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POPULAR SPECIES OF EDIBLE MUSHROOMS AS A GOOD SOURCE OF ZINC TO BE RELEASED TO ARTIFICIAL DIGESTIVE JUICES

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Because fruiting bodies of edible mushrooms accumulate elements very effectively, in this study for the first time we aimed at determining the degree of the release of zinc(II) ions to artificial digestive juices imitating the human gastrointestinal tract from freeze-dried popular edible mushroom fruiting bodies, such as *Agaricus bisporus*, *Boletus badius* and *Cantharellus cibarius*. For the analysis, anodic stripping voltammetry method was used. The amount of zinc released to artificial saliva within 1 minute ranged from 0.03 to 1.14 mg/100 g d.w. In gastric juice, the amounts were higher and ranged from 0.75 to 2.07 mg/100 g d.w. depending on the incubation time. After incubation of the freeze-dried edible mushroom fruiting bodies for 1 minute in artificial saliva, 15 in artificial gastric juice and then 150 minutes in artificial intestinal juice, it was found that the concentration of the released zinc in artificial intestinal juice was the highest and amounted to 6.44 mg/100 g d.w. The total average amount of zinc released from *Boletus badius* was the highest and this was estimated at 4.13 mg/100 g d.w. For the remaining two investigated species of *A. bisporus* and *C. cibarius*, the total amounts of zinc released into artificial digestive juices were only slightly lower and were estimated at 2.23 and 3.29 mg/100 g d.w. on average, respectively. It was demonstrated for the first time that mushrooms release zinc to artificial digestive juices imitating conditions in the human digestive tract and are a good source of this element.

Key words: *zinc, trace elements, stripping voltammetry, freeze-dried fruiting bodies of mushrooms, artificial saliva, gastric juice*

INTRODUCTION

Zinc plays a basic role in many biochemical reactions. It is necessary during the growth of immune cells, because it constitutes part of the metalloenzymes, which are involved in the synthesis of DNA, RNA and proteins (1). Moreover, zinc exhibits antioxidant activity and therefore prevents the oxidation of unsaturated fatty acids, is involved in the storage and release of insulin from pancreas, and also enhances its action and thereby facilitates glucose transport into cells (2-5). Zinc is absorbed in human digestive system from food or drinking water. This also occurs to a lesser extent in the stomach and large intestine than in the small intestine. Zinc can also enter through the lungs (zinc dust) and can pass directly through the skin, but in relatively small amounts. The concentration of this element increases in blood and zinc may remain in the bone for many days after exposure. The concentration of this element in human serum is a mean of 1 mg/L and the amount in blood cells is ten times higher. It is removed from the body in urine and feces (6-8).

Zinc is also one of the antagonists of the glutamate system, which is involved in antidepressant activity. This element crosses the blood-brain barrier as well as the blood-cerebrospinal fluid barrier, thanks to histidine and divalent metal transporter 1 (DMT1). In the brain, thanks to the appropriate transporters, zinc is moved into the cytoplasm, and its concentration is regulated by metallothioneins (9, 10). Zinc is also important in the prevention of Alzheimer's disease, involved in immune processes affecting the immune system and increases maintenance of normal levels of vitamin A in plasma (11). The proper functioning of skin and mucous membranes and the process of heavy metal detoxification, e.g. cadmium and lead, are also zinc-dependent.

This element is involved in the activation of over 300 enzymes, among others, alcohol dehydrogenase involved in the metabolism of alcohol, carbonic anhydrase, which is involved in the production of bicarbonate (HCO_3^-) ions, and histidine deaminase, which catalyzes the deamination reaction (12). Zinc exhibits antioxidant properties, because it is present in superoxide dismutase responsible for the elimination of free radicals (13, 14). Studies have shown that zinc supplementation causes a reduction of T cell activation and a decrease in tumor necrosis factor (TNF-α) release. In the future, it may affect the treatment of autoimmune diseases (15). This element is also required to treat ulcers, because it promotes the healing of wounds (5) and the normalization of serum Zn/Cu ratios may be useful in the treatment of depression (16). The daily requirement for zinc in a healthy adult human is dependent on age and is about 15 mg.

Mushrooms growing in a natural environment have always been consumed by humans due to their unique taste and aroma

(17). The medicinal use of mushrooms has a very long tradition (18, 19). Fruiting bodies of edible mushrooms are a source of many important organic and inorganic substances, such as proteins, polysaccharides, phenolic compounds, terpenoids, indole compounds, vitamins and bioelements, which exhibit a key physiological role in the human body (20, 21). Some of the metabolites are used to treat such serious diseases as cardiovascular diseases, diabetes, atherosclerosis, cancer and exhibit antioxidant, antiviral and antibacterial activity (22-26).

In many studies, qualitative and quantitative analyses of bioelements have been carried out including the analysis of zinc occurring in fruiting bodies of edible mushrooms (27). Mushrooms constitute a good material for studies, because they have the ability to accumulate elements from the soil and surrounding environment, acting as environmental bioindicators (18, 27, 28). Zinc concentrations in fruiting bodies of edible mushrooms are considerably higher than those in plants (herbs, fruits, vegetables, crops) (29). Only sea foods (oyster) are a better source of this element than mushrooms (WHO/FAO). Mushroom fruiting bodies, as well as their spores have the highest ability to accumulate micro- and macronutrients. The most important mechanism of accumulation of elements in mushrooms is based on binding by metallothionein - a low-molecular-weight protein, which has an affinity especially for metals. The accumulation of elements in fruiting bodies of mushrooms is influenced by fungal structure, ontogenetic development of the fungus, biochemical composition, decomposition activity, environmental factors, for example metal concentration in air and soil and its pH, organic matter and the bioavailability of metals (30-32). Increased concentration of these micronutrients in the soil, and then in the mushroom is associated with its place of location (28, 33, 34).

Many studies confirming the very good accumulation of elements in mushrooms have been carried out; hence due to the occurrence of this phenomenon, it is advisable not only to investigate their amount, but also to study the actual quantities released into the artificial digestive juices. This will allow an evaluation of their usefulness as a source of zinc supplied with food. In order to determine the bioavailability of zinc from freeze-dried edible mushrooms such as *Agaricus bisporus*, *Boletus badius*, *Cantharellus cibarius*, artificial digestive juices imitating those naturally occurring in the digestive tract of humans were used. These species were selected due to their popularity among consumers and documented medicinal properties (34-36).

As far as we know, the present study is the first to evaluate the release of zinc into artificial gastric juices in standard conditions (25°C), which constitutes the first step in the investigation of the bioavailability of this element (37). In addition, as another objective of the current study, the total amount of released zinc was established. Therefore, the same batch of freeze-dried material was placed successively in the artificial saliva solution, and after the centrifugation of the extract designed for the analysis of the amount of released zinc, the material was placed in a solution of artificial gastric juice, and finally in a solution of artificial intestinal juice.

This procedure was designed to mimic conditions occurring in the human digestive tract.

MATERIALS AND METHODS

Reagents

Citric acid, NaOH, K_2HPO_4, Na_2HPO_4, and $KHCO_3$ were from Polish Company of Chemistry (Gliwice, Poland); $NaHCO_3$, and NaCl were from PPH Golpharm (Cracow, Poland); $MgCl_2$ was from Chempur (Cracow, Poland); $CaCl_2$ was from Pharma Zentrale GmbH (Germany); bile salts and pepsin were from BTL (Lodz, Poland); spleen extract, HCl, KCl, HNO_3 concentrated Suprapur®, KNO_3 Suprapur®, and H_2O_2 30% were from Merck (Darmstad, Germany); Zn(II) standards were from OUM-7 Lodz, Poland. Quadruple-distilled water with a conductivity of less than 1 µS cm^{-1} was obtained with the use of an S2-97A2 distillation apparatus (Chemland, Stargard Szczecin, Poland).

Mushroom material

In this study, fruiting bodies of selected edible mushrooms were used: *Agaricus bisporus* (JE Lange) Imbach (White bottom mushroom) of commercial origin (supermarket), and collected from the natural environment in mixed forests of South Poland (in the vicinity of Nowy Sacz and Alwernia) between 2012 and 2013; *Boletus badius* Pers. (Bay bolete); and *Cantharellus cibarius* Fr. (Chantarelle). Taxonomic identification of the young sporocarps was conducted according to online keys (http://www.mycokey.com) and to Knudsen and Vesterholt (38). Representative voucher specimens were deposited at the Department of Pharmaceutical Botany, Jagiellonian University Medical College, Cracow, Poland. Mushroom materials were frozen and lyophilized (Freezone 4.5. Labconco; temperature: –40°C) to obtain the mushroom samples for further analyses.

Sample preparation

Freeze-dried mushrooms were ground in a porcelain mortar, followed by the preparation of weighed portions of approximately 500 mg. They were placed in flasks containing 10 mL of artificial saliva solution according to Arvidson's recipe (39), shaken for 1 minute (shaking time in artificial saliva similar to that in the remaining artificial digestive juices: gastric and intestinal, results from the assumed average storage time of food in the oral cavity). The suspension was then centrifuged and decanted, and the residue - mushroom fruiting bodies after digestion in artificial saliva solution were placed in 10 mL of gastric juice. Samples were shaken in 10 mL of artificial gastric juice for 15, 30, 60, 90, and 120 minutes, respectively.

The solution was again centrifuged and decanted, and 10 mL of artificial intestinal juice solution were added to the recovered fruiting bodies of investigated species and the mixture was shaken for 150 minutes. Shaking was carried out using a type 327 Universal Shaker type, and the decanted solution was centrifuged for 30 minutes in a MPW-223e centrifuge. Solutions prepared according to such a method were filtered through membrane filters (Millex, Millipore Corporation, USA). The resulting filtrates from artificial saliva solution and artificial gastric juice were mineralized by the addition of 1 mL of nitric acid Suprapur® in a UV Mineral R-8 Power Supply 8 mineralizer equipped with a UV lamp for 24 hours. Mineralization of artificial intestinal juice filtrate was carried out after the addition of 1 mL of nitric acid Suprapur® and 50 µL dihydrogen peroxide for 24 hours under the same conditions (apparatus and time). In order to determine zinc(II) ions by the anodic stripping voltammetry method, mineralized samples were neutralized by adding the appropriate volume of 0.1M NaOH solution.

Preparation of artificial digestive juice solutions

Artificial saliva

Liquid imitating conditions which are present in the oral cavity was prepared according to Arvidson's model. Artificial saliva with a pH about 6.7 was prepared by mixing 100 mL 25

mM KH_2PO_4, 100 mL 24 mM Na_2HPO_4, 100 mL 150 mM $KHCO_3$, 100 mL 100 mM NaCl, 100 mL 1.5 mM $MgCl_2$, 6 mL 25 mM citric acid and 100 mL 15 mM $CaCl_2$ with quadruple-distilled water. In this model, no digestive enzymes (α-salivary amylase, lipase salivary) present in saliva were included (39).

Artificial gastric juice

In the stomach, pH values range from 1.0 to 3.5, but in most artificial gastric juice models pH is 2.0. The solution of this artificial body fluid was prepared according to Polish Pharmacopoeia IX by dissolution of 2.0 g NaCl and 3.2 g pepsin in quadruple-distilled water. Then, 80 mL of 1M hydrochloric acid was added to adjust the pH, followed by supplementation with quadruple-distilled water to 1000 mL (37).

Artificial intestinal juice

Artificial intestinal juice used in the model for *in vitro* studies was prepared by dissolving 5 mL of pancreatic extract (4 g/L) and bile salts (25 g/L) in 0.1 M $NaHCO_3$ solution, followed by supplementation with quadruple-distilled water to 1000 mL (40).

Validation of the differential pulse anodic stripping voltammetry (DP ASV) method for the determination of zinc in artificial digestive juices

The method for the determination of zinc concentration was validated. For this purpose, parameters such as accuracy, precision, linearity, limit of detection and limit of quantification were determined.

Accuracy

In order to evaluate the degree of accuracy of the obtained results in comparison to the reference value for a sample containing zinc(II) ions of known concentration, increasing amounts of the reference standard were added at a concentration of 1 µg/mL which corresponded to 50, 100 and 150% of the amount of zinc ions in the sample. After each addition of the standard, the voltammetric curve was recorded three times (*Fig. 1*). The average recovery was 98.23%.

Precision

The precision of the method was determined by multiple (n = 3) determinations of zinc ion concentration in the investigated samples using an internal standard and adopting relative standard deviation as a criterion (*Table 1*).

Linearity

Using the applied voltammetric method, the relationship between current intensity and voltage applied to the electrodes was investigated. For the determined sample, which contained the basic electrolyte KNO_3 at a concentration of 0.1 M, each 10 µL of Zn(II) (1 mg/mL) standard was added in three repetitions. After each standard addition, determination was performed in three repetitions. It was established that the curve describing the relationship between current intensity and Zn(II) concentration is linear in the range of 0.8 – 11.2 µg/mL. The linear equation is presented as follows: $y = -2.43 \times +0.85$. The established Pearson correlation coefficient is $r = 0.9997$.

Limit of detection (LOD) and limit of quantification (LOQ)

Limit of detection and limit of quantification were determined from the linearity in the concentration range 0.8 – 11.2 µg/mL. For the calculations, formulas such as $LOD = 3.3\ S_y/a$, $LOQ = 10\ S_y/a$ were used, where S_y is standard error of estimate, a is slope of the line. The determined limit of detection and limit of quantification were: LOD = 0.64 µg/mL and LOQ = 1.92 µg/mL, respectively.

Statistical analysis

The results were expressed as mean values with standard deviations (S.D.). All the analyses were conducted using Statistica 10 (StatSoft, Poland). Statistical significance was tested at $P \leq 0.05$ and at $P \leq 0.01$.

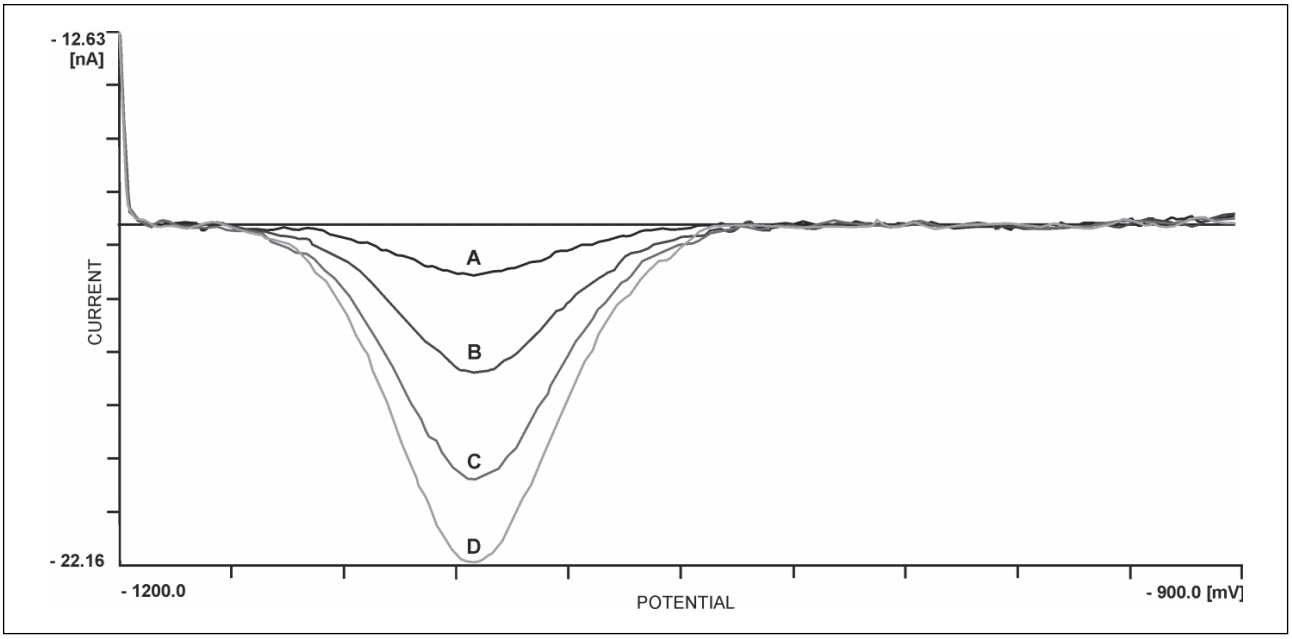

Fig. 1. Voltammogram obtained for a sample of *Agaricus bisporus* species after incubation in saliva for 1 minute, and in gastric juice for 60 minutes. The study of zinc release in gastric juice for sample (A) and standard addition (B - D).

RESULTS

The use of a sample preparation procedure for the analysis, as well as the application of the differential pulse anodic stripping voltammetry (41-43) method for the determination of zinc(II) ions released from the fruiting bodies of *A. bisporus, B. badius, C. cibarius* enabled the precise, relatively easy and fast determination of zinc in artificial digestive juices.

In the study, the degree of zinc ion release from freeze-dried edible mushroom fruiting bodies to artificial saliva, artificial gastric juice and artificial intestinal juice was determined. For this purpose, a method of sample preparation allowing for the determination of zinc ions released to artificial body fluids was established. Based on the obtained results, it was shown that zinc is released to any type of artificial digestive juice independently of the mushrooms species.

The analyses showed a relationship between the type of digestive juice; the time during which the material was tested; mushrooms species investigated, and the degree of zinc release. To mimic the action of the gastrointestinal tract, the release of zinc was determined from the freeze-dried edible mushroom fruiting bodies from immersion to artificial saliva solution, introduction to artificial gastric juice solution, and finally introduction to artificial intestinal juice solution with simultaneous mechanical stirring and thus mimicking the peristaltic movement in this section of the gastrointestinal tract. After incubation of the freeze-dried edible mushroom fruiting bodies for 1 minute in artificial saliva, for 15 and 30 minutes in artificial gastric juice and then for 150 minutes in artificial intestinal juice, it was found that the concentration of the released zinc was the highest in artificial intestinal juice.

However, maintaining the same incubation time in artificial saliva, and prolonging the incubation period in artificial gastric juice to 60, 90 and 120 minutes, it was observed that after transfer of samples to the artificial intestinal juice for a period of 150 minutes a reduction in the concentration of released zinc was reported due to extended residence of the samples in artificial gastric juice. The amount of zinc released within 1 minute to artificial saliva ranged from 0.03 to 1.14 mg/100 g d.w. (*Table 1*). In the gastric juice, the amounts were higher and ranged between 0.75 – 2.07 mg/100 g d.w. depending on the incubation time. In the intestinal juice, the minimum value of the total zinc released was the highest, and the differences in relation to the maximum values were greater in comparison to artificial gastric juice (ranging from 0.41 to 6.44 mg/100 g d.w.). *B. badius* was the species for which the total amount of released zinc(II) ions in mg/100 g d.w. was the highest (the sum of the amount obtained in artificial saliva, gastric and intestinal juice). Zinc amount released from dried fruiting bodies of this species to artificial gastric juice, depending on the incubation time, and its amount was the highest for 15 minutes (1 minute in the artificial saliva and 150 minutes in the artificial intestinal juice), and the lowest for 60 minutes, which corresponded to 6.44 mg/100 g d.w., and 0.41 mg/100 g d.w., respectively, at the same incubation conditions for the remaining residence periods of the material in artificial saliva and intestinal juice. The total average amount of zinc released from this species was the highest and accounted for 4.13 mg/100 g d.w.

For the remaining two other investigated species, namely *A. bisporus* and *C. cibarius*, the total amount of zinc released into artificial digestive juices was only slightly lower, and amounted to 2.23 and 3.29 mg/100 g d.w. on average, respectively. Moreover, the amount of zinc released from wild-growing *B. badius* and *C. cibarius* species was greater than that from *A. bisporus* of commercial origin (*Fig. 2*). It should also be noted that the highest amounts of zinc in all the investigated fruiting bodies were released into artificial intestinal juice. This may be explained by the etching of mushroom material by enzymes, and the pH of artificial digestive juice as well.

DISCUSSION

There are numerous reports on the qualitative and quantitative composition of elements occurring in the fruiting bodies of edible mushrooms, which confirm their high accumulation in mushroom fruiting bodies (17-19). But this report has demonstrated for the first time that mushrooms release zinc to artificial digestive juices imitating conditions in the human digestive tract and allowed an estimation of the

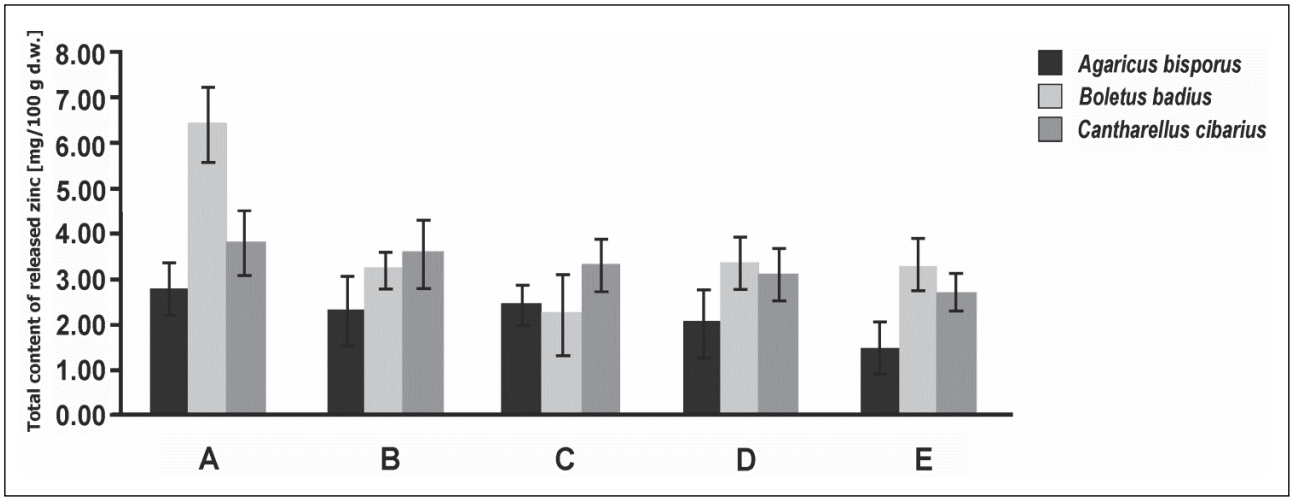

Fig. 2. Total concentration of zinc released [mg/100 g d.w.] from edible mushrooms species: *Agaricus bisporus, Boletus badius, Cantharellus cibarius* in artificial digestive juices where:
A - artificial saliva 1 minute of incubation, gastric juice 15 minutes of incubation and intestinal juice 150 minutes of incubation;
B - artificial saliva 1 minute of incubation, gastric juice 30 minutes of incubation, and intestinal juice 150 minutes of incubation;
C - artificial saliva 1 minute of incubation, gastric juice 60 minutes of incubation and intestinal juice 150 minutes of incubation;
D - artificial saliva 1 minute of incubation, gastric juice 90 minutes of incubation and intestinal juice 150 minutes of incubation;
E - artificial saliva 1 minute of incubation, gastric juice 120 minutes of incubation and intestinal juice 150 minutes of incubation.

Table 1. The amount of zinc released to artificial digestive juices from edible mushroom species (mg/100 g d.w).

Digestive juice	Incubation time (min)	The amount of zinc released (mg/100 g d.w)
Agaricus bisporus		
Artificial saliva	1	0.03 ± 0.06 0.06 ± 0.04 0.08 ± 0.02 0.06 ± 0.01 0.08 ± 0.17
Artificial gastric juice	15 30 60 90 120	1.05 ± 0.08 0.77 ± 0.01* 1.31 ± 0.07 1.10 ± 0.69 0.75 ± 0.05
Artificial intestinal juice	150	1.71 ± 0.09 1.51 ± 0.14 1.08 ± 0.06 0.88± 0.03* 0.69 ± 0.49
Cantharellus cibarius		
Artificial saliva	1	1.14 ± 0.09 1.01 ± 0.04* 1.09 ± 0.06 0.78 ± 0.14 0.89 ± 0.07
Artificial gastric juice	15 30 60 90 120	0.82 ± 0.02* 0.91 ± 0.00** 0.77 ± 0.02* 1.02 ± 0.04* 1.00 ± 0.02*
Artificial intestinal juice	150	1.79 ± 0.04* 1.68 ± 0.02* 1.44 ± 0.06* 1.30 ± 0.15 0.80 ± 0.21
Boletus badius		
Artificial saliva	1	0.88 ± 0.01* 0.71 ± 0.06 0.85 ± 0.02* 0.97 ± 0.07 0.84 ± 0.01*
Artificial gastric juice	15 30 60 90 120	1.09 ± 0.06 1.00 ± 0.09 0.92 ± 0.11 2.02 ± 0.03* 2.07 ± 0.02**
Artificial intestinal juice	150	6.44 ± 0.01** 1.49 ± 0.04* 0.41 ± 0.26 0.46 ± 0.02* 0.50 ± 0.04

Data are presented as the mean ±S.D. (standard deviation); n = 3 repetitions; * $P \leq 0.05$;** $P \leq 0.01$; by Statistica 10 (StatSoft, Poland).

usefulness of fruiting bodies as a source of alimentary zinc. The highest amount of zinc is released from fruiting bodies to artificial intestinal juice.

Zinc is a trace mineral and it is not produced in the human body, so it must be supplied with food. The daily requirement for this element in a healthy adult human is dependent on age and is about 15 mg (44). Meat rich in proteins is the main source of zinc and contains from 0.40 to 6.77 mg per 100 g. Cereal is the major plant food source of this element and contains from 0.30 to 2.54 mg/100 g, but vegetables have 0.12 to 0.60 mg/100 g, and fruits have 0.02 to 0.26 mg/100 g. The level of zinc absorption was found to be greater from food rich in proteins than in carbohydrates and this is the reason why vegetarians suffer from a lack of zinc (45). Comparing this result with the standards for daily zinc requirements in humans, the intake of 100 g of dried fruiting bodies of mushrooms (for example *B. badius*) provides about half of the daily zinc requirement. So, edible mushrooms rich in proteins which contain essential amino acids (typical for animal protein) and dietary fiber (chitin and chitosans) could be an alternative for a vegetarian diet (46).

Edible mushrooms may constitute a good source of this element, because they are a better source of zinc than plants

(according to WHO/FAO standards) (47). Because the mechanism for the take up of metals from the environment, wild growth mushrooms and those of commercial origin are not only a very popular food stuff, but also an effective additive for the diet.

Conflict of interests: None declared.

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