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Research article

Squalene in fruiting bodies of true mushrooms changes in the process of vegetation

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Abstract
The paper presents the results of the search for raw sources of squalene among real mushrooms. Lipophilic extracts obtained from fruiting bodies of 22 species were analyzed, 21 of which belonged to the class of Agaricomycetes of the orders Pezizales, Polyporales, Cantharellales, Russulales, Boletales, and one species to the class of Pezizomycetes of the order Pezizales. It was established that the content of squalene in lipophilic extracts from the fruiting bodies of *Tyromyces chioneus, Fomitopsis betulina, Pleurotus ostreatus* and *Postia stiptica* can exceed the content of squalene in fatty oil from the seeds of *Amaranthus caudatus*. However, the mass of the lipophilic extract obtained from the dried fruiting bodies of these mushrooms is insignificant. Also, the content of squalene in fruiting bodies at different stages of development, as shown by studies conducted on aphyllophoroid mushrooms *Tyromyces chioneus, Laetiporus sulphureus, Pleurotus ostreatus, Fomitopsis betulina, Mucidula múcida,* decreases as the fruiting bodies of these mushrooms ripen. At the same time, the yield of lipophilic extract from the fruiting bodies of *M. múcida*, is abnormally high, 9–15% and increases during their ripening and, although the squalene content in them decreases as the mushrooms grow, this decrease is much smaller than in other mushrooms.

Keywords
Squalene, Aphyllophoroid fungi, *Tyromyces chioneus, Mucidula múcida*, lipophilic extracts

Introduction

From a chemical point of view, squalene is a compound with 6 double bonds - 2,6,10,15,19,23-hexamethyl-2,6,10,18,22-tetracosahexaene of the general formula $C_{30}H_{50}$. In its pure form, squalene is a colorless, odorless and tasteless oil. Squalene is quite chemically stable with a high boiling point. The richest source of squalene is fat from the liver of deep-sea sharks, where its content can reach 60-90% (Popa et al., 2015). Fatty oils from plant seeds are an alternative source of squalene. One of the richest squalene oils is amaranth seed oil, where the squalene content can reach 5–8% (Baraniak

and Kania-Dobrowolska, 2022; Korabel et al., 2022). In other vegetable oils, the content is lower and rarely exceeds 1% (Mendes et al., 2022). Squalene is a component of mammalian subcutaneous fat. It is known from the literature that the highest content of squalene in human tissues is found in skin lipids (about 500 µg g-1) and adipose tissue (≈ 300 µg g-1) (Tsimidou, 2010), where it plays an important physiological role. It easily penetrates through the skin into the body, and is a powerful immunostimulant, while being non-toxic and safe. The emollient and hydrating properties of squalene and its biocompatibility with human skin have led to its introduction into a range of cosmetic products (Huang et al., 2009). As part of cosmetics, it acts as an effective wound-healing agent, and also protects the skin from free radical damage and prevents its aging, which occurs due to lipid peroxidation under the influence of UV radiation (Huang et al., 2009). In mammals and humans, squalene is synthesized in the liver and is an intermediate link in the synthesis of cholesterol and steroid substances. Squalene has been used as a cholesterol-lowering drug, in dermatology, and as an adjuvant in vaccines (Popa et al., 2015). The squalene-based oil-in-water emulsion vaccine adjuvant MF59 has been administered to more than 100 million people in more than 30 countries for patients with influenza, hepatitis B, malaria, and shingles (Kim et al., 2020). The production of squalene has become especially relevant during the global Covid-19 pandemic (Mendes et al., 2022). Due to the fact that deep-sea shark fat is a scarcely available source of squalene, the search for more accessible sources of squalene remains relevant. In addition, the presence of cholesterol in the oils from the liver of marine animals significantly complicates the purification of squalene. During the analysis of the hexane extract obtained from the fruiting bodies of *Tyromyces chioneus* (Fr.) P. Karst which belongs to aphyllophoroid fungi, we found the content of squalene exceeded that of amaranth oil, using GC-MS (unpublished data, see Table 2). Therefore, we decided to investigate the content of squalene in a number of other phylogenetically close mushrooms, and to investigate how its amount changes during the vegetation of the mushroom fruiting bodies.

Materials and Methods

Raw materials

Thirty fruiting bodies of 21 species were collected on the outskirts of the Lviv city mainly in the summer-autumn period. Aphyllophoroid mushrooms were dried in the laboratory at room temperature whereas fleshy fruiting bodies were dried in a drying cabinet at 55 °C. The speciments was identified by the taxonomical keys used to identify mushroom species (Kuo, 2022). A voucher specimen has been deposited in the Herbarium of the Botanical Garden of Danylo Halytsky Lviv National Medical University (Lviv, Pekarska St., 73, 79010, Ukraine).

Reagents

The following analytical grade reagents were used in the work: petroleum ether 40-70°C, hexane, cyclohexane, diethyl ether, chloroform, methanol. All of the reagents were purchased from "Sfera Sim" (Lviv, Ukraine). Silica gel for column chromatography (40/160 mesh) was purchased from Chemapol (Czech Republic). Squalene (purity ≥ 98) (Sigma-Aldrich, USA) and Silufol UV plates (Kavalier, Czech Republic) were also used.

Preparation and purification of the extract

After drying the fruiting bodies, they were crushed in an electric grinder and sieved through a sieve with a mesh of 0.5 mm. The crushed powder (30–50 g) was extracted in a Soxhlet apparatus with petroleum ether with a boiling point of 40–70 °C for an hour. Next, the solvent was distilled off, and the residue was dried in an oven at 55 °C. The obtained residue was weighed and dissolved in hexane or cyclohexane (depending on further actions) in the ratio mushroom extract - cyclohexane 1:10. If it was impossible to analyze the hexane extract using GC-MS, it was divided into fractions by chromatography on silica gel. The cyclohexane solution was centrifuged and the clear supernatant was applied to a silica gel column (40/160 mesh, Chemapol company, Czech Republic), pre-washed with cyclohexane. After the solution entered the gel, the substances adsorbed on the column were eluted with cyclohexane, and those substances that were not eluted under the specified conditions were eluted with chloroform. The solvent was evaporated, and the residue was weighed. Squalene was eluted in the first fractions, then these fractions were combined and analyzed using gas chromatography-mass spectrometry (GC-MS).

Chromatography on Silufol plates

Solutions of lipophilic fractions of the studied fungi and 1% solution of squalene were applied to Silufol UV plates measuring 14 × 12 cm. Hexane - chloroform (1:1) was used as the solvent system, which gave the best results among the tested solvent systems. After raising the solvent system to a height of 11–11.5 cm, the plate was removed from the chromatographic chamber, dried in a drying cabinet at + 60 °C until the solvents weathered, and then placed in a chamber with iodine. Squalene appeared as yellow spots with Rf = 0.9.

Gas chromatography-mass spectrometry (GC-MS)

The analysis was carried out using a mass spectrometer 6C/MS Agilent Technologies 6890 N/5975 B attached to a chromatographic column (model HP-5MS, length 30 m, diameter 0.25 mm, filler: 95% dimethylpolysiloxane + 5% diphenylpolysiloxane); the carrier gas was helium with a constant flow of 1.5 mL min^{-1}). The column was washed with methanol. The gas chromatography was programmed to increase the temperature by 15 °C min^{-1} from 75 to 300 °C. The initial temperature was maintained for 1 min, and the final temperature was maintained for 8 min. A mass-selective detector with an interface temperature T = 250 °C was used. Ionization was carried out by electron impact, the ionization energy was 70 eV, the temperature of the ion source was 230 °C and the quadrupole temperature 150 °C. The NIST05 and WILEY 2007 mass spectra library with a total number of spectra of more than 470,000 were used for component identification in conjunction with AMDIS identification computer programs. The degree of reliability was calculated by the % of coincidence with the substances that are in this data library. The relative error of determining the above components does not exceed 10% with a confidence probability of 0.95.

Statistical analysis

Each experiment was performed in triplicate. The average values were recorded. Chromatography on silica gel column was also performed three times. The data were evaluated statistically using Student's t-test, and a value of $p \leq 0.05$ was considered to be statistically reliable.

Results and Discussion

The mass of the total residue of lipophilic substances obtained after distillation of the solvent (petroleum ether or hexane) and drying in an oven was determined by weighing and the results are presented in Table 1.

Table 1 – The percentage content of lipophilic substances extracted from the fruiting bodies.

Mushroom species	Order/Family	Mass of lipophilic substances in dried raw materials (%)
	Pezizales	
Sarcoscypha coccinea (Scopoli) Saccardo	Sarcoscyphaceae	1.14 ± 0.09
	Polyporales	
Tyromyces chioneus (Fr.) P. Karst	Polyporaceae	1) 0.45 ± 0.04 2) 0.30 ± 0.05
Pycnoporus cinnabarinus (Jacq.) P. Karst	Polyporaceae	0.59 ± 0.06
Coriolus versicolor (L.) Quél	Polyporaceae	0.26 ± 0.05
Polyporus badius (Pers.) De	Polyporaceae	0.44 ± 0.06
Polyporus squamosus (Huds.) Fr.	Polyporaceae	1.21 ± 0.12
***Postia** stiptica (Pers.) Jülich*	Fomitopsidaceae	1.46 ± 0.14
Fomitopsis betulina (Bull.) B.K. Cui, M.L. Han & Y.C. Dai	Fomitopsidaceae	1) 1.35 ± 0.12 2) 0.85 ± 0.09
Laetiporus sulphureus (Bull.) Murrill	Fomitopsidaceae	1.89 ± 0.12
	Agaricales	
Mucidula múcida (Schrad.) Höhn	Physalacriaceae	1) 9.29 ± 0.52 2) 14.72 ± 0.62
Pleurotus ostreatus (Jacq. ex Fr.) P. Kumm. wild strain collected in nature	Pleurotaceae	1) 1.28 ± 0.12 2) 0.96 ± 0.11 3) 0.91 ± 0.12
Pleurotus ostreatus (Jacq. ex Fr.) P. Kumm. sort Euromycel PL132	Pleurotaceae	1) 0.21 ± 0.03 2) 0.14 ± 0.03 3) 0.17 ± 0.05
Lycoperdon pyriforme Schaeff.	Agaricaceae	0.75 ± 0.08
Lycoperdon perlatum Pers	Agaricaceae	1.12 ± 0.13
Flammulina velutipes (Curtis) Singer	Physalacriaceae	1.03 ± 0.11
	Cantharellales	
Hydnum repandum L.	Hydnaceae	1) 3.44 ±0.29 2) 2.74 ± 0.24
***Craterellus cornucopioides* (L.) Pers.**	Cantharellaceae	3.82 ± 0.37
***Cantharellus cibarius* Fr.**		2.41 ± 0.22
	Russulales	
Russula virescens (Schaeff.) Fr.	Russulaceae	2.48 ± 0.23
	Boletales	
Boletus subtomentosus L.	Boletaceae	3.28 ± 0.33
Suillus luteus (L.) Roussel	Suillaceae	4.76 ± 0.43
Scleroderma aurantiacum L. ex Pers	Sclerodermataceae	1.22 ± 0.32

The obtained results made possible to establish that the total amount of lipophilic substances contained in most studied mushrooms was small and ranged from 0.15 to 1–2%. At the same time, it fluctuates within very large limits, from 0.14% in *Pleurotus ostreatus* to 14.72% in *Mucidula múcida* (a hundredfold difference). In addition, their amount in raw material samples collected from different places varied greatly (Table 1). According to our observations, it most likely depends on the

mushroom growth, but we failed to objectively assess this parameter. It is also possible that the quantitative and qualitative composition of extractive substances is affected by various environmental factors such as air temperature, humidity, mineral composition of the soil, intensity of solar radiation. There is relevant literature on the influence of these factors on growth cultivated mushrooms, in particular, on *P. ostreatus* (Garuba et al., 2017; Aghajani et al., 2018; Hultberg et al., 2020).

The analysis of mushroom extracts was carried out using GC-MS. The mushroom extracts from which the mixture was not too complex, it was possible to obtain results without additional treatments. Such extracts were from mushrooms belonging to the Polyporales order, while hexane extracts of other mushroom orders (Cantharellales, Russulales, Boletales) in most cases could not be analyzed without additional separation. These data are presented in Table 2.

Table 2 – Squalene content in lipophilic mushroom extracts.

Mushroom species	Squalene content (%)	Degree of reliability*
Fomitopsis betulina	1) 0.57	96
	2) 6.06	96
Pleurotus ostreatus	1) 7.73	96
	2) 2.23	98
Hydnum repandum	0.46	91
Craterellus cornucopioides	0.51	98
Mucidula múcida	2.70	95
Lycoperdon pyriforme	2.81	80
Scleroderma aurantiacum	5.03	87
Lycoperdon perlatum	1.05	93
Pycnoporus cinnabarinus	2.51	93
***Postia** stiptica*	5.19	97
Tyromyces chioneus	12.59	98
Coriolus versicolor	3.75	94
Polyporus badius	3.25	99

*In Tables 2 and 3, the degree of reliability is calculated by the computer program NIST05 and WILEY 2007

For the analysis of lipophilic extracts of mushrooms not listed in Table 2, we enriched them for squalene by preliminary separation into fractions by column chromatography on silica gel. After that, these fractions were analyzed by gas chromatography-mass spectrometry. The method of purification of squalene from amaranth oil was taken as a basis (He et al., 2002), but without the step of saponification of fatty acids with alkali.

The application of saponification procedure for lipophilic extracts from mushrooms is not desirable due to the possible formation of resins from some of their components in alkaline conditions. Separation of lipophilic mushroom extracts was carried out on a silica gel column followed by analysis of the separated fractions using GC-MS. In all cases, squalene was contained in the first eluate fractions. As an example, we present a graph of the separation of the lipophilic extract of *Sarcoscypha coccinea* bright red on a silica gel column (Fig. 1).

After elution from the silica gel column, the first fractions were applied to silica gel plates and detected squalene after developing with iodine vapors. In addition, pure squalene has a high refractive index value ($n^{20}/_{D}$ =1.494), which was also measured. A thin-layer chromatogram of some lipophilic mushroom extracts and pure squalene (standard) is presented in supplementary Fig. S1. However, quantitative determination of squalene content in lipophilic extracts is much more convenient and more accurate to determine by GC-MS method. The results of tests on fractions enriched with squalene are presented in the Table 3.

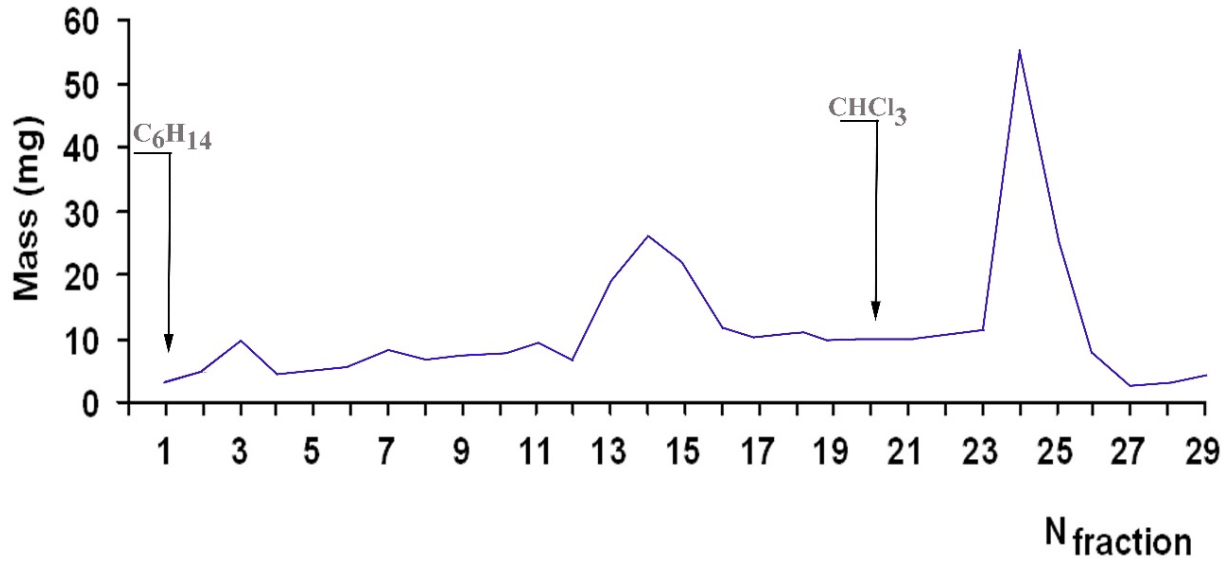

Fig. 1. – Separation of the lipophilic extract of *Sarcoscypha coccinea* on a silica gel column.

According to GC-MS, squalene was not found in the lipophilic extracts of *Russula virescens, Boletus subtomentosus*, *Suillus luteus* and ***Cantharellus cibarius*** **fruiting bodies.** Thus, from the point of view of obtaining squalene, mushrooms belonging to the Orders Polyporales and Agaricales are of greatest interest because the content of squalene in their lipophilic extract is often higher than in the oil of most plants, including amaranth oil (4.5–7.0%) (Korabel et al., 2022). Based on the obtained results, lipophilic extracts of *M. múcida*, *L. sulphureus*, *F. betulina*, *T. chioneus* and *P. ostreatus* can be promising sources for obtaining squalene. However, it should be noted that the data presented in Tables 2 and 3 were mostly based on the analysis of only one raw material sample, or, when obtained from two samples, they differed greatly. The most likely reason for this condition is the collection of fruiting bodies at different stages of their development. Therefore, we conducted additional studies in order to establish the dependence of the amount of squalene in the lipophilic extract on the developmental stage of *M. múcida*, *L. sulphureus*, *F. betulina*, *T. chioneus*, *P. ostreatus* and *L. perlatum* fruiting bodies.

Table 3 – Squalene content in lipophilic fractions of mushrooms.

Mushroom species	Squalene content (% in the enriched fraction)	Squalene content (% in the extract)*	Degree of reliability
Flammulina velutipes	16.08	1.81	99
Sarcoscypha coccinea	18.08	1.14	97
Laetiporus sulphureus	33.45	2.86	97
Polyporus sqamosus	8.12	0.53	95

*conversion to the entire mass of the lipophilic extract.

Weekly observations were made on the potential growth sites of these mushrooms. In the same place of collection, young fruiting bodies were harvested a week after their appearance, ripe fruiting bodies after two week and overripe fruiting bodies after three weeks. Besides considering the harvesting time, the division into young, ripe and overripe fruiting bodies was additionally made by assessing their morphological state. For example, a young birch sponge was pure white, soft and easy to cut with a knife. Ripe fruiting bodies were more difficult to cut with a knife. Overripe fruit bodies

became woody and they were even more difficult to cut with a knife. In the case of the *Lycoperdon perlatum*, the division into three groups according to the degree of maturity was carried out after cutting the fruit bodies and assessing the color of their inner part. Young fruiting bodies were pure white, ripe ones were yellowish inside, and overripe ones acquired a grayish color. Selected fruiting bodies, sorted into groups according to the degree of development, were used separately to obtain lipophilic extracts, then analyzed by GC-MS (Table 4).

Table 4 – Changes in the amount of lipophilic extract and its squalene content depending on the degree of maturity of fruiting bodies.

Source of lipophilic extract	Stage of maturation	Yield of lipophilic extract (%) from the mass of raw materials	Squalene content (% in the lipophilic extract)*
Tyromyces chioneus	young	0.72	11.34
	ripe	0.64	6.00
	overripe	0.61	1.38
Laetiporus sulphureus	young	1.24	2.86
	ripe	0.95	1.39
	overripe	0.24	0.32
Pleurotus ostreatus	young	0.38	8.42
	ripe	0.17	2.09
	overripe	0.08	1.78
Fomitopsis betulina	young	2.04	5.34
	ripe	1.48	1.23
	overripe	0.64	0.83
Mucidula múcida	young	9.43	4.24
	ripe	10.31	3.01
	overripe	15.82	1.78
Lycoperdon perlatum	young	1.34	1.02
	ripe	0.99	1.07
	overripe	0.63	1.70

*according to GC-MS data

The results presented in Table 4 show that for all mushroom species (except for *L. perlatum*) the squalene content decreased as the fruiting bodies aged. We observed the highest content of squalene in the lipophilic extract of *T. chioneus*. The yield of the amount of lipophilic extract decreased as the fruiting bodies aged (except for *M. múcida*). However, in order to evaluate the profitability of obtaining squalene from mushroom raw materials, taking into account the different mass yield of lipophilic extract, it is necessary to take into account how much squalene can be obtained from 100 g of dried mushrooms (Table 5).

Thus, analyzing the data in the table 5, it can be concluded that only *M. mucida* can seriously compete with amaranth seeds as a source of squalene. This mushroom is characterized by a very high content of lipophilic substances, the amount of which increases during the growth of the fruit bodies, unlike all other analyzed mushroom species. Although the concentration of squalene in older *M. mucida* fruiting bodies decreases, this decrease is not as strong as in other mushrooms.

Tyromyces chioneus and *F. betulina* also deserve attention. The young fruiting bodies of these mushrooms have a fairly significant content of squalene. It is higher than that of *M. mucida*, but the total amount of extracted lipophilic substances is 5–25 times lower. Due to this, the specific content of squalene in this raw material is lower. In addition, during the aging of the fruiting bodies of these mushrooms, a 10-fold decrease in the total amount of squalene is observed, while in *M. mucida* such

a decrease is only 30%. According to GC-MS data, the bulk of the lipophilic extract from *M. mucida* fruiting bodies consists of higher fatty acids (Table 6).

Table 5 – Theoretical yield of squalene (in mg) from 100 g of dried fruiting bodies.

Source of lipophilic extract	Stage of maturation	Yield of the residue (%) raw material dried mass	Squalene content (% in the extract)*	Theoretical yield of squalene (mg) from dried raw material (100 mg)
Tyromyces chioneus	young	0.72	11.34	81.6
	ripe	0.64	6.00	38.4
	overripe	0.61	1.38	8.4
Mucidula mucida	young	9.43	4.24	399.8
	ripe	10.31	3.01	310.3
	overripe	15.82	1.78	281.6
Laetiporus sulphureus	young	1.24	2.86	35.5
	ripe	0.95	1.39	13.2
	overripe	0.24	0.32	0.8
Fomitopsis betulina	young	2.04	5.34	108.9
	ripe	1.48	1.23	18.2
	overripe	1.23	0.83	10.2
Pleurotus ostreatus (cultivated, variety Euromycel PL132)	young	0.38	8.47	32.2
	ripe	0.17	2.09	3.6
	overripe	0.08	1.78	1.4
Lycoperdon perlatum	young	1.34	1.02	13.7
	ripe	0.99	1.07	10.6
	overripe	0.63	1.70	10.7
Amaranthus caudatus seeds**		4.2	4.5 – 7.0	189 – 294

*according to GC-MS data; **(Korabel et al., 2022).

Unsaturated fatty acids (oleic and linolenic) make up about half of the entire assortment of these acids. As fruiting bodies age, the content of unsaturated fatty acids decreases. There is also a gradual decrease in the amount of squalene from young to overripe mushrooms.

Conclusions

Our research shows that among analysed mushrooms, the highest content of squalene is observed in aphilophoroid mushrooms. Aphyllophoroid fungi are representatives of true fungi of the division Basidiomycota. For the vast majority of representatives of this group, the substrate is wood or living tissues of trees and shrubs (Safonov, 2003). Compared to cap mushrooms, the fruiting bodies of many species of aphyllophoroid fungi can exist for quite a long period of time, and their formation is less dependent on external moisture. These are wood-destroying fungi, which have the main role in the destruction of forest litter and, as a result of this process, the circulation of substances in nature. They clean the soil from numerous fallen leaves, needles, twigs, destroy tree stumps, dead, broken and wind-blown trunks, logs left in the forest at the place of felling. Most of them are not of nutritional interest, but may be of interest as a source of other biologically active substances. In recent decades, they have been actively studied from the point of view of application in medicine, as they synthesize a wide range of different biologically active compounds such as polysaccharides (Hamidi et al., 2022), enzymes (Daou et al., 2019), organic acids, triterpene and steroid substances (Kurchenko et al., 2022), antibiotics (Asgharpour et al., 2020).

Table 6 – Chemical composition of fruit bodies of *Mucidula múcida* according to GC-MS data.

Developmental stage	Total number of detected substances	The most important substances	% content
young mushroom	16	Undecylenic acid	-
		Dodecanoic (Lauric) acid	23.34
		Hexadecanoic (Palmitinic) acid	11.46
		9-Octadecenoic (Oleic) acid	37.52
		Octadecanoic (stearic) acid	4.88
		9,12-Octadecadienoic (Linoleic) acid	15.78
		Squalene	4.24
ripe mushroom	26	Undecylenic acid	3.90
		Dodecanoic (Lauric) acid	11.64
		Hexadecanoic (Palmitinic) acid	10.50
		9-Octadecenoic (Oleic) acid (Z)	22.77
		9,12-Octadecadienoic (Linoleic) acid	15.38
		Octadecanoic (Steric) acid	4.24
		Eicosane	3.67
		Docosane +Tricosane + Heptadecane	7.40
		Squalene	3.01
overripe mushroom	18	Undecylenic acid	1.78
		Dodecanoic (Lauric) acid	15.91
		Hexadecanoic (Palmitinic) acid	11.41
		9-Octadecenoic (Oleic) acid (Z)	45.52
		9,12-Octadecadienoic (Linoleic) acid	-
		Octadecanoic (Steric) acid	5.24
		Eicosane	4.35
		Docosane +Tricosane + Heptadecane	3.99
		Squalene	1.78

The finding of squalene in some representatives of this group of mushrooms in quantities greater than in most vegetable oils opens up the prospects of using this raw material for its production. As our research has shown, squalene can be obtained from this raw material using a simple method. This allows you to save valuable amaranth oil and use low-value aphyllophoroid mushrooms that can be cultivated on dead wood. Today, the technology of cultivating a number of mushrooms that have food or medicinal uses on the trunks of dead trees is well developed, in particular, oyster mushroom (*P. ostreatus*) (Landínez-Torres et al., 2021), *Auricularia auricula-judae* (Regis and Geösel, 2024), shiitake (*Lentinula edodes*) (Kobayashi et al., 2020). Therefore, if necessary, this cultivation technology can be used to develop a technology for growing other wood-destroying fungi.

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