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In Vitro Culture of *Boletus badius* as a Source of Indole Compounds and Zinc Released in Artificial Digestive Juices

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Abstract The objective of this study was to obtain the *in vitro* cultures of *Boletus badius* under controlled conditions and investigate the release of indole compounds and zinc from the mycelium of *B. badius* to artificial digestive juices under conditions similar to those in the human gastrointestinal tract. Biomass was obtained from cultures grown using both only the Oddoux medium as well as the same medium with added zinc hydroaspartate and zinc sulfate. The release of 5-hydroxy-L-tryptophan, L-tryptophan, and serotonin from the *B. badius* biomass extracts to the artificial digestive juices was determined. Differential pulse anodic stripping voltammetry was used to demonstrate that zinc is released from each of the extracted materials. The total amount of zinc in the materials under study was estimated to be between 7.12 and 44.15 mg/100 g dry weight. It was demonstrated that *in vitro* cultures of *B. badius* grown using appropriately selected media may supplement zinc and indole compounds.

Keywords: artificial digestive juice, *Boletus badius,* indole compounds, mycelial culture, zinc supplementation

Introduction

In recent years, more information on the therapeutic and dietary substances that are present in edible mushrooms has been reported. Previous studies have shown that the composition of free and bound amino acids present in mushrooms is comparable to that of the amino acids present in animal proteins (1-4). Biologically and therapeutically active components of mushrooms are used in the treatment of various lifestyle diseases such as cardiovascular disease, diabetes, atherosclerosis, and cancer (5-8). *Boletus badius* (Bay bolete) is a species that can be observed during autumn in the coniferous and mixed forests of Europe and North America (Eastern Canada, Minnesota, South and North Carolina). *B. badius* is a popular edible species and is widely known for its flavor, which is similar to that of *B. edulis* (King bolete).

The intense brown color of the cap of this species is owing to the presence of the so-called badion dye, which contains cesium in a chromogenic system. *B. badius* mycelium was contaminated by the explosion of the reactor Chernobyl in 1986, which primarily emitted radioactive cesium. When comparing the studies on the accumulation of individual phenolic and cinnamic acids in mushrooms, it should be noted that this species contains protocatechuic, *p*-hydroxy benzoic, *p*-coumaric, and cinnamic acids in the highest amounts [21.38, 1.28, 13.91, and 8.73 mg/kg dry weight (D.W.), respectively] (9-11).

The above mentioned results show that the extracts of this species exhibit high total antioxidant activity. Elmastas *et al*. (12) reported that the percent inhibition of methanolic extracts from the dried fruiting bodies of *B. badius* at a concentration of 100 μg/mL in an oxidation test of linoleic acid was 99.2%. In the fruiting bodies of this species, indole compounds, which are the precursors of neuro-transmitters, were detected: L-tryptophan (0.68 mg/100 g D.W.), serotonin (0.52 mg/100 g D.W.), and tryptamine (0.47 mg/100 g D.W.) (13). The fruiting bodies of *B. badius* are particularly rich in free amino acids such as tryptophan, cystine, methionine, lysine, aspartic acid, and glutamic acid; furthermore, these bodies contain high amounts of fatty acids such as linoleic (approximately 70%), palmitic (20%), oleic, lauric, myristic, and arachidonic acids (5,13).

Mushrooms play an essential role in the existence of ecosystems by converting organic matter available to plants into water-soluble inorganic matter. Moreover, knowledge about their therapeutic and dietary properties has increased. Over the past 20 years, more studies have been conducted on the composition of primary and secondary metabolites and bioelements in wildly grown mushrooms. However, there are no studies on the accumulation and release of

these substances in humans. Therefore, the objective of this study was to obtain the *in vitro* cultures of *B. badius* in controlled conditions and investigate, for the first time, the release of indole compounds and zinc from the mycelium of *B. badius* to the *in vitro cultures* of artificial digestive juices under conditions of the human gastrointestinal tract.

In terms of zinc ions, one should consider the type of salt added (organic and inorganic) and concentrations of salts added to the culture medium. Indole compounds and zinc were selected for the analysis owing to their antioxidant potential and antidepressant and anti-inflammatory activities, which may be important in the prevention of lifestyle diseases (14,15). The objective of this study was to demonstrate that *B. badius* is a valuable source of zinc and indole compounds and is important for humans.

Materials and Methods

Mushrooms material In this study, the fruiting bodies of *B. badius* (Fr.) Kuhn. ex Gilb. (Bay bolete) were used *in vitro*. The fruiting bodies of this species were collected from the natural environment of the mixed forests of South Poland (close to Nowy Sącz and Alwernia) between 2012 and 2014. Taxonomic identification of the young sporocarps was made according to online keys (http://www.mycokey.com) and a study conducted by Knudsen and Vesterholt (16). Representative voucher specimens were deposited at the Department of Pharmaceutical Botany, Jagiellonian University Collegium Medicum, Kraków, Poland. Mushroom material were frozen and lyophilized (Freezone 4.5. Labconco; temperature: −40°C) to obtain the mushroom samples for further analyses.

A procedure established to set up *in vitro* cultures and achieve maximum biomass growth was as follows: mycelial cultures were derived from explants from the hymenium of *B. badius* fruiting bodies. The explants were degreased using 70% ethanol for 15 s followed by sterilization for 5 min using 15% sodium hypochlorite. After repeated washing using sterile redistilled water, fragments of the fruiting bodies were transferred to Oddoux medium solidified using agar under the conditions of a laminar air flow.

Experimental cultures Cultures from the solid medium were used to further set up experimental cultures, which were grown using a modified liquid medium according to the method reported by Oddoux in 1957 (17). The starting inoculum of the solid medium weighed 0.1 g. The resulting biomass from cultures grown on a solid medium was subjected to passage in Erlenmeyer flasks (500 mL) containing liquid medium (250 mL). The purpose of culturing using liquid medium was to maximize the biomass yield for further analyses. The biomass was obtained from the cultures grown on both the Oddoux medium alone (control) as well as the same medium with added zinc hydroaspartate at concentrations of 100 and 200 mg/L and zinc sulfate at concentrations of 87.23 and 174.47 mg/L. For all variants, five cultures of *B. badius* were set up. Biomass from *in vitro* cultures was separated from the liquid medium using a Büchner funnel and by rinsing with distilled water four times. The resulting biomass was immediately frozen and dried by lyophilization (Freezone 4.5, Labconco lyophilizer; temperature: −40°C).

Reagents Citric acid, NaOH, K_2HPO_4, Na_2HPO_4, $KHCO_3$, and $ZnSO_4 \cdot 7H_2O$ were obtained from the Polish Company of Chemistry (Gliwice, Poland). $NaHCO_3$ and NaCl were obtained from Przedsiębiorstwo Produkcyjno-Handlowe Galfarm (Kraków, Poland). $MgCl_2$ was obtained from Chempur (Kraków, Poland). $CaCl_2$ was obtained from Pharma Zentrale GmbH (Germany). Bile salts and pepsin were obtained from BTL (Łódź, Poland). Spleen extract, HCl, KCl, concentrated HNO_3, Suprapur®, and KNO_3 Suprapur® were obtained from Merck (Darmstad, Germany). Zn(II) standards were obtained from Okręgowy Urząd Miar-7 Łódź, Poland. Zinc dihydroaspartate was obtained from Farmapol (Poznań, Poland). Standards of indole compounds (L-tryptophan, 5-hydroxy-L-tryptophan, 5-methyl-tryptophan, serotonin, melatonin, tryptamine, 5-methyl-tryptamine, indoleacetic acid, indoleacetonitrile, indole, and indoleacetamide) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were of HPLC grade. Methanol, acetic acid, petroleum, ether, and dichloromethane were obtained from Merck and were of analytical grade. Quadruple-distilled water with a conductivity of less than 1 μS cm^{-1} was obtained using an S2-97A2 distillation apparatus (ChemLand, Stargard Szczecin, Poland).

Preparation of artificial digestive juice solutions

Artificial saliva: Liquid imitating the conditions of the oral cavity was prepared according to Arvidson's model, wherein artificial saliva with a pH of approximately 6.7 is prepared by mixing with quadruple-distilled water, 100 mL of 25 mM KH_2PO_4, 100 mL of 24 mM Na_2HPO_4, 100 mL of 150 mM $KHCO_3$, 100 mL of 100 mM NaCl, 100 mL of 1.5 mM $MgCl_2$, 6 mL of 25 mM citric acid, and 100 mL of 15 mM $CaCl_2$. In this model, no digestive enzymes (e.g., α-salivary amylase and salivary lipase) present in saliva were included (18).

Artificial gastric juice: The pH of the stomach ranges from 1.0 to 3.5, but in most artificial gastric juice models, the pH is 2.0. An artificial body fluid solution was prepared according to Polish Pharmacopeia X by dissolving 2.0 g NaCl and 3.2 g pepsin in quadruple-distilled water. Then, 80 mL of 1 M hydrochloric acid was added to adjust the pH followed by the addition of quadruple-distilled water to attain a volume of 1,000 mL (19).

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 the addition of quadruple-distilled water to 1,000 mL (20,21).

Sample preparation Freeze-dried biomass from *in vitro* culture of *B. badius* was ground in an agate 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 model (18), shaken for 1 min in Gastroel-2014 -an apparatus specially constructed for this type of experiments at the Jagiellonian University. In the device, gastrointestinal track activity was simulated by imitating natural digestive conditions: temperature, the order and the type of applied digestive juices, digestive juice movements in the system and the assumed average storage time of food in the digestive track parts. The suspension was then centrifuged, decanted, and the residues, i.e., mushroom fruiting bodies after digestion in artificial saliva solution, were placed in 10 mL of artificial gastric juice. Samples were shaken in 10 mL of artificial gastric juice for 15, 60, and 120 min in Gastroel-2014 -apparatus at 37°C. The solution was again centrifuged and decanted, and for the recovered fruiting bodies of the investigated species, 10 mL of artificial intestinal juice solution was added and shaken for an additional 150 min in Gastroel-2014 -apparatus. The decanted solution was centrifuged for 30 min in an MPW-223e centrifuge (120×*g*). Solutions prepared according this method were filtered through membrane filters (Millex; Millipore Corporation, Billerica, MA, USA) and were subject for indole compound and zinc analysis.

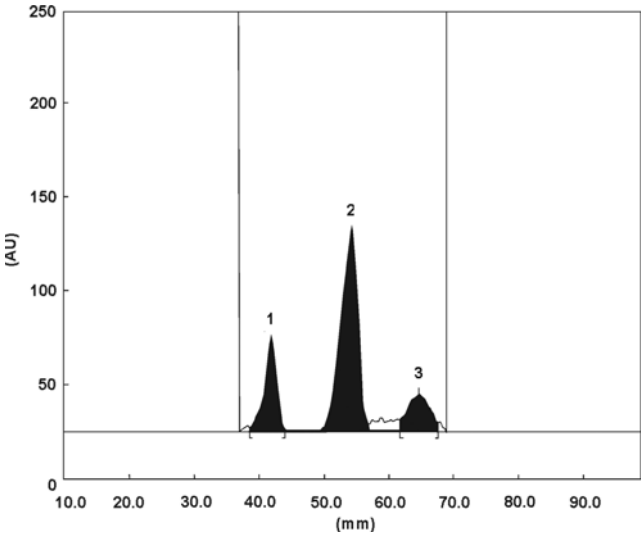

Fig. 1. Exemplary densitogram of the material grown using medium supplemented with 100 mg/L of zinc hydroaspartate after 150 min of incubation in the artificial intestinal juice, after developing mobile phase; 5-hydroxy-L-tryptophan (peak 1), L-tryptophan (peak 2), and serotonin (peak 3).

TLC analysis coupled with densitometric detection of indole compounds Thin layer chromatography (TLC) analyses with densitometry were performed using aluminum-packed silica gel 60 plates (Art. No. 1.055540001; Merck) and Camag apparatus (Muttenz, Switzerland), Linomat IV sample applicator, and Camag TLC Scanner 3 densitometer (Muttenz, Switzerland) with winCATS software.

Based on the preliminary qualitative analysis of the 10 analyzed indole compounds, the presence of three compounds was detected. Reference solutions in methanol were prepared at the following concentrations: L-tryptophan=0.048 mg/mL, 5-hydroxy-L-tryptophan =0.100 mg/mL, and serotonin=0.050 mg/mL.

In the first stage of the study, separation conditions for L-tryptophan, 5-hydroxy-L-tryptophan, and serotonin were optimized. A mobile phase comprising isopropanol, 25% NH_3 and water (8:1:1, v/v/v) was used to obtain a good resolution of the analytes: 5-hydroxy-L-tryptophan, R_F ~0.44; L-tryptophan, R_F ~0.57; and serotonin, R_F ~0.68 (Fig. 1). The spots on the chromatographs were analyzed using densitometry at a wavelength of 280 nm; this wavelength was selected based on the absorption spectra of the chromatogram (Fig. 2). On TLC plates (10×10 cm), 3 μL of reference solution and 20 μL of sample solution were applied in the form of bands with a length of 8 mm. Chromatograms were developed to a height of 9.5 cm in a chromatographic chamber saturated with the mobile phase and then dried at room temperature. Densitometry analysis was performed at a wavelength of 280 nm. Qualitative analysis was performed by standard procedure after the addition of an appropriate standard solution to the sample. Increase in the analytical signal was observed. Quantitative analysis was performed by comparing the peak area of the investigated sample with the peak area of the appropriate standard solution. Test results were calculated per 100 g D.W. and are summarized in Table 1.

Validation of TLC analysis coupled with densitometry detection of indole compounds Validation of the method was performed by the determination of accuracy, precision, linearity, limit of detection, and limit of quantification (22) (Table 1).

Accuracy: Accuracy was determined based on the sample analysis of known concentrations and comparing the results obtained by a validated method with true values, followed by calculation of the recovery percentage. Recovery percentage of the method was determined at the three concentration levels 80, 100, and 120% corresponding to the concentrations 0.375, 0.473, and 0.575 mg/mL for 5-hydroxy-L-tryptophan; 0.628, 0.792, and 0.926 mg/mL for L-tryptophan; and 0.232, 0.283, and 0.345 mg/mL for serotonin.

Precision: Precision of the method was determined at the three concentration levels 50, 100, and 150% corresponding to the concentrations 0.213, 0.431, and 0.645 mg/mL for 5-hydroxy-L-tryptophan; 0.386, 0.785, and 1.192 mg/mL for L-tryptophan; and 0.130, 0.271, and 0.384 mg/mL for serotonin.

Linearity: Linearity was determined by comparing the relationship between peak area (mm^2) and an amount of test substance applied to the plate (mg/spot). Two series of assays in the following concentration ranges were made for each substances: from 0.048 to 0.720 mg/spot for 5-hydroxy-L-tryptophan, from 0.100 to 2.000 mg/spot for L-tryptophan, and from 0.050 to 1.600 mg/spot for serotonin.

***Limit of detection (LOD) and limit of quantification (LOQ)*:** The LOD and LOQ were determined from the linearity in the following concentration ranges: from 0.048 to 0.432 mg/spot for 5-hydroxy-L-

Table 1. Validation of the developed methods with statistical evaluation for TLC analysis coupled with densitometric detection of indole compounds

Validation parameters	5-hydroxy-L-tryptophan	L-tryptophan	serotonin
R_F[1)]	~0.44	~0.57	~0.67
LOD (mg/spot)	0.016 a=8529.1 S_y=41.61	0.116 a=4309.4 S_y=151.8	0.017 a=6556.4 S_y=33.48
LOQ (mg/spot)	0.049	0.352	0.051
Recovery 80% (%)	$\bar{x}$=98.27 S_x=0.764 RSD=0.88%	$\bar{x}$=96.33 S_x=1.000 RSD=1.04%	$\bar{x}$=97.03 S_x=0.569 RSD=0.59%
Recovery 100% (%)	$\bar{x}$=98.23 S_x=0.961 RSD=0.98%	$\bar{x}$=98.47 S_x=1.220 RSD=1.24%	$\bar{x}$=98.73 S_x=0.551 RSD=0.56%
Recovery 120% (%)	$\bar{x}$=97.57 S_x=0.504 RSD=0.52%	$\bar{x}$=98.20 S_x=1.010 RSD=1.03%	$\bar{x}$=97.57 S_x=0.473 RSD=0.48%
Precision 50% concentration levels (mg/mL)	$\bar{x}$=0.241 S_x=0.0027 RSD=1.12%	$\bar{x}$=0.386 S_x=0.0045 RSD=1.17%	$\bar{x}$=0.130 S_x=0.0015 RSD=1.18%
Precision 100% concentration levels (mg/mL)	$\bar{x}$=0.451 S_x=0.0026 RSD=0.58%	$\bar{x}$=0.785 S_x=0.0056 RSD=0.71%	$\bar{x}$=0.271 S_x=0.0066 RSD=2.42%
Precision 150% concentration levels (mg/mL)	$\bar{x}$=0.615 S_x=0.0075 RSD=1.22%	$\bar{x}$=1.192 S_x=0.0105 RSD=0.88%	$\bar{x}$=0.384 S_x=0.0055 RSD=1.43%
Linearity	*p*=8086.7·m+241.7 *r*=0.9966	*p*=3422.0·m+148.9 *r*=0.9932	*p*=5352.2·m+483.6 *r*=0.9983

[1)] R_F, retardation factor; $\bar{x}$, mean value; S_x, standard deviation; RSD, relative standard deviation; a, the slope of regression line; S_y, standard error of the estimate; m, mass; *p*, confidence interval; *r*, correlation coefficient (mg/spot)

tryptophan, from 0.100 to 0.800 mg/spot for L-tryptophan, and from 0.050 to 0.400 mg/spot for serotonin. The following equations were used:

$$LOD=3.3\times S_y/a \quad (1)$$

$$LOQ=10\times S_y/a \quad (2)$$

where S_y is the estimation error and a is the slope.

The validation results presented in Table 1 indicate that the proposed method is characterized by high sensitivity. LOD for 5-hydroxy-L-tryptophan was 0.016 mg, L-tryptophan was 0.116 mg, and serotonin was 0.017 mg. The LOQ was estimated at 0.049, 0.352, and 0.051 mg for 5-hydroxy-L-tryptophan, L-tryptophan, and serotonin, respectively. Percentage recovery of the studied compounds was presented as mean values for the three concentration levels and was high (range, 96.33-98.73%). Satisfactory precision was determined for the three concentration levels and was confirmed by the values of variability coefficients RSD, which were in the range of 0.58-2.42%. Linearity for 5-hydroxy-L-tryptophan, L-tryptophan, and serotonin was preserved in a wide range, i.e., from 0.048 to 0.720 mg/spot, from 0.100 to 2.000 mg/spot, and from 0.050 to 1.600 mg/spot, respectively.

Zinc analysis The resulting filtrates from artificial saliva solution, gastric juice, and intestinal 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 h. To determine zinc(II) ions by the anodic stripping voltammetry method, mineralized samples were neutralized by adding an appropriate volume of 0.1 M NaOH solution. An example voltammogram obtained for a sample of *B. badius* species after incubation in saliva for 1 min and in gastric juice for 120 min is presented in Fig. 3.

The differential pulse anodic stripping voltammetry (DP ASV) method for the determination of zinc in artificial digestive juices was validated. For this purpose, parameters such as accuracy, precision, linearity, limit of detection, and limit of quantification were determined.

Validation of DP ASV method for the determination of zinc

***Accuracy*:** To evaluate the degree of accuracy of the results obtained when comparing the reference value to the value obtained for the sample containing zinc(II) ions of known concentration, increasing amounts of standard solution were added at a concentration of 1 μg/mL, which corresponded to 50, 100, and 150% of the amount

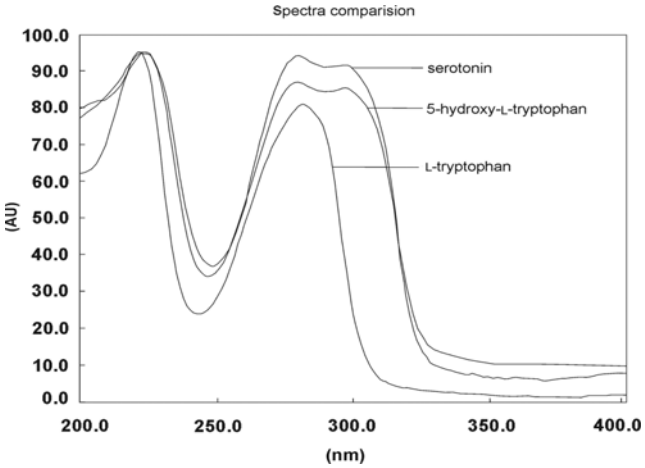

Fig. 2. Absorption spectra of L-tryptophan, serotonin, and 5-hydroxy-L-tryptophan registered directly from the chromatogram.

of zinc ions in the sample. Each time when the standard was added, the voltammetry curve was recorded. The average recovery value was 98.23%.

Precision: Precision of the method was determined by multiple (5 repetitions in triplicate) determinations of the concentration of zinc ions in the investigated samples using internal standard and adopting relative standard deviation as a criterion.

Linearity: The relationship between the current intensity and voltage applied to the electrodes was investigated by voltammetry. To the test sample, which contained basic electrolyte KNO_3 at a concentration of 0.1 M, 10 μL of Zn(II) (1 μg/mL) standard was added. Each time the standard was added, the voltammetry curve was recorded in triplicate. In the range of 1 to 100 μg/L, the curve describing the relationship between current intensity and Zn(II) concentration was established to be linear. The linear equation is presented as follows:

$$y=0.104x+0.016 \quad (3)$$

The established Pearson correlation coefficient was r=0.996.

***LOD and LOQ*:** LOD and LOQ were determined from the linearity curve obtained in the concentration range of 1-100 μg/L. For the calculations, equations (1) and (2) were used. The calculated LOD and LOQ were 0.36 and 1.2 μg/L, respectively.

Statistical analysis The results were expressed as mean values and standard deviations (SD). Statistical analyzes were conducted using the commercially available package GraphPad InStat Software using Tukey's post-hoc test. Values were considered significant at $p<0.05$.

Results and Discussion

The use of thin layer chromatography and densitometry within the UV range allows the simultaneous identification and quantification of 5-hydroxy-L-tryptophan, L-tryptophan, and serotonin in extracts obtained by leaching with artificial digestive juices under conditions of the human gastrointestinal tract.

A mobile phase that comprised isopropanol (25% NH_3) and water (8:1:1, v/v/v) was used to analyze the compounds present in the sample, which allowed for the separation and identification of the tested compounds (Fig. 1). Significant differences in the retention factor R_f were obtained, which facilitated the identification of the individual components. High-resolution peaks were obtained directly from chromatograms by densitometry at a wavelength of 280 nm, enabling further quantitative analysis (Fig. 1).

We have established that good mycelial mass growth for *B. badius* could be obtained in solid cultures and agitating liquid cultures on modified Oddoux (17) medium at 25±2°C under a 16-h photoperiod (900 lx/8 h dark). Twenty-fold fresh biomass growth in cultures in liquid medium was obtained within a 14-day growth cycle. The biomass growth in the initiated cultures averaged 9.0 g D.W. per liter of medium. The obtained biomass increments and dynamics of *B. badius* mycelium growth did not differ from the results that we obtained in our earlier studies for *Tricholoma equestre* (L.: Fr.) Kumm., *Xerocomus badius* (Pers.; Fr.) Fr., *Sarcodon imbricatus* L., and *Cantharellus cibarius* Fr. cultures (23-25).

The amount of zinc and indole compounds released from the lyophilized biomass of *B. badius in vitro* cultures grown using both the Oddoux medium as well as the same medium with added zinc hydroxyaspartate and zinc sulfate to the artificial digestive juices under temperature of the human body (37°C) was determined. Activity of the human gastrointestinal tract was simulated by natural digestive conditions, taking into account criteria such as temperature, the order of digestive juices applied, movements in the Gastroel-2014 – apparatus release system (designed for the purpose of this experiment), and the residence time of the contents of the artificial digestive juices.

Based on the results obtained from previous studies, it has been shown that in the fruiting bodies of *B. badius*, which were not thermally treated, L-tryptophan and serotonin were determined at amounts of 0.68 mg/100 g D.W. and 0.52 mg/100 g D.W., respectively, while after thermal treatment, the amount of L-tryptophan increased to 1.74 mg/100 g D.W. and serotonin was not determined. However, the presence of 5-methyl-tryptophan and melatonin was observed (26). In further experiments, Muszyńska *et al*. (27) studied the release of indole compounds from the fruiting bodies of *B. badius in vitro* cultures into artificial gastric juice and determined that 5-hydroxy-L-tryptophan, L-tryptophan, serotonin, and 5-methyl-tryptamine were released.

In the fruiting bodies, we did not determine the amount of 5-hydroxy-L-tryptophan. The amount of L-tryptophan ranged from 3.1 to 8.3 mg/100 g D.W. The content of 5-hydroxy-L-tryptophan in *in vitro* cultures ranged from 28 to 75 mg/100 g D.W. The amount of L-tryptophan was determined to be between 0.6 and 15.8 mg/100 g D.W., whereas serotonin was determined in only one sample at an amount of 0.4 mg/100 g D.W. The total amount of indole compounds

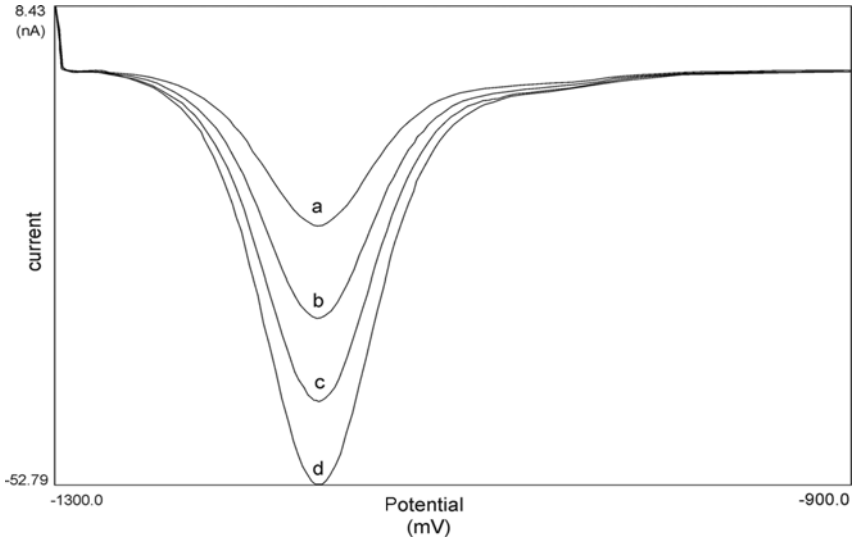

Fig. 3. Voltammogram obtained from a sample of *B. badius* species after incubation in saliva for 1 min and in gastric juice for 120 min. The study of zinc release into gastric juice for sample (a) and standard addition (b, c, and d).

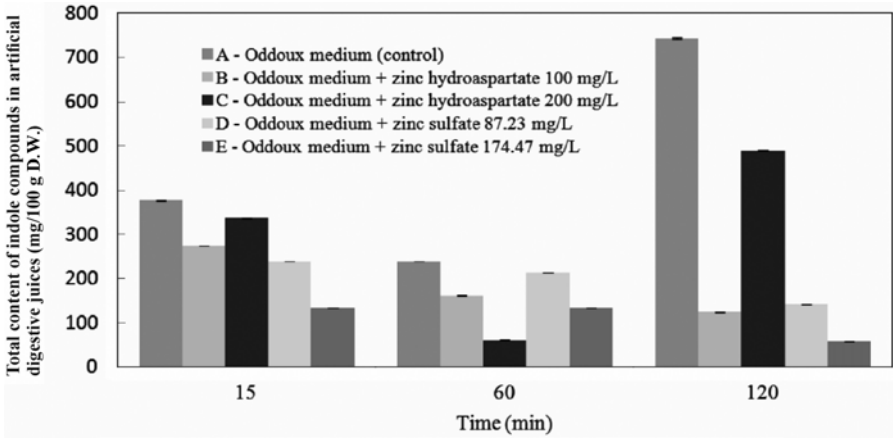

Fig. 4. Histogram representing the total amount of indole compounds (5-hydroxy-L-tryptophan, L-tryptophan, and serotonin) released from the biomass of *B. badius* to *in vitro* cultures into artificial digestive juices (saliva, gastric, and intestinal juice) depending on the incubation time.

in fruiting bodies was 123 mg/100 g D.W. and in *in vitro* cultures was 62 mg/100 g D.W., whereas that of the compounds in cultures enriched with zinc salts was estimated at up to 212 mg/100 g D.W. in the biomass of the mycelial cultures grown on the Oddoux medium only (28).

In this study, among 5-hydroxy-L-tryptophan, L-tryptophan, and serotonin, serotonin was determined in the largest number of samples (6.79 mg/100 g D.W. in the material etched in artificial gastric juice and up to 378.85 mg/100 g D.W. in the material etched in artificial saliva). In a large number of samples, 5-hydroxy-L-tryptophan was also determined to be present and the amounts released from the material investigated was determined between 10.41 mg/100 g D.W. in *in vitro* cultures incubated in artificial intestinal juice up to 57.86 mg/100 g D.W. in the material incubated in artificial saliva solution (Table 2).

L-tryptophan was determined in a lesser number of samples and its presence was not confirmed in any sample extracted in artificial saliva. Higher quantities of L-tryptophan were observed in samples extracted in artificial intestinal juice solution compared with those extracted in simulated gastric juice, whereas higher amounts of serotonin and 5-hydroxy-L-tryptophan were released in artificial gastric juice. For indole compounds that were determined, no relationship between the incubation time of the material in the artificial gastric juice and quantities of these compounds extracted into artificial intestinal juice was observed. Similarly, as observed in previous studies conducted Muszyńska in 2015, the highest total amounts of indole compounds were released from *B. badius in vitro* cultures grown in the Oddoux medium only (Fig. 4).

The most preferred incubation time of material in the artificial gastric juice for further release of indole compounds was 15 min for three of out of five types of materials (materials grown using medium enriched with zinc hydroaspartate at a concentration of 100

mg/L and zinc sulfate at concentrations of 87.23 and 174.47 mg/L), whereas for the remaining two, the time was set at 120 min (biomass from the Oddoux medium only and the same medium with added 200 mg/L of zinc hydroaspartate).

Analyzing the results of zinc release, it was demonstrated that zinc is released from each of the etched materials in all variants of time (Table 3). Previous studies showed the presence of 44.27 mg/100 g D.W. of zinc in the fruiting bodies and 17.21 mg/100 g D.W. in *in vitro* cultures, which simultaneously are not different from the results obtained in this experiment (13,29). In this study, the total amount of zinc determined ranged from 7.12 mg/100 g D.W. in the material obtained from the Oddoux medium only to 44.15 mg/100 g D.W. in the material from the same medium with added 174.47 mg/L zinc sulfate(VI).

The amounts of zinc released into artificial saliva were substantially lower than those determined in the extract of artificial gastric or intestinal juice (from 0.27-0.52 mg/100 g D.W. for artificial saliva to 1.95-29.42 mg/100 g D.W. for artificial gastric and intestinal juice). An apparent relationship between the etching time of the material in the artificial gastric juice and the amount of zinc released can be observed. For each time period, the highest amount of zinc was released into artificial gastric juice after 60 min of previous incubation; however, no clear relationship between the release of zinc after 15 and 120 min of material incubation in the artificial gastric juice was observed. In the artificial intestinal juice with addition of lower amounts of zinc salts (zinc hydroaspartate and zinc sulfate at

Table 2. The content of indole compounds in lyophilized biomass of *B. badius in vitro* cultures extracted into artificial digestive juices

Artificial juice	Artificial saliva (mg/100 g D.W.)	Artificial gastric juice (mg/100 g D.W.)			Artificial intestine juice (mg/100 g D.W.)		
Time (min)	1	15	60	120	150	150	150
Materials		(after 1 min in artificial saliva)			(after incubation in gastric juice)		
5-hydroxy- L-tryptophan							
Oddoux medium+zinc hydroaspartate 100 mg/L	52.05±0.50[1)]	54.95±1.01			15.90±0.19[a]	22.04±0.59[ab]	16.31±0.09[b]
Oddoux medium+zinc hydroaspartate 200 mg/L	57.86±0.75				14.30±0.38[a]	23.28±0.71[a]	
Oddoux medium+zinc sulfate(VI) 87.23 mg/L	33.43±0.53	56.50±0.80				22.13±0.49[b]	16.78±0.22[b]
Oddoux medium+zinc sulfate(VI) 174.47 mg/L	39.53±0.48					26.66±0.29[b]	18.86±0.26[b]
Oddoux medium (control)	47.04±0.61		23.98±0.34[b]	12.49±0.09[b]		21.26±0.21[b]	10.41±0.30[b]
L-tryptophan							
Oddoux medium+zinc hydroaspartate 100 mg/L			87.97±0.36		41.23±0.60		
Oddoux medium+zinc hydroaspartate 200 mg/L					41.23±0.64[a]		78.86±0.51[a]
Oddoux medium+zinc sulfate(VI) 87.23 mg/L			7.85±0.08[b]	3.34 ± 0.08[b]	37.90±0.86		
Oddoux medium+zinc sulfate(VI) 174.47 mg/L			8.64±0.17[b]	3.43±0.08[b]	36.32±0.54		
Oddoux medium (control)					35.05±0.59	.	.
Serotonin							
Oddoux medium+zinc hydroaspartate 100 mg/L	207.70±3.08	87.42±0.13[a]	14.93±0.24[ab]	31.05±0.70[ab]	15.01±0.10[a]		17.73±0.13[a]
Oddoux medium+zinc hydroaspartate 200 mg/L	219.06±2.63	58.00±0.88[a]	37.60±0.27[ab]	40.55±1.11[ab]	21.20±0.53		19.35±0.61
Oddoux medium+zinc sulfate(VI) 87.23 mg/L	119.95±0.62	78.73±0.58			23.40±0.12[a]		6.79±0.04[a]
Oddoux medium+zinc sulfate(VI) 174.47 mg/L	75.96±0.34	15.46±0.14			20.93±0.51		
Oddoux medium (control)	378.85±5.78		15.37±0.25[b]	139.25±1.68[b]	57.19±0.25[a]		42.02±0.36[a]

[1)]Data are presented as the mean±SD; Tukey-Kramer post-hoc test was used to reveal the differences between paired groups of elements in rows, the same letters are marked for which the content differences are statistically significant (for *p* values below 0.05) (GraphPad InStat).

Table 3. Zinc content in lyophilized biomass of *B. badius in vitro* cultures extracted into artificial digestive juices

Artificial juice	Artificial saliva (mg/100 g D.W.)±SD	Artificial gastric juice (mg/100 g D.W.)±SD			Artificial intestine juice (mg/100 g D.W.)±SD		
Time (min)	1	15	60	120	150	150	150
Materials		(after 1 min in artificial saliva)			(after incubation in gastric juice)		
Oddoux medium+zinc hydroaspartate 100 mg/L	0.47±0.03[1)]	10.87±0.53[a]	12.94±0.66[ab]	9.49±0.52[ab]	1.95±0.18[a]	4.30±0.15[ab]	23.11±2.10[ab]
Oddoux medium+zinc hydroaspartate 200 mg/L	0.35±0.03	12.32±0.77[a]	15.87±1.35[ab]	13.25±0.27[b]	20.04±1.91[a]	9.19±0.53[ab]	2.75±0.16[ab]
Oddoux medium+zinc sulfate (VI) 87.23 mg/L	0.38±0.03	7.74±0.55[a]	12.05±0.47[ab]	7.20±0.33[b]	5.01±0.28[a]	5.84±0.53[ab]	7.90±0.68[ab]
Oddoux medium+zinc sulfate (VI) 174.47 mg/L	0.52±0.05	26.45±1.26[a]	29.42±2.27[ab]	16.63±1.48[ab]	17.18±0.73[a]	7.09±0.43[ab]	2.10±0.13[ab]
Oddoux medium (control)	0.27±0.02	4.13±0.29[a]	9.36±0.38[ab]	3.78±0.28[b]	15.37±0.61[a]	4.15±0.10[ab]	3.07±0.21[ab]

[1)]Data are presented as the mean±SD; a Tukey-Kramer post-hoc test was used to reveal the differences between paired groups of elements in rows; the same letters are marked for which the content differences are statistically significant (for *p* values below 0.05) (GraphPad InStat).

concentrations of 100 and 87.23 mg/L, respectively), the lowest amount of zinc was released after 15 min of previous sample incubation in artificial gastric juice, whereas the highest was after 120 min.

In the biomass from the Oddoux medium only and the same medium with added zinc hydroaspartate and zinc sulfate at concentrations of 200 and 174.47 mg/L, respectively, a different situation was observed. The lowest amounts of zinc were released into artificial intestinal juice after 120 min of previous biomass incubation in the artificial gastric juice, whereas the highest was after 15 min. The results of the experiment confirm the ability to accumulate the elements from the environment; after 60 and 120 min of biomass incubation in the artificial gastric juice, the lowest total amount of zinc was released from the material grown on the control medium according to Oddoux (material after 15 min of incubation in the artificial gastric juice was an exception). The addition of zinc salt to the culture medium affected the accumulation of this element from the medium, leading to increased release into the majority of artificial digestive juices.

In conclusion, this study analyzed the amount of zinc and indole compounds in mushroom material obtained from controlled conditions of *in vitro* cultures for the first time. It was demonstrated that *B. badius in vitro* cultures are capable of accumulating and releasing zinc, and synthesis and release of indole compounds, into three types of artificial gastric juices simulating natural condition in the human digestive tract. This study clearly demonstrated that *B. badius in vitro* cultures grown on appropriately selected media may serve as a good source of zinc and indole derivatives for humans. Because the content of the analyzed indole compounds is comparable to the content present in meat and simultaneously taking into account that zinc is better absorbed from protein-rich foods, mushrooms may constitute a meat alternative for vegetarians.

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