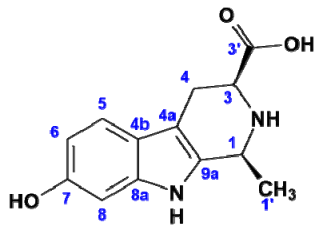
SZENT ISTVÁN UNIVERSITY

THESIS OF DOCTORAL (PH.D) DISSERTATION

ANTIOXIDANT BIOACTIVE COMPOUNDS OF BASIDIOMYCETOUS MUSHROOMS



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1. BACKGROUND AND OBJECTIVES

The first written remarks about mushrooms, dating back about 2000 years ago, show that they served not only nutritional but also medical purposes. The research on fungi has also begun, with the cultivation of mushrooms. The main direction of the research - to show the nutritional value of mushrooms and to increase the potential of cultivation - was given by economic interests (CHEUNG, 2010).

Nowadays, mycological biochemistry has become a completely new science field, with around 100 species of mushrooms (FERREIRA et al. 2009). In general we could say, that the tests are focusing on effects of extracts from the fruiting body (for example, the cap and stipe). Mushroom research has showed a large number of bioactive substances, the effects of which are wide-ranging, for example antioxidant, antimicrobial, anti-inflammatory, immune-stimulant and other specific effects (WASSER, 2011).

Therapeutically useful compounds are generally small-molecule peptides or macromolecules (e.g.: polysaccharides). The phenolic and flavonoid derivatives which are found in mushrooms – essentially for preventive purposes – can be important in preserving health and preventing diseases. Such compounds have been discovered in both cultivated and wild mushroom species (ALVAREZ-PARRILLA et al. 2007; LI et al., 2012). Our knowledge about secondary metabolites of mushrooms is far from complete, even for a single species. The current trends focus exactly on these active ingredients of mushrooms.

Besides the scientific interest and diversity, the subject has another important and not negligible aspect as well. Some fungi can now be consumed in the form of powders, capsules and tablets. For example the species *Ganoderma lucidum, Hericium erinaceus* and *Lentinula edodes* are favorite and still consumed mushrooms. Quality of these products cannot be accomplished without standardized values for active ingredients.

In today's world, the number of special mushrooms and the number of newly discovered mushroom compounds increases day by day. Mycotherapy (the therapeutic use of mushroom substances) is gaining ground, which is due to the Internet, globalized commerce and the pursuit of a healthy life.

Further research of the subject may contribute to better understanding and more conscious use of mushrooms, often called "super foods", which are increasingly important in our today's diet.

The aims of our study:

1. to develop a method for sampling and preparation, generally suitable for mushrooms;
2. to introduce a more detailed fractionation (cap skin – cap meat – gills – stipe) of mushroom fruiting bodies instead of the classical one (cap and stipe) and to study of their weight relations;
3. to evaluate the biochemical potential of different mushroom species, cultivated mushroom strains and their fruiting body parts, based on three measured parameters;
4. to measure the effect of conservation methods on the examined parameters of different fungal species,
5. to perform various chromatographic tests: profile analysis, determination of chromatographic parameters of antioxidant components and development of an isolation protocol for mushrooms' antioxidant substances.

2. MATERIALS AND METHODS

2.1. Mushroom species

Several mushrooms and cultivated mushroom strains were obtained for the tests. The mushroom species are: **Button mushrooms** (*Agaricus bisporus* – 8 white and 6 brown strains), **Poplar mushroom** (*Agrocybe cylindracea*), **Enokitake** (*Flammulina velutipes*), **Lion's mane** (*Hericium erinaceus*), **Slate bolete** (*Leccinum duriusculum*), **Shiitake** (*Lentinula edodes* – 3 strains), **King oyster mushroom** (*Pleurotus eryngii*), **Oyster mushroom** (*Pleurotus ostreatus* – 17 strains). I introduced a more detailed fractionation of mushroom fruiting bodies instead of the classical fractions (cap and stipe) (FERREIRA et al., 2007). After fractionation, 4 morphologically distinct parts are obtained, such as cap skin, cap meat, gills and stipes.

Steps of preparation of extracts

- The freeze-dried and powdered mushroom sample (1g) was extracted with 10 ml (96%) methanol;
- This mixture was shaken for 1 hour at 60 °C at 175 rpm (*Laberte Vibrotherm, LE 204/2*);
- afterwards it was filtered through Whatman No. 4 filter paper;
- the residue was placed back in the flask and the procedure was repeated;
- the combined extracts were evaporated to final volume 10 ml in a drying cabinet at 45 ° C;
- before the various chromatographic procedures, the samples were filtered through a teflon syringe filter of 0.22 μm pore size.

2.2. Spectrophotometric measurements

Determination of total phenolic content – (SINGLETON & ROSSI, 1965)

- 1 ml mushroom extract + 4 ml of Folin-Ciocalteau reagent (10 *V/V*%) + 5 ml of Na_2CO_3 (7,5 m/m%)
- 30 minutes incubation, then measuring at 765 nm
- Calibration: 20-150 µg gallic acid (10 µg scale)

Final data is given in gallic acid equivalents (mg GAE / g d.m.)

Determination of total flavonoid content – (GURSOY et al. 2009)

- 1 ml mushroom extract + 1 ml of methanol + 2 ml of $AlCl_3$ (2 m/m%)
- 10 minutes incubation, then measuring at 415 nm
- Calibration: 5-60 µg quercetin (5 µg scale)

Final data is given in quercetin equivalents (mg QE / g d.m.)

Determination of antioxidant activity – (BLOIS, 1958)

- 1 ml mushroom extract + 3,1 ml methanol + 0,9 ml DPPH (1 mM) – for cuvette
- 50 µl mushroom sample + 150 µl methanol + 50 µl DPPH (1 mM) – for microplate
- 15 minutes incubation, then measuring at 517 nm

The DPPH radical scavenging activity (RSA %) was calculated by the following equation:

Radical scavenging activity (*%*)= $[(A_0-A_1)/A_0]*100$

where A_0 is the absorbance in the absence of sample the and A_1 is the absorbance in the presence of sample, respectively.

Preservative treatments – Some mushroom strains: *Agaricus bisporus* 'K-145', *Pleurotus ostreatus* 'P80' and *Lentinula edodes* 'KST-67' were used for various preservation methods.

Table 1. – The different parameters of preservation methods

Treatments	Type	Period of time	Temperature
Chilling methods	chilling	3 days	5 °C
	chilling	7 days	5 °C
	freezing	3 months	-20 °C
	freezing	6 months	-20 °C
Drying methods	drying on sticks	48 hours	20-32 °C
	room temperature	48 hours	24,5 °C
	drying cabinet	48 hours	35 °C
	drying cabinet	24 hours	60 °C
	drying cabinet	24 hours	90 °C
Chemical methods	blanching	-	100 °C
	salty	-	100 °C
	pickles	-	100 °C
	nature	-	100 °C
Microwave treatments	800 W	5 minutes	-
	800 W	15 minutes	-

The effect of different preservative treatments on the overall antioxidant activities, phenolic and flavonoid contents was measured using the above described methods.

2.3. Separation methods

Thin-layer chromatography and solid-phase extraction (SPE) - The best resolution for the separation of antioxidants was given by the ACN-H_2O-CH_3COOH 75:25:3 (V/V) solvent mixture. The developed TLC plates were allowed to air-dry, then immersed in 1mM DPPH solution. Areas with the DPPH scavenging activity were observed as yellow bands on a bluish background. Active components were determined by derivatization methods. Solid phase extractions (SPE) were also done, trying to reduce the complexity of the samples, so that the active component could be isolated at higher concentration.

Instrumental Analysis

HPLC-DAD-ESI-MS – Methods based a high performance liquid chromatographic separation were used for four different tests:

1. Detection of antioxidant components by a DPPH-HPLC-DAD method;

2. Characterization of the active substances, purity of chromatographic peaks by a HPLC-DAD-MS technique;

3. Isolation of the active compound from SPE eluates;

4. Quantitative comparison of the active substances of different fungal species and fractions.

In all four cases, the same separation parameters and HPLC equipment were used.

ESI-TOF-MS – To have the formula, it is necessary to determine the exact mass of the isolated material. The isolated sample was tested by positive and negative ESI ionization. Based on the data obtained the exact mass, the chemical formula and the structural properties were determined.

NMR (nuclear magnetic resonance spectroscopy) – NMR is a fundamental tool for chemical structure examination. The isolated sample was dissolved in deuterated methanol and measured in standard NMR tubes of 5 mm diameter. The data was collected using by *VnmrJ 3.2C software*.

CD (circular dichroism spectroscopy) – NMR studies were supplemented by circular dichroism spectroscopy (CD). The circularly polarized light (circularly polarized to the right and left) passes through the chiral sample to varying degrees. This serves to choose the right chiral structure, as for this task NMR spectroscopy is not suitable.

3. RESULTS

3.1. Differences between mushroom species and cultivated mushroom strains

Mushroom species

The highest level of phenolics was measured in the poplar mushroom, while the smallest amount was detected in the enokitake. However, most of the flavonoids were found in the enokitake and this is due to its colour. Of the cultivated mushrooms, brown button mushroom contained significant amounts of phenolics, while in the oyster mushroom only one third was detected (**Fig. 1.**).

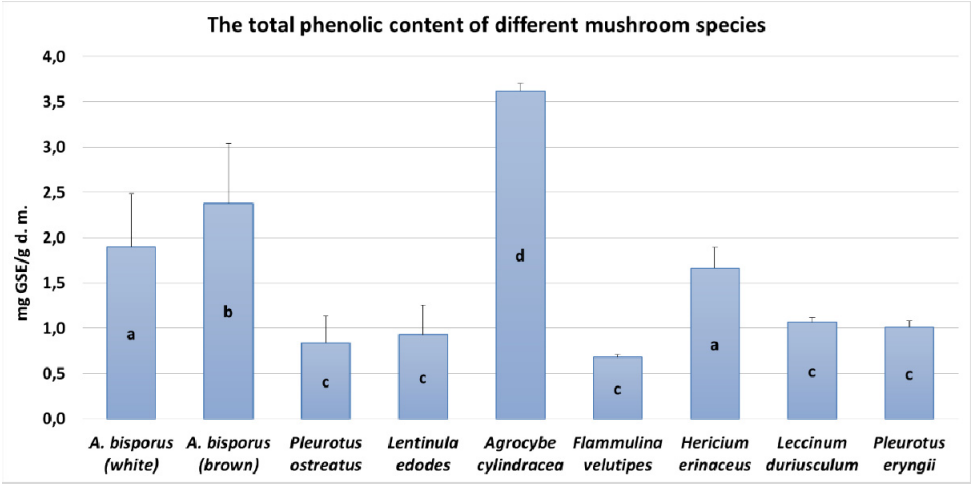


Fig. 1. – Contents of total phenolics in the mushroom species.
Each value is expressed as a mean ± standard deviation
In each bar different letters mean significant differences ($p < 0.05$)

Among the wild mushrooms, the lion's mane contained high phenolic besides poplar mushroom. The results for button mushroom are consistent with the findings of BARROS et al. (2008). The results of quantities are not comparable with the results of other studies (with other preparation, extraction

or measurement protocols), but the ratios between species (FERREIRA et al., 2009) are very similar to my results.

The flavonoid content is only approximately 5 to 10 percent of the total phenolic content (**Fig. 2**). This phenolics to flavonoid ratio has been confirmed by other researchers (JAYAKUMAR et al., 2009).

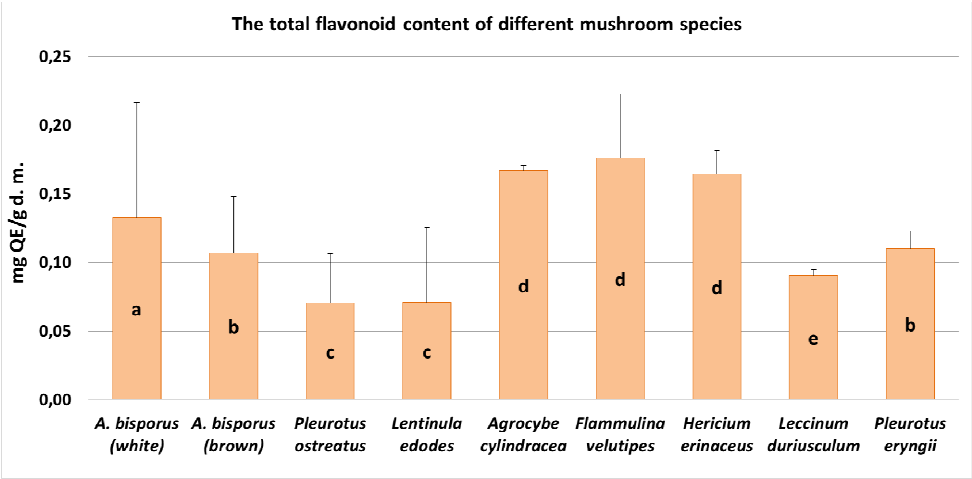


Fig. 2. – Content of total flavonoids in the mushroom species.

Each value is expressed as a mean ± standard deviation

In each bar different letters mean significant differences ($p < 0.05$)

The amount of flavonoids in some of the wild species (poplar mushroom, enokitake, lion's mane) is outstanding compared to other species. The flavonoid contents of the oyster and brown-cap button mushrooms are the same, while the poplar mushroom contains less. It is surprising that the white-cap button mushroom has higher flavonoid values than those of brown-cap button mushroom. The examined fungi are balanced on the basis of the amount of bound free radicals. The two endpoints are represented by *Pleurotus* mushrooms. While the radical-scavenging activity of the oyster mushroom is the weakest, the king oyster mushroom has the highest capacity to bind radical molecules.

In order to get an overall picture of antioxidant activity I have determined the EC_{50} values of mushroom extracts (**Table 2.**).

Table 2. – Radical-scavenging activity and EC_{50} values of the examined mushroom species

Mushroom species	*A. bisporus* (white)	*A. bisporus* (brown)	*Pleurotus ostreatus*	*Lentinula edodes*	*Agrocybe cylindracea*
Radical-scavenging activity (%)	81,8 ± 7,36[a]	80,8 ± 8,51[a]	60,9 ± 18,43[b]	86,4 ± 9,26[a]	86,6 ± 0,33[a]
EC_{50} values (µg/ml)	144,7 ± 2,1	90,3 ± 0,06	256,6 ± 5,78	287,7 ± 3,9	69,2 ± 0,06
Gombafaj	*Flammulina velutipes*	*Hericium erinaceus*	*Leccinum duriusculum*	*Pleurotus eryngii*	Gallic acid (1 mg/ml)
Radical-scavenging activity (%)	86,92 ± 0,26[a]	89,7 ± 0,21[a]	90,4 ± 0,20[a]	92,2 ± 0,13[a]	88,0 ± 0,44[a]
EC_{50} values (µg/ml)	671,1 ± 24,9	429,8 ± 2,0	295,5 ± 2,8	344,9 ± 1,3	7,2 ± 0,8

In each cells different letters mean significant differences ($p < 0.05$).

Based on the EC_{50} values can be distinguished 3 different groups:

- the first group includes highly active extracts (e.g.: the *Agaricus bisporus* and *Agrocybe cylindracea*)
- The second group contains a single definitive antioxidant component in the extracts (such as white rot mushrooms – *L. edodes, Pleurotus* spp.)
- The third group contains extracts of very small impact (e.g.: *Flammulina velutipes, Hericium erinaceus*)

Cultivated mushroom strains

Among the button mushroom strains, the phenolic content of the strain 'S-856' is outstanding. The smallest amount of phenolics was measured in the brown 'Heirloum' button mushroom strain. Among the oyster mushroom strains, strain '1-0-30' had the highest phenolic content, while the lowest values were given by strains '357-145' and 'HK-35'.

Between the shiitake strains, the strain '40-80 'proved to be the most valuable while the' KST-70 'strain contained the least phenolics.

Based on the flavonoid content, one of the most outstanding strains is the white button 'A-15', the other strains contain half or third of this amount. The flavonoid content of the oyster strains is minimal, exception is strain '1-0-30' and the smallest values being given by strain '480'. Of the shiitake strains, '40 -80' contains the smallest, while 'KST-67' the largest amount. As regards antioxidant activity, button mushroom strains vary largely, they are able to bind radical molecules between 67-90%. Antioxidant activity of oyster strains are even more dispersed, the difference between the worst ('BL') and the best strain ('H1') is approximately 62%.

There is no significant difference between the 'KST' strains, but the extract of strain '40 -80 accounts for nearly 20% less free radical molecules.

I classified each cultivated strains of button and oyster mushrooms. The cultivated mushroom strains (brown, distinct from white button) were collected in a summary table (**Table 3**.). The classification was based on the quartiles of the three parameters examined in all strains. Data on shiitake mushrooms was not included in the table, because only 3 shiitake strains data was available.

Table 3. – Classification of mushroom strains based on the 3 parameters tested

White button

Strain	Phenolics	Flavonoids	RSA (%)	Quality
'A-15'	2,47 ± 0,04	0,328 ± 0,006	87,2 ± 0,2	outstanding
'S-737'	2,71 ± 0,06	0,128 ± 0,016	90,4 ± 0,3	high
'K-145'	2,09 ± 0,06	0,135 ± 0,010	88,6 ± 0,2	high
'S-800'	1,86 ± 0,06	0,134 ± 0,005	76,6 ± 0,2	normal
'L-901'	1,4 ± 0,03	0,093 ± 0,026	86,3 ± 0,3	normal
'S-512'	2,07 ± 0,1	0,092 ± 0,04	71,5 ± 0,3	normal
'A-XXX'	1,32 ± 0,04	0,055 ± 0,001	80,8 ± 0,3	low
'L-927'	1,25 ± 0,1	0,099 ± 0,005	73,0 ± 07	low

Brown button

Strain	Phenolics	Flavonoids	RSA (%)	Quality
'S-856'	3,48 ± 0,01	0,164 ± 0,010	89,9 ± 0,2	outstanding
'Brown'	2,66 ± 0,07	0,050 ± 0,005	86,7 ± 0,2	high
'K-165'	2,54 ± 0,03	0,127 ± 0,009	86,9 ± 0,2	high
'Broncho'	1,86 ± 0,03	0,127 ± 0,003	78,1 ± 0,3	normal
'A-δ'	2,61 ± 0,14	0,176 ± 0,014	67,7 ± 1,7	normal
'Heirloum'	1,14 ± 0,05	0,099 ± 0,003	75,5 ± 0,2	low

Oyster mushroom

Strain	Phenolics	Flavonoids	RSA (%)	Quality
'1-0-30'	1,41 ± 0,07	0,180 ± 0,009	85,3 ± 0,4	outstanding
'G24'	1,33 ± 0,05	0,091 ± 0,002	78,5 ± 1,6	outstanding
'357-213'	1,17 ± 0,07	0,064 ± 0,005	65,2 ± 1,1	high
'P80'	0,77 ± 0,04	0,068 ± 0,008	79,7 ± 3,6	high
'H1'	0,69 ± 0,02	0,072 ± 0,009	90,2 ± 0,1	high
'LSZ'	0,85 ± 0,04	0,090 ± 0,004	50,7 ± 2,1	high
'BL'	1,33 ± 0,06	0,054 ± 0,003	58,4 ± 4,7	normal
'HK-35'	0,59 ± 0,03	0,104 ± 0,005	59,8 ± 1,2	normal
'H7'	0,64 ± 0,02	0,068 ± 0,010	47,4 ± 2,4	normal
'480'	0,66 ± 0,06	0,023 ± 0,006	79,2 ± 0,5	normal
'ZN'	0,69 ± 0,03	0,050 ± 0,003	66,2 ± 5,6	normal
'Po462'	0,62 ± 0,06	0,080 ± 0,006	45,0 ± 1,0	normal
'BAK'	0,84 ± 0,13	0,039 ± 0,005	28,2 ± 3,1	low
'DSN'	0,61 ± 0,05	0,050 ± 0,003	57,6 ± 4,3	low
'VL-80'	0,62 ± 0,08	0,053 ± 0,006	31,0 ± 0,9	low
'Spoopo'	0,72 ± 0,04	0,037 ± 0,002	41,7 ± 2,7	low
'357-145'	0,61 ± 0,02	0,046 ± 0,006	52,7 ± 2,2	low

3.2. The parts of mushrooms fruiting body

Among the fractions, the cap skin and the gills provide significantly higher values than the whole fruiting body, the primordia and the other mushroom parts (**Fig. 3.**). The phenolics concentrations of primordia and fruiting bodies are almost identical, and similar conclusions were drawn by other researchers (BARROS ET AL. 2007; SOARES ET AL. 2009) **too.** During development, processes take place in the mycelium that accumulate bioactive and other primary substances in sufficient quantities. Thus, primordia formation and the "explosive" formation of fruiting body can be equivalent to the flow of water and the pre-produced materials. The reason for the different phenolic contents of the fruiting body parts is presumably to be found in the physiology of the mushroom. The cap skin is the outermost layer that is in contact with the environment. The primary tasks of the gills are evolving, maturing and storing the spores. After the formation of the fruiting body, gills decay rapidly, therefore the phenolic compounds can protect the spores in the gills.

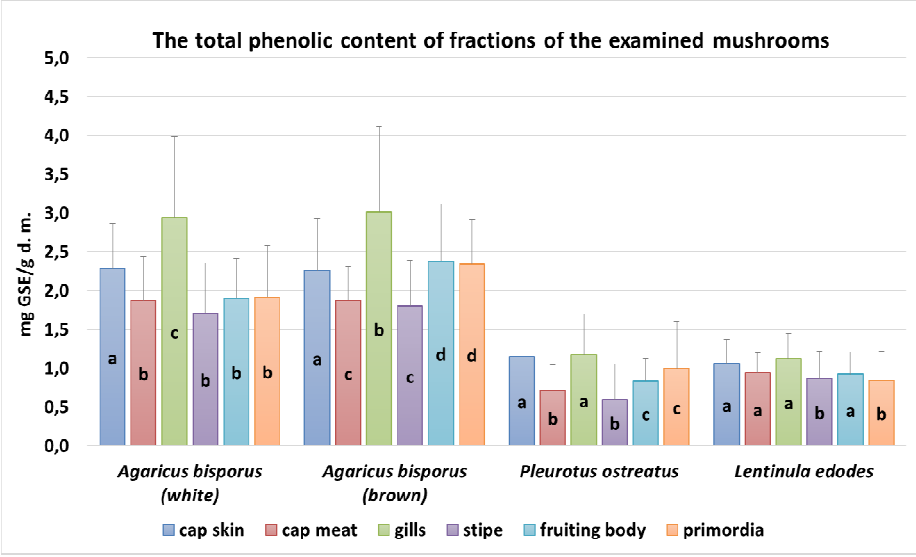


Fig. 3. – Total phenolic content of the cultivated mushroom fruiting body parts.

Each value is expressed as a mean ± standard deviation

In each bar different letters mean significant differences ($p < 0.05$)

Examination of the fruiting body parts of the wild mushroom species reveals a similar relation as in the case of cultivated species. The flavonoid content of the stipe is very low for all tested mushroom species. We observed slight differences between the fruiting body and primordia (the primordia contains a little more flavonoids, on average by 10 %, in each mushroom species).

Radical scavenging activities of different cap parts (cap skin, cap meat, gills) are balanced, but the cap meat of some species are of lower quality (e.g.: *Pleurotus ostreatus*, white *Agaricus bisporus*). All the examined mushroom species showed high antioxidant activity (above 75%), except for the oyster mushroom. The following order was established for the antioxidant activity between the fruiting body parts: **gills> cap skin> fruiting body> primordia> stipe> cap meat**.

3.3. Results of preservative treatments

The preservation methods influenced the phenolic, flavonoid contents and antioxidant activity of the fungal samples in different ways.

Based on the examination of the different preservative processes, the best preservation method is 60-90 °C drying. The amount of phenolic substances increases slightly with increasing temperature, except for *Agaricus bisporus*. The amount of flavonoids showed a fourfold increase at the higher temperature, typically at 90 ° C, for all three mushrooms.

Cooling and freezing processes - apart from *Pleurotus ostreatus* - do not affect antioxidant activity. However, phenolics and flavonoids are reduced in some cases, depending on the species and duration.

The various chemical preservation methods and blanching (*A. bisporus* is well suited to blanching) significantly reduced all parameters of the mushrooms.

3.4. TLC-DPPH

Thin layer chromatographic assays were performed with the most valuable cultivated strains and all wild species. The antioxidant profile of the mushroom species, as obtained by TLC-DPPH, is shown in **Figure 4**.

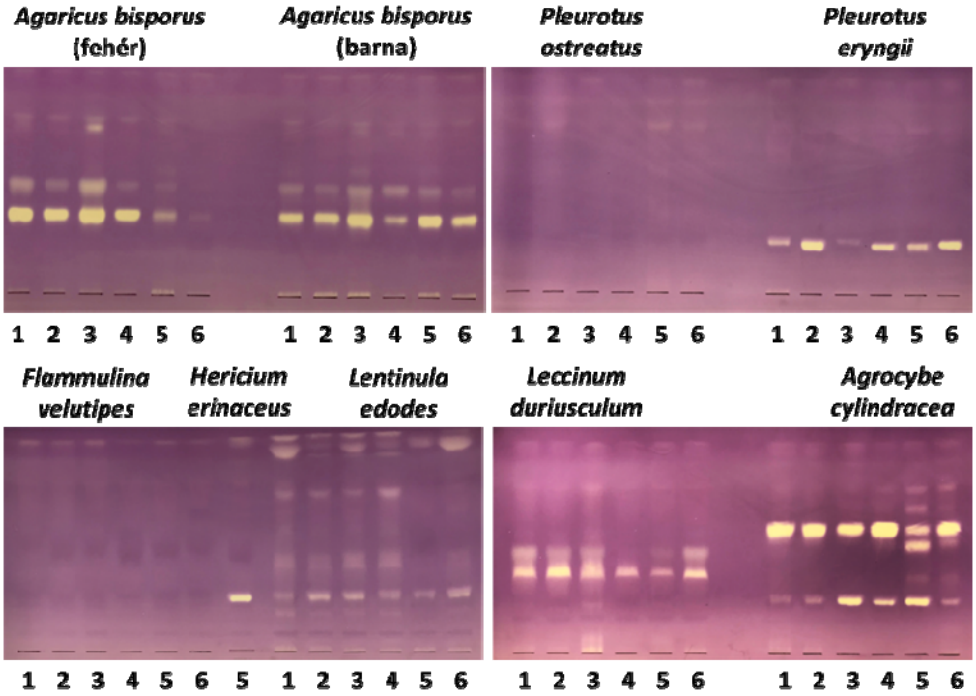


Fig. 4. – Thin-layer chromatographic antioxidant profile analysis of the fungi (**1-cap skin, 2-cap meat, 3-gills, 4- stipe, 5-fruiting body, 6-primordia**)

Antioxidant components were not detected in two mushrooms species (*Pleurotus ostreatus* and *Flammulina velutipes*). It means that the antioxidant compound is under the detection's threshold in the examined mushroom. Possibly, the antioxidant compounds are not stable on the layer. Based on the results of the derivatization methods, the active compounds have phenolic character, are in some cases glycosylated and/or contain amine groups. This conclusion can be deduced in other mushroom species as well.

3.5. HPLC-DPPH

Mushroom samples were analyzed by an off-line DPPH-HPLC method, i.e., before injection, DPPH solution or pure methanol (control) was added to the sample at an appropriate ratio (RIETHMÜLLER et al., 2016).

Based on my results, I consider the DPPH-HPLC method as useful also for the examination of basidiomycetous mushrooms. Thereby it can be deployed to look for compounds with antioxidant activity, even in the case of an unknown sample, provided that a suitable chromatographic separation is found. The revealed properties coincided with thin layer chromatographic (TLC-DPPH) results for each mushrooms. Based on the antioxidant profiles, there is no large difference within genera, while there are differences between the different genera. I found a compound in most of the examined mushroom species.

3.6. The results of instrumental examination of the isolated compound (ESI-TOF-MS, NMR and CD)

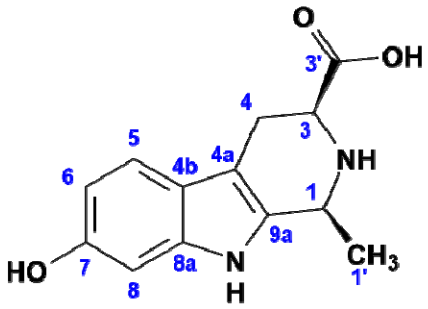
Based on the derivatization tests, I have focused on the extracts of white button, poplar and slate bolete species. The instrumental tests were performed with those parts that produced a larger spot size in thin-layer. Thus, the isolations were carried out primarily from the gills. A successful SPE procedure resulted from the unified (cap skin + cap meat + gills + stipe extracts) poplar mushroom sample, since the individual parts contained the same active substances. I isolated 2.3 milligrams of material, its R_f-value was 0.57.

We determined the m/z values of the isolated compounds, based on the spectra recorded in the positive and negative modes. The main component of the sample is a compound of molecular mass **246 Da**. The mass of the protonated molecular ion on the basis of accurate mass measurement was $[M+H]^+$= 247,1084 Da; Chemical formula $[M+H]^+$= $\mathbf{C_{13}H_{15}N_2O_3^+}$. Theoretical mass: 247.1083 Da (deviation 0.4 ppm).

Structural properties that can be deduced from negative and positive mass spectra and fragmentation data:

- In the neutral molecule (**M=246 Da**, molecular formula: $\mathbf{C_{13}H_{14}N_2O_3}$), the total number of rings and double bonds is 8,
- presence of a probable indole skeleton,
- presence of a probable NH_2-group,
- presence of a probable carbonyl-group
- presence of probable one more ring or a double bond

The isolated component is the diastereomer of the *Brunnein-B* compound. Diastereoisomers are non-reflective, non-interlacable molecules (**Fig. 6.**).



Number	^{1}H (δ, ppm)	^{13}C (δ, ppm)
1	4.65 (q, J = 7.0 Hz, 1H)	51.2
1'	1.71 (d, J = 6.7 Hz, 3H)	17.2
3	3.94 (dd, J = 12.1, 4.9 Hz, 1H)	59.7
3'	-	173.7
4	3.38 (dd, J = 15.6, 4.9 Hz, 1H) 2.98 (ddd, J = 15.6, 12.1, 2.5 Hz, 1H)	24.4
4a	-	107.7
4b	-	121.4
5	7.28 (d, *J* = 8.5 Hz, 1H)	119.6
6	6.62 (dd, *J* = 8.5, 2.1 Hz, 1H)	110.7
7	-	154.9
8	6.76 (d, J = 2.1 Hz, 1H)	97.8
8a	-	139.7
9a	-	129.6

Fig. 6. – Structure, numbering and total ^{1}H and ^{13}C NMR signalling of the isolated compound in deuteriated methanol at 600 MHz

Brunnein-B was first discovered and described in the brown webcap mushroom (*Cortinarius brunneus* (Pers.) Fr. 1838) (TEICHERT et al., 2007). The isolated novel material is a yellow crystalline compound which is well-soluble in water and MeOH, is thermostable and it has a strong antioxidant activity. Its radical scavenging activity is 91.86 % and the EC50 value is 119.04 µg/ml.

3.7. NEW SCIENTIFIC RESULTS

The new scientific results of this research of the dissertation are achieved:

I. The sample preparation methods for mushroom species were compared and some standardization options were revealed. I introduced a new sample preparation method for mushrooms with pilothecium (type of fruiting body): the fractionation. The emphasis have been placed on the four morphologically well-differentiated body parts of fruiting bodies, instead of the traditional methodology (cap – stipe**).** The four distinct parts are: **cap skin, cap meat, gills and stipe**.

II. The strains of the cultivated mushrooms (*A. bisporus* – eight strains, brown *A. bisporus* – six strains, *P. ostreatus* – seventeen strains, *L. edodes* – three strains) were examined and compared based on three biochemical parameters. A new classification system for mushroom strains was created (which does not exclude the possibilities of expansion).

III. The effects of preservative processes on antioxidant substances were studied in **three** cultivated mushroom species. Among the various methods, the most gentle were the drying ones, while the largest decrease of antioxidants was caused by chemical preservation processes.

IV. A thin layer chromatographic procedure was developed for mushrooms and an isolation protocol was elaborated for solid phase extraction. I have determined the antioxidant profile of the mushroom species' fruiting bodies and their parts.

V. I transferred and optimized a DPPH-HPLC method - originally developed for plant specimens - for mushroom samples. I have determined the chromatographic characteristics of the active substances in the examined mushrooms and compared their quantity.

VI. The phenolic and flavonoid profiles, the antioxidant activity and the EC_{50} values of different body parts of the poplar mushroom (*Leccinum duriusculum*) were described firstly. One of the most important antioxidant compounds of the *Agrocybe cylindracea syn. Agrocybe aegerita* was isolated and we have successfully determined the chemical structure. Its molecular formula is $\mathbf{C_{13}H_{14}N_2O_3}$, and it is the diastereomer of the *Brunnein-B* molecule.

CONCLUSIONS AND PROPOSALS

A more detailed fractionation of mushroom fruiting bodies was introduced instead of the classical fractions (cap and stipe) (FERREIRA et al., 2007). After fractionation, four morphologically distinct parts are obtained, such as cap skin, cap meat, gills and stipe (KALAC, 2009).

Some extraction methods (from literature) were used and examined for different aspects. Based on my results, I came to the conclusion that the extraction methods using methanol are capable of extracting significantly more antioxidant substances than those using other solvents (SMITH et al., 2015). My method (mechanical shaking with heating) has a lower demand for raw material and time than most of the other extraction processes.

The cap skin and the gills are the most useful parts of the fruiting body, based on their phenolic content and antioxidant activity. The cap meat is roughly equivalent to the stipe, but for some taxa (pl.: *Leccinum duriusculum, Lentinula edodes*) its value is not negligible.

A novel eluent was developed (ACN-H_2O-CH_3COOH, 75: 25: 3) instead of eluents known from the literature, which provides a suitable resolution for testing methanol extracts and analysing the antioxidant profile of particular mushroom species (CAI et al., 2013). Based on these results, there is no significant difference between the antioxidant profiles within a genus, but there are such differences between the genera. Further compounds can be isolated and tested based on the TLC-DPP profile of the examined mushroom species.

It has been verified that the antioxidant content of the fruiting body is equivalent to the weighted average of its different parts. The antioxidant compositions were determined for all the examined mushrooms: the individual parts of fruiting bodies have different antioxidant makeup - mostly quantitatively, but in some species also qualitatively, too.

One common compound has been detected, which is found in five mushroom species, moreover, it is a determinative one in two species. One of the antioxidant compounds of *Agrocybe cylindracea* was determined by instrumental analysis (HPLC, TOF-MS, NMR, CD) as diastereomer of *Brunnein-B* (**M=246 Da**), $\mathbf{C_{13}H_{14}N_2O_3}$.

The main three cultivated mushroom species in Hungary: the button mushroom (brown and white cap), oyster mushrooms and shiitake strains were investigated. The button mushroom strains proved to be the best antioxidant-containing fungi, they were followed by shiitake strains, while oyster mushroom strains had the weakest antioxidant activity. A qualification system of cultivated strains was developed for antioxidant effects, based on the results of the cultivated mushroom strains and a database was set up with data of 34 macrofungal strains, based on examined parameters of the fruiting body and its parts. Data and results of the mentioned qualification system will also be useful for mushroom breeding.

The best preservation methods (for antioxidants) were the dryings between 60-90 °C. The amount of phenolic materials increased for increasing drying temperatures, except for *A. bisporus*. The flavonoid contents increased fourfold at higher temperatures (typically 90 °C) for all examined mushrooms. Cooling and freezing processes did not affect antioxidant activity (except *P. ostreatus*), while the different chemical preservations decreased all examined parameters of the mushrooms drastically (FERNANDES et al., 2013; JAFRI et al., 2013).

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