_{50}	Se	Zn
2,5-Dihydroxybenzoic acid	−0.988	0.989	0.987
4-Hydroxybenzoic acid	−0.946	0.950	0.957
Protocatechuic acid	−0.946	0.954	0.945
Vanillic acid	ns	ns	ns
p-Coumaric acid	−0.926	0.951	0.929
Ferulic acid	−0.953	0.959	0.952
t-Cinnamic acid	−0.984	0.983	0.977
Naringin	−0.902	0.902	0.892
TPC	−0.978	0.995	0.993
TFC	−0.882	0.882	0.873
AAC	−0.999	0.999	0.999
EC_{50}		−0.999	−0.998
Se	−0.999		0.999
Zn	−0.998	0.999	

TPC, total phenolic content; TFC, total flavonoid content; AAC, ascorbic acid content

activity, because of the limited availability of free radicals for donation [27, 63]. On the other hand, high content of metals in enriched mushroom can result in bioavailability of the metals. Thus, higher content of Zn and other metals bioavailable in mushrooms may result in lower complexation with polyphenols. In this context, the increase in antioxidant activity (represented by $DPPH^{\cdot}$) observed in our experiment could be the result of the fact that the formation of complexes was reduced or other compounds exhibiting higher antioxidant activity were formed. Also interaction between Se and Zn could affect the bioavailability of metals and consequently the formation of complexes. Despite the reduction of antioxidant properties of some elements including Zn [27], our results with simultaneous supplementation with Zn and Se provide the opportunity to improve antioxidant properties and antioxidant contents in enriched mushrooms.

To conclude, the enrichment is a good practice to enhance the mineral content in mushrooms. Additionally, the obtained results demonstrated that simultaneous enrichment with micronutrients with contrary effects on antioxidant properties can activate synthesis of phenolic compounds and ascorbic acid. The micronutrient supplementation of substrates caused improvement of the antioxidant properties and increased content of phenolic compounds in enriched fruiting bodies of *P. ostreatus* and *P. eryngii* in comparison with the controls.

The present study is part of experiments with simultaneous enrichment of mushrooms, which focuses on estimating the impact of minerals on antioxidant properties of edible mushrooms. The investigation is the first study evaluating the effect of addition of two elements to the substrate at the same time on antioxidant properties of mushrooms. Further studies on Se and other micronutrients are in progress.

Compliance with ethical standards

Conflict of interest None.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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