ХИМИЯ РАСТИТЕЛЬНОГО СЫРЬЯ. 2020. №3. С. 67–72.
DOI: 10.14258/jcprm.2020037277

UDC 582.284 + 58.088 + 58.04 + 577.13 + 577.115 + 577.115

EXTRACTIVE SUBSTANCES OF SAPROTROPHIC MUSHROOMS *LENTINULA EDODES* AND *PHOLIOTA SQUARROSA.* HEAVY METAL CONTENT

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The article is devoted to comparing the chemical composition of extractive substances of the legs and caps of two types of saprotrophic fungi *Lentinula edodes* and *Pholiota squarrosa*. The content of metals: mercury, cadmium and iron in different parts of the mushrooms is compared in the article. The amount of substances recovered by various solvents is determined. The amount of ester-soluble substances is more found in caps than in stems. Water soluble substances are more extracted from P. squarrosa. Isopropyl alcohol extracts more substances from shiitake. Qualitative and quantitative analysis of neutral compounds, acids, and compounds of isopropanol extract was carried out by chromatography-mass spectrometry. Isopropyl alcohol mainly extracts disaccharides and sugar alcohols. It was revealed that the main sugar alcohols of *L. edodes* are mannitol and ribitol. Sugar alcohols content differs in different parts. Ribitol predominates in the stems. Shiitake caps contain mannitol and ribitol in equal amounts. Sugar alcohols are present in trace amounts in *P. squarrosa*, but trehalose (disaccharide) is the main component of the alcoholic extract. The compositions of fatty acids, sterols, carbohydrates and sugar alcohols in various parts of mushrooms were determined. Ergosterol predominates among sterols, while linoleic acid prevails among acids. Caps have a more diverse sterol composition. Both types of fungi contain polysaccharides consisting of glucose residues. It grows in *P. squarrosa*; mercury and cadmium accumulate in vivo. Wood-based shiitake grown in a greenhouse contains almost no mercury and cadmium, but contains iron. The amount of cadmium and mercury in the caps is greater than the stems. Metals are not extracted with isopropyl alcohol and are practically not extracted with hot water.

Keywords: Lentinula edodes, Pholiota squarrosa, extractive substances, sterols, fat acids, carbohydrates and sugar alcohols, heavy metal content

Introduction

Shiitake mushroom (Japanese forest mushroom, lentinula edodes, king mushroom) (*Lentinula edodes Berk.) Pegler* belongs to the Marasmiaceae family, *Lentinula* genus [1]. Shiitake grow in groups on decaying deciduous trees. These mushrooms grow naturally in the warm and humid climate of South-East Asia. This mushroom is actively cultivated and eaten in many Asian countries, as well as in Europe. Shiitake is a biological species with medical properties that is used directly in traditional Chinese medicine, including the treatment of oncology [2].

The content of carbohydrates in the dry matter of the fruiting part is 67.5–78% [3].

The content of simple sugars is 15.9%. There are fatty acids: linoleic, oleic, palmitic, with a relative content: 67%, 5.5%, 16%, respectively. Antioxidant properties of mushrooms are due to the phenolic compounds: tocopherols and catechin [2]. Mushrooms contain sterols, among which ergosterol predominates. Ergosterol is present in free form, in the form of complexes with polysaccharides and in the form of peroxides and esters with glycosides of higher fatty acids. Ergosterol is part of the cell membranes of fungi. Ergosta-7,22-dienol, ergosta-7,5-dienol and fungisterol are contained also in *L. edodes* [4].

It is considered that the healing and immunostimulatory properties are due to the polysaccharide-lentinan. Lentinan is widely used as a means of

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metabolism improving and as a food additive around the world [5]. Lentinan is soluble in water and aqueous solutions, insoluble in alcohol.

Caps of mushrooms are used in restaurants in Russia. The stems of the mushroom is not in demand because of its stiffness and can be a raw material for the extraction.

The *P. squarrosa* mushroom (*Pholiota squarrosa* (Oeder) P. Kumm) belongs to the Strophariaceae family, genus Pholiota [6]. Similar to shiitake, Pholiota squarrosa grows in groups on dead, mostly deciduous trees (Fagus, Salix, Fraxinus, Malus, Betula, Populus, etc.), as well as on living trees. *P. squarrosa* are widespread throughout the forest zone of the Russia, Europe, North America, China, including Japan. Vitamins, amino acids, lipids and polysaccharides are found in these mushrooms [7]. The anti-inflammatory, immunostimulating, and antitumor properties of are also associated with polysaccharides. Mannoglucan is the main polysaccharide of *P. squarrosa*. This heteropolysaccharide consists of mannose, glucose and galactose in a ratio of 50 : 33 : 18, respectively [7].

L. edodes prefers a subtropical climate, so mushroom cultivation in Russia causes certain difficulties. In this regard, the aim of our work was to obtain preliminary data on the extractive substances of the saprotrophic fungus *P. squarrosa* and to compare its composition with *L. edodes* , as well as to clarify the differences in the composition of the parts of the fruiting bodies of these fungi, since the lower and upper parts are often used in different ways. Since it is known that fungi often accumulate heavy metals, the contents of mercury, cadmium and iron in different parts of the fungi were determined.

Experimental

L. edodes mushrooms were grown on sawdust of birch in the All-Russian Research Institute of Agricultural Microbiology. *P. squarrosa* were collected on October 15, 2019 in the forest of Kirishi district, Leningrad region, 17 km south of Kirishi, 700 m from the highway Zuyevo-Kirishi.

The samples were washed from the substrate residues, and then dried for 14 hours at a temperature 40 °C with the use of an electric dryer to the humidity of 10–15%. The initial humidity was 80–90%. The particle size of mushrooms was 0.3–3 mm after grinding the dried mushrooms.

The mushrooms were first extracted with isopropanol (IPS), then with hot water or 1% sodium hydroxide. The isopropanol extract was evaporated and the residue was extracted with methyl tert-butyl ether. The methyl tert-butyl (MTBE) extract was separated into acids and neutrals by washing with an aqueous solution of 1% NaOH. The substances were analyzed by gas chromatography-mass spectrometry (GC-MS). Hot water extraction was carried out at a temperature of 90 °C for 3 hours and the ratio of mushrooms and water 1 to 30.

Qualitative and quantitative analyzes of neutral compounds, acids and compounds of isopropanol extract were carried out by GC-MS. The device is an 6850A Agilent GC/MS Instrument (Agilent Technologies, Inc.) with a model G2629A gas chromatograph equipped with a model G2577A HP5973 Network selective mass spectrometry detector. The ionizing energy was 70 eV. The temperatures of a separator and an ion source were 280 and 230 °C, respectively. To fractionate samples, Rxi®-5 Sil MS column (30000 × 0.18 mm ID) with a 0.10 μm (low polarity crossbond® silarylene phase; similar to 5% phenyl/95% dimethyl polysiloxane) was used. The thermostat temperature was programmed to increase from 100 to 250 °C at a rate of 5 °C per min. The evaporator temperature was 270 °C. The flow rate of the carrier gas (helium) was 1 cm^3 per min. The dosed volume was 0.1 μL. The gas chromatographic retention indices of the analyzed substances were determined using the retention indices of *n*-alkanes as standard compounds (Aldrich). The standard compounds were chosen so that the retention times of the studied substances fell between those of the reference alkanes. Retention indices were calculated following the determination of the coefficients of the following equation: $I = a\tau^2 + b\tau + c$, where I and τ represent the retention index and retention time, respectively. All calculations were performed using an Advanced Grapher program (version 2.08).

Determination of the double bond position in fatty acids with one double bond was carried out after preliminary dimethyl desulfurization [8]. Sugar alcohol analysis, carbohydrates and other hydroxyl-containing compounds was performed after preliminary silylation with a mixture of hexamethyldisilazane, trimethylchlorosilane and pyridine [9]. The belonging of the peak to a certain compound was determined by the addition of the corresponding compound. The analysis of acids was carried out after preliminary methylation by diazomethane [10].

The aqueous extract was evaporated at 40 degrees, dried in a vacuum drying box at the same temperature, and 0.04 g of material was taken from the residue to determine the presence of polysaccharides in the aqueous extract. The weighed portion was placed in a 100 ml flask; 50 ml of 2% HCl was poured into the flask. The solution was

boiled for 30 min. The hydrolyzate was evaporated at 40 °C in a vacuum drying box. The residue was silylated as described above for GC-MS analysis. The metal content was determined in the initial mushrooms, as well as in the mushrooms after extraction with isopropanol and after extraction with isopropanol and water.

The mercury content was determined on the mercury analyzer RA-915M (Pyro). The content of cadmium and iron was determined using an atomic absorption spectrometer with electrothermal atomization and Zeeman correction of nonselective absorption "MGA-915MD" with preliminary sample preparation using the mineralizer "Minotavr 2" (Lumex 2009). The total amount of the analyzed material was 5 samples from 2 species of mushrooms. Analyzes were done in triplicate.

Results and discussion

Extractive substances. Water and alkali-soluble substances are more in the caps of the mushrooms, both in *P. squarrosa* and *L. edodes* (Tab. 1). Substances soluble in MTBE are also more in caps. *P. squarrosa* contains more water-soluble compounds.

The ratio of neutral substances and acids of MTBE extract is different. *L. edodes* caps have more neutral substances than stems, more acid in both parts. *P. squarrosa* has more neutral substances than acids, and the stems are slightly larger than the caps. The qualitative composition of the neutral substances of *L. edodes* and *P. squarrosa* caps (Tab. 2) is more diverse than the stems. Ergosterol – is the main sterol that is found in the studied mushrooms.

The compositions of fatty acids of fungi and their parts were almost the same (Tab. 3). Linoleic acid turned out to be more in the caps of both types of mushrooms. There is more palmitic acid in *L. edodes* caps than in stems, and vice versa in *P. squarrosa*.

The main differences in the qualitative composition of the compounds were observed in the isopropanol extract, after the removal of ether-soluble substances. The main components of *L. edodes* alcohol extracts are ribitol and mannitol. Ribitol in the stems of the mushroom contains more than 80%, caps contain more than 40% (18% of the analyzed stems, 8.6% of the analyzed caps) (tab. 4). The main components of alcoholic extracts of *P. squarrosa* are mannitol and disaccharide – trehalose, both in the stems and in caps contains more than 40%.

Mushroom water extracts were hydrolyzed and glucose was identified as part of the hydrolysates. Thus, polysaccharide consisting of glucose units is extracted from with water from *L. edodes* and *P. squarrosa.*

Heavy metals (Hg, Cd, Fe). The analysis of the content of heavy metals was carried out in the mushrooms before and after extraction with isopropyl alcohol and water in order to assess whether heavy metals are isolated to a solvent. Cd and Hg are usually the most toxic heavy metals for basidiomycetes [26, 27]. Edible mushrooms, especially wild ones, can contain metals, such as Cd and Hg, at levels significantly higher than in other food products [28]. Mushrooms collected in uncontaminated areas accumulate Hg in much higher concentrations than plants [29]. Iron analysis was done to compare differences in solubility of compounds containing metals upon extraction.

The content of mercury, cadmium, and iron in *P. squarrosa* (Tab. 5) is lower than the content of the corresponding elements determined in Finland (0.62 mg/kg – Hg, 4.4 Cd, 130 Fe) [30]. The mercury content in *L. edodes* coincides with the analysis carried out in Spain [31], cadmium is much less than 0.392 [30], 1.98 [32]. The iron content is less than 37 mg/kg [32].

Isopropyl alcohol does not extract compounds containing Cd, Hg and Fe (Tab. 5). Caps contain more heavy metals, which is probably due to the fact that the stems are actually a continuation of the mycelium and conduct water with dissolved substances, and the caps accumulate them. But iron is more in the stems of *L. edodes*. A significant part of shiitake mercury goes into water. These data are somewhat contrary to the report that mercury accumulated in mashrooms bodies is mainly stored in the mushrooms body during cooking and pickling [33]. Perhaps the results are affected by a small mercury content in cultivated mushrooms.

Table 1. Group composition of extractives in various parts of mushrooms

Extractives extracted with	*L. edodes*		*P. squarrosa*	
	Stem	Cap	Stem	Cap
	Extract yield, % of dry matter			
MTBE composed of	1.3	3.2	0.5	1.4
neutral sybstances	25	31	33	38
and acids	75	68	67	62
IPS	9.8	9.6	5.9	2.8
Hot water	30.6	36.1	39.0	38.4
1% NaOH	31.0	48.0	46.4	45.7

Table 2. GC-MS neutral substances of *L. edodes* and *P. squarrosa*

Compound Molecular mass	Retention parameters min/I	Quantitative content,%			
		L. edodes		*P. squarrosa*	
		Cap	Stem	Cap	Stem
Linoleic acid monoglyceride M=354	30.0/2593	–	–	4.3	–
Oleic acid monoglyceride M=356	30.1/2595	–	–	6.6	–
Unidentified comp.	31.8-35.5	–	–	1.0	–
(22E)-Ergosta-5,7,9,(11),22-tetraen-3β-ol $C_{28}H_{42}O$ M=394	36.05/3116 (3150 for DB-5 [11])	9.6	2.2	11.9	–
Ergosterol $C_{28}H_{44}O$ M=396	36.6/3183 (3152 for DB-5 [11])	74.6	79.9	37.7	80.1
Ergosta-7,22-dien-3β-ol $C_{28}H_{46}O$ M=398	36.8/3212 (3202 for DB-5 MS [11])	5.0	1.5	7.2	6.5
Neoergosterol, M=380	36.9/3214	0.3	–	3.5	3.0
Unidentified. M=394	37.0/3215	–	2.0	–	3.0
Unidentified.M=398	37.5/3217	–	–	–	5.6
Ergost-7-en-3β-ol M=400	37.7/3246 (3220 for Methyl Silicone [12])	–	4.2	–	1.6
β or γ-sitosterol M=414	38.1/3280	–	–	0.1	–
Unidentified. M=426	38.5/3314	–	–	0.3	1.5
Unidentified	38.7/3316	–	–	0.1	–
Anthreergostatetraenol M=394	38.8/3317	0.5	0.6	1.0	–
Unidentified.M=394	39.9/3319	–	2.0	2.0	–
Unidentified.	40.0/3320	–	0.6	0.1	–
Unidentified.M=410	40.4/3361	–	–	0.1	–
Unidentified.M=428	40.9/3374	–	–	14.2	–
Stigmast-5,22-dien-3-ol M=454	41.6/3414 (3244 for DB-5[11])	4.7	6.2	8.0	–
Unidentified	42.1/3416	–	–	0.2	–
Unidentified	42.6/3420	–	0.4	1.4	–
Unidentified	44.5/3572	–	0.4	0.2	–
Unidentified	46.2/3708	–	–	0.05	–
Unidentified	47.7/3828	–	–	0.1	–

Table 3. GC-MS Fatty acids *L. edodes* and *P. squarrosa*

Compound	Retention parameters min/I*	Quantitative content,%			
		L. edodes		*P. squarrosa*	
		Cap	Stem	Cap	Stem
5 Unidentified comp.	1.3 - 2.3/ less than 1100	0.3	0.3	–	–
Benzoic acid	2.9/1125 (1170 for DB-5 [13])	–	–	–	2.3
Butanedioic acid (succinic acid)	5.3/1275	–	–	–	4.5
p-hydroxybenzoic acid (4-hydroxybenzoic acid)	8.6/1417 (1558 for HP-5 MS[14])	–	–	0.8	10.7
1,4-benzenedicarboxylic acid (terephthalic acid)	9.5/1452	–	–	–	3.4
Unidentified	10.9/1545	–	–	0.5	12.9
Tetradecanoic acid (myristic acid)	13.4/1767 (1780 for HP-5 [15])	–	–	0.2	–
Pentadecanoic acid	15.4/1820 (1833 for DB-5 [16])	1.3	2.2	0.2	–
9Z-Hexadecenoic acid	16.9/1883 (1898 for DB-5 [16])	–	–	0.9	–
11Z-Hexadecenoic acid	18.1/1915 (1938 for DB-5 [17])	0.3	0.2	–	–
Hexadecanoic acid (palmitic acid)	18.4/1928 (1942 for DB-1 [17])	16.6	12.2	13.7	15.6
6,9,12-Octatriene acid (γ-linolenic acid)	21.1/2052	0.6	1.4	–	–
Octadeca-9Z,12Z-dienoic acid (linoleic acid)	21.5/2086 (2095 for HP-5MS [18])	70.4	74.0	62.8	39.1
9Z-Octadecenoic acid (oleic acid)	21.6/2094 (2095 for DB-5 [19])	2.2	2.2	11.0	–
11Z-Octadecenoic acid	21.7/2101 (2110 for DB-5 [20])	1.2	0.4	1.8	–
Octadecanoic acid (stearic acid)	22.1/2122 (2137 for HP-1 [21])	1.2	1.4	2.9	2.9
Unidentified	23.1/2166	0.2	0.2	–	1.9
Unidentified	24.3/2240	0.6	0.6	1.5	–
Eicosanoic acid (arachidic acid)	25.6/2330 (2359 for HP-5MS [22])	–	0.3	0.2	–
Docosanoic acid (behenic acid)	28.7/2543 (2567 for HP-5MS [22])	0.6	0.2	0.2	–
Tetracosanoic acid (lignoceric acid)	31.7/2745 (2760 for HP-5 [22])	0.6	0.6	1.1	–
2-Hydroxy tetracosanoic acid	33.5/2856	1.1	0.7	0.4	–
6 Unidentified comp.	34.0-36.2/ less than 3000	2.7	0.5	–	–

*The retention parameters of methyl esters are given.

Table 4. Monosugar, disaccharides and sugar alcohols

Compound	Retention parameters min/I*	Quantitative content,%			
		L. edodes		*P. squarrosa*	
		Cap	Stem	Cap	Stem
Phosphoric acid	5.1/1262 (1280 for HP-5 [23])	–	–	–	0.3
Hydroxybutanedioic acid	9.4/1447	–	–	–	0.2
Threitol	9.5/1452 (1529 for HP-5[23])	–	–	0.1	0.06
Erythritol	9.7/1462 (1535 for HP-5[24])	–	–	0.08	0.1
Xylitol	13.1/1712 (1748 H for P-5[25])	0.4	0.4	0.6	0.3
Arabitol	13.4/1729 (1760 for HP-5[24])	0.4	0.2	0.5	0.1
Ribitol	13.5/1735 (1766 for HP-5[24])	48.4	83.0	0.2	0.03
Glucosa	16.8, 18.6/1894, 1989 (1926 for DB-5[6], 2031 for HP-5[24])	–	–	3.4	7.9
Mannitol	17.6/1944 (1975 for HP-5[24])	48.3	16.4	45.6	40.3
Sorbitol	17.7/1946 (1980 for HP-5[24])	–	–	0.9	3.4
Tregalosa	30.9/2776 (2816 for DB-5MS[25])	–	–	48.3	47.0

*The retention parameters of silyl esters are given.

Table 5. Metals content (mg/kg dry weight) in *P. squarrosa* and *L. edodes*

Sample	Hg	Cd	Fe
L. edodes stems	0.048 ± 0.003	0.015 ± 0,001	10.705 ± 0.201
caps	0.047 ± 0.005	0.014 ± 0.001	5.824 ± 0.220
L. edodes stems after extraction with IPS	0.054 ± 0.003	0.017 ± 0,001	11.951 ± 0.119
stems after extraction with water	0.043 ± 0.009	0.002 ± 0.001	16.770 ± 0.206
caps after extraction with IPS	0.053 ± 0.006	0.023 ± 0.003	6.436 ± 0.115
caps after extraction with water	0.043 ± 0.001	0.007 ± 0.004	11.497 ± 0.379
P. squarrosa stems	0.210 ± 0.010	0.080 ± 0.005	9.500 ± 0.345
caps	0.510 ± 0.015	0.280 ± 0.020	11.001 ± 0.612
P. squarrosa stems after extraction with IPS	0.236 ± 0.009	0.100 ±0. 0.006	10.557 ± 0.657
stems after extraction with water	0.390 ± 0.022	0.050 ± 0.008	7.990 ± 1.625
caps after extraction with IPS	0.544 ± 0.017	0.295 ± 0.005	11.375 ± 0.585
caps after extraction with water	0.734 ± 0.290	0.401± 0.194	16.071 ± 3.044

It can be assumed that mushrooms grown under artificial conditions contain less heavy metals than mushrooms grown under natural conditions, as reported by other authors. The content of heavy metals in cultivated mushrooms is usually lower than in forest mushrooms, which is most likely due to soil composition and pollution, as well as the age of mycelium (part of the fungus growing below the surface of the earth), which can be several years in forest mushrooms. compared with several months in cultivation [28]. Garbage can become the main way to load mercury in humus in places with a high mercury content in the air due to long-distance transport [34].

Conclusions

The main differences in the studied mushrooms are observed in the composition of sugar alcohols, carbohydrates and the content of heavy metals. The *P. squarrosa* contains tregalosa (disaccharide), while *L. edodes* contains mannitol and ribitol. Ribitol is the main sugar alcohol of the *L. edodes* stems. As it turned out, the caps of both mushrooms are more saturated with various neutral substances than the stems. Ergosterol predominates among sterols in all parts of the mushrooms. Oxidized forms of sterols are contained in the *P. squarrosa* caps. As for acids, no differences were found, except for the content of aromatic acids in the *P. squarrosa*. The main fatty acid in both types of mushroom is linoleic acid. Its content is approximately the same in all parts, with the exception of the stems of the *P. squarrosa*, which contain less. The composition of the water-soluble substances of the studied mushrooms contains water-soluble polysaccharides consisting of glucose residues. The content of Hg, Cd is greater in mushroom caps. Hg, Cd, Fe are not released by isopropyl alcohol. Cd and Hg are removed from the shiitake by hot water, but not from the flake. The metal content in the *L. edodes* grown under artificial conditions is negligible. In the caps of mushrooms there are more heavy metals than in the stems.

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Received January 9, 2020

Accepted March 10, 2020

For citing: Bakanov V.V., Vedernikov D.N., Khabarova L.S. *Khimiya Rastitel'nogo Syr'ya*, 2020, no. 3, pp. 67–72. (in Russ.). DOI: 10.14258/jcprm.2020037277.