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Biologically Active Aldehydes in Extracts of *Lactarius Pergamenus (Fr.) Fr* Fresh Fruiting Bodies

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The biologically active aldehydes in extracts of fungi of the genus Lactarius were identified. It's established that these substances are unstable, they are found in fresh and frozen fungi, but are absent in dried mushrooms and interact with 1,4-phenylenediamine to form a colored compound. Methylene chloride is the best extragent for these substances. TLC on silufol plates showed that there were several substances in Lactarius pergamenus fruiting bodies and they had varying degrees of stability. For selection of these substances, methylene chloride extract was separated on a column of silica gel. Fraction, which gave the most expressive reaction with 1,4-phenylenediamine on thin-layer chromatograms were analyzed by GC-MS both in the absence and in the presence of 1,4-phenylenediamine. As a result, it was found that 1,4-phenylenediamine or other aromatic amines interacted with highly active aldehydes, that were present in fruiting bodies. Among them 2,2-dimethylocta-3,4-dienal was the most stable and was present in the biggest quantity. This substance very rarely occurs in the vegetable kingdom and in fungi extracts of Lactarius genus wasn't previously described. Its possible function in fungi is prevention of damage by parasites and eating by animals.

Keywords: *Lactarius pergamenus (Fr.) Fr*, aldehydes, identification, 1,4-phenylenediamine, gas chromatography-mass spectrometry, 2.2 dimethylocta-3,4-dienal

Біологічно активні альдегіди в екстрактах свіжих плодових тіл *Lactarius pergamenus (Fr.) Fr*

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Метою дослідження було виявлення біологічно активних альдегідів в екстрактах грибів роду Lactarius. Встановлено, що ці речовини нестабільні, вони містяться у свіжих та заморожених, але відсутні у сушених грибах та взаємодіють із 1,4-фенілендіаміном, утворюючи кольорову сполуку. Метиленхлорид - найкращий екстрагент для цих речовин. ТШХ на пластинках з силуфолу показала, що в плодових тілах Lactarius pergamenus є кілька таких речовин і вони мають різний ступінь стійкості. Для відбору цих речовин метиленхлоридний екстракт розділяли на колонці з силікагелем. Фракцію, яка дала найбільш виражену реакцію з 1,4-фенілендіаміном на тонкошарових хроматограмах, аналізували ГХ-МС у відсутності та у присутності 1,4-фенілендіаміну. В результаті було встановлено, що 1,4-фенілендіамін або інші ароматичні аміни взаємодіють з високоактивними альдегідами, які були присутні у плодових тілах. Серед них 2,2-диметилокта-3,4-діеналь був найбільш стійким і у найбільшій кількості. Ця речовина дуже рідко зустрічається у рослинному царстві, а у екстрактах грибів роду Lactarius раніше не описана. Імовірна його функція у грибах - це запобігання ураження паразитами та поїдання тваринами.

Ключові слова: *Lactarius pergamenus (Fr.) Fr*, альдегіди, ідентифікація, 1,4-фенілендіамін, газова хроматографія-мас-спектрометрія, 2,2-диметилокта-3,4-діеналь

Polyphenol oxidase (tyrosinase, 1.10.3. PPO) is a metalloprotein that contains copper in its molecule. In the presence of oxygen, this enzyme is able to oxidize the ortho-diphenols to the corresponding quinones. PPO is widely distributed in animal tissues, plants, fungus and bacteria. It is responsible for melanin production in animals and causes browning of damaged plant tissues and plant juice in air [1]. The physiological function of PPO in plants and fungus still remains unclear. It is believed that the enzyme can perform the protective function in embryophytes and fungi. It is proved that o-quinones formed by the enzymatic action protect plants against pathogens and insects. In most basidiomycotic fungi, the activity of PPO is insignificant. However, it was found that in 7 of 8 species of fungi of the genus *Lactarius* it is high [2]. These fungi genus differ from the other by the presence of milky juice which protects them from being eaten by insects, molluscs and warm-blooded animals, as well as protects against microorganisms and other fungi. Some species of *Lactarius,* in particular *Lactarius pergamenus*, contain a milky juice with a very pungent taste that deters animals. Possibly, the high level of PPO is a component of protection system against pathogens and insects, furthermore the phenolic compounds (which are inherent to plants) were not found in these fungi. There are studies that suggest that *Lactarius* fungi exude this enzyme into the environment [3].

At the investigation of polyphenol oxidase activity in extracts of plants commonly used method of determining, based on the fact that this enzyme in the presence of molecular oxygen oxidizes o-diphenol (e.g., catechol) to the quinone, which then reacts with 1,4-phenylenediamine to give a compound, the color intensity of which is measured colorimetrically or spectrophotometrically [4].

The content of the enzyme in the extract is proportional to the rate of formation of color. Determination of polyphenol oxidase can be carried out in the absence of 1,4-phenylenediamine, watching just catechol oxidation, but then significantly reduced sensitivities of the reaction using this method.

At the investigating of polyphenol oxidase activity in extracts of fungi fruiting bodies of the genus *Lactarius*, particularly in *Lactarius pergamenus, L. quetus* and *L. vellereus* we marked that the substances of these extracts themselves can interact with 1,4-phenylenediamine to form a compound painted in orange color [5]. Because of this fact the use of this technique is unacceptable for the determination of the enzyme activity. A similar reaction analogue of 1,4-phenylenediamine also gives a close substance - o-dianisidine (3.3-dimetoxybensidine), but color is developing faster and it is more intense. It can be notes, that 1,4-phenylenediamine or o-dianisidine is also used in determining of amino oxidase [6, 7] and therefore for detection of mushroom's aminooxidase in the above method as such can not be used.

Previously, we have investigated the methanol and methylene chloride extracts from fresh and dried of Lactarius pergamenus fruit bodies by gas-chromatography–mass spectrometry (GC/MS), thin-layer chromatography (TLC), and column chromatography on silica gel. It has been shown that fresh extracts of the fruiting bodies of *Lactarius pergamenus* are rich in very labile compounds, the main ones being aldehydes [8].

These substances disappear quickly when mushrooms were dried and its components of latex contains ingredients that protect fruiting bodies from damage by parasites and from eating by animals.

The aim of this work was to identify biologically active aldehydes contained in extracts of fungi of the genus *Lactarius* and test the possibility of using for this purpose the reaction with 1,4-phenylenediamine.

Materials and methods

Materials. Mushrooms fruiting bodies of *Lactarius* species were collected in the mixed forest in the Skole district, Lviv region. Gathered mushrooms were brought to a lab for investigation during 24 h. Reagents used in the work were analytically pure.

Extraction conditions. 520 g of frozen *L. pergamenus* mushrooms (stored for one month in the refrigerator at -18 °C) were defreezed, crushed and homogenized in a blender with 1.0 liter of water. To the obtained homogenate 500 ml of methylene chloride was added and the mixture was stirred for 18 hours. The resulting mixture was centrifugated, the bottom layer of organic solvent was separated, filtered through anhydrous sodium sulfate. The solvent evaporated to a volume of ≈ 20 ml, methylene chloride residue was evaporated in a drying oven at +45 °C. 1.0 g of obtained extract was dissolved in 5.0 ml of hexane. The solution was placed in a refrigerator for the freezing of stearic acid. The supernatant were applied onto chromatography column (silica gel, height 15 cm, diameter 29 cm, L40/160). After that, the substances from column were subsequently eluted with the following solvents:

- Hexane: ethyl acetate -8: 1 (90 ml);
- Hexane: ethyl acetate: methanol - 4: 2: 1 (70 ml);
- Hexane: ethyl acetate: methanol- 2: 1: 2 (50 ml);
- Methanol (50 ml).

Fractions of 1.5 ml volume from the column were collected in pre-weighed tubes. They were analyzed by the reaction with 1,4-phenylenediamine, then they were evaporated and weighed. After that, graph of the dependence of mass from member of a fraction was built.

The fractions which gave a strong color reaction with 1,4-phenilendiamine were combined and subjected to repeated column chromatography on silica gel (height 8.2 cm, 0.8 cm diameter, L40/160), substances were subsequently eluted following mixtures of solvents:

- Benzene (10 ml)
- Benzene: acetic acid - 20: 1 (20 ml)

As in the previous chromatography, fractions from the column was collected in pre-weighed tube volume of 1.5 ml, which were analyzed by reaction with 1,4-phenylenediamine, then evaporated and weighed. Then, graph of the dependence of mass from position of fractions were built

Gas chromatography-mass spectrometry assay. For identification of a compound that is oxidized by polyphenol oxidase in *Lactarius pergamenus* extracts, extract obtained from a fresh mushroom was divided into two portions. To the first portion of the extract immediately after receiving three volumes of methanol were added (for precipitation of polyphenol oxidase, proteins and other compounds), and the second portion of the extract was left at room temperature during 24 and 72 hours for oxidation of substances by enzyme, that contained in the extract. Three volumes of methanol was added to these portions after 24 and 72 hours. Precipitates were removed by centrifugation, and the obtained in supernatant matter was studied using gas-chromatography-mass spectrometry. One microliter injections were applied to the HP-5 column (30 m length and 0.25 mm ID), filled with of 5% phenyl, 95% dimethylpolysiloxane stationary phase. Helium was used as gas carrier at 1.5 ml/min flow rate. The column was washed with methanol. The GC oven program was isothermal at 75°C, then a ramp at 15°C /min to 300°C, then again isothermal at 300 °C for 8 min. The mass-selective detector interface temperature was set at 250°C. The ion source was operated by electron ionization, the ionizing energy was 70 eV, ion source temperature 230°C, quadrupole temperature 150°C. The mass spectrometer was operated in scan mode. The identification of compounds in samples was performed using the GC-MS libraries.

The evaluation of the burning taste. The evaluation of the burning taste was carried out organoleptically. For this purpose, small pieces of fresh, frozen and dried fruit bodies were applied to the tip of the tongue. After 2-3 seconds, a burning taste was recorded, which was assessed on a three-point scale (0 - no sensation, 1 - weak sensation, 2 - medium sensation, 3 - strong sensation).

Evaluation of the burning taste of substances on chromatograms from fresh mushrooms was carried out after viewing TLC in UV light and marking the light spots with a pencil. Burning taste was determined by applying the tongue to them.

Results and Discussion

For purification substances of aldehyde nature that interact with 1,4-phenilendiamine, collected fruiting bodies were dried in a desiccator at +52°C, frozen at -18°C and used freshly. These examples were extracted by water and organic solvents (methanol and methylene chloride). To the resulting extract was added solution of 1,4-phenylenediamine and after 10 min extinction was measured at 400 nm. It was found that the reaction of extracts of fresh mushrooms was the most intense, and reaction from dried mushrooms extracts with 1,4-phenylenediamine was not observed. Consequently, these substances are unstable, and obviously they do not withstand heat or easily oxidized by atmospheric oxygen. It is also shown that methylene chloride extracted most of these substances, although aqueous extracts of fresh mushrooms also give a reaction. Therefore as, the main method of treatment was selected extraction with methylene chloride, was chosen as the main object of study peppery milk cap (*Lactarius pergamenus*).

After extraction the solvent was evaporated and substances were analyzed by chromatography on Silufol plates (Fig. 1).

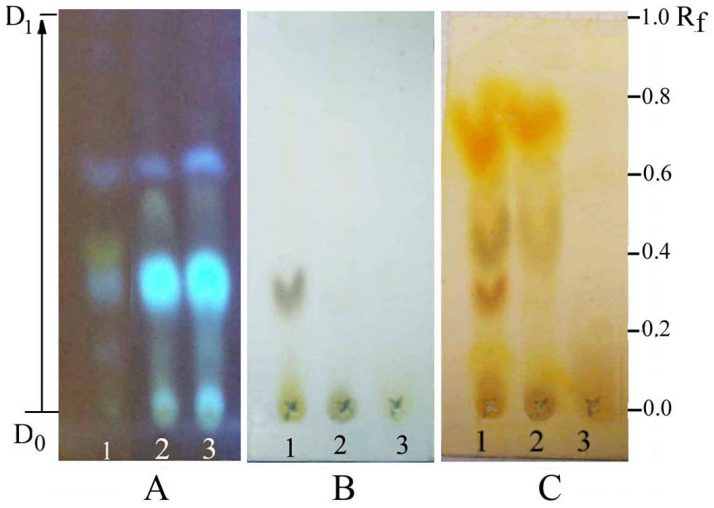
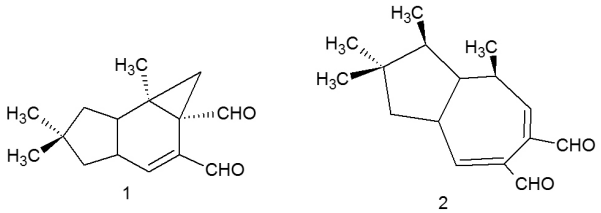
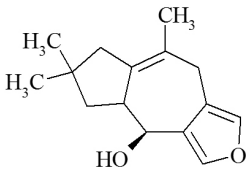


Fig. 1. TLC of the extracts (**1** – fresh mushrooms with methylene chloride; **2** – fresh mushrooms with methanol and petroleum ether; **3** – dried mushroom pomace with methylene chloride) isolated from *L. pergamenus* fruit bodies Hexane : ethyl acetate (8:1) solvent system. (**A** - UV light; **B** - visible light 22 h after chromatography; **C** - after treatment with 0.3 % o-dianisidine solution).

As can be seen from the chromatograms the largest number of substances that interact with 1,4-phenylenediamine is detected in extracts obtained from fresh mushrooms. In frozen mushrooms such substances are less and in dried mushrooms these substances are absent or almost absent. Fresh *L. pergamenus* fruiting bodies have a burning taste. It should be explained release of very burning taste sesquiterpene dialdehide isovelleral (1) and velleral (2) from stearic acid esters if mushrooms are injured [9]. These dialdehides possess high antimicrobial, cytotoxic and antifeedant activity.





In their oxidation in air less active sesquiterpenes are produced. In particular, the substance of yellow-green fluorescence (Fig. 1A track 2 and 3, R_f = 0.28), which we have identified as 3,14,15-trymetylfuranolactaran-8-ol (3) [10]. This substance (3) is much more stable, but its biological activity is weaker [10]. In frozen mushrooms the dialdehides (1 and 2) are absent because have not burning taste. Some changes can be also seen in the chromatogram. Thus, in Fig. 1B (track 1, R_f = 0.28), the substance obtained by chromatography of the extract from fresh fruiting bodies, after 22 hours of exposure of chromatograms on air, is oxidized and the formation of substances that acquire a black color on the chromatographic plate. Such substances are absent on the chromatograms of extracts from frozen and dried extracts of *L. pergamenus* (Fig. 1B, tracks 2 and 3). This substance is aldehyde and interacts with 1,4-phenylenediamine and o-dianazidine to form a compound colored brown (Fig. 1C track 1, R_f = 0.28).

Most likely, the other two compounds detected on chromatograms by spraying 1,4-phenylenediamine also have aldehyde nature, but they are more stable and can withstand freezing fruiting bodies of *L. pergamenus*. Among them, in our opinion, the most stable substance is one that have the highest Rf (Fig. 1C track 2, R_f = 0.75).

To establish the chemical nature of this substance we used mushrooms frozen at -18 °C. Then, fruiting bodies were unfreezed and extracted by methylene chloride as described previously (see section Materials and Methods). After removing the solvent 1.917 g a substance was obtained from 520 g fruiting bodies with similar to the ointment consistency. For the purification 1.0 g of residue was used for chromatography analysis. Graph of the dependence of mass from position of fractions is shown in Fig. 2.

Fractions, that give color reaction with 1,4-phenylenediamine (NoNo 35-48), were combined and then applied to a column of silica gel and re-chromatographed on silica gel in the system of benzene: acetic acid - 20 : 1 (Fig. 3).

For further research fraction (No 7) was selected. This fraction given a color reaction without additional spots accordance TLC.

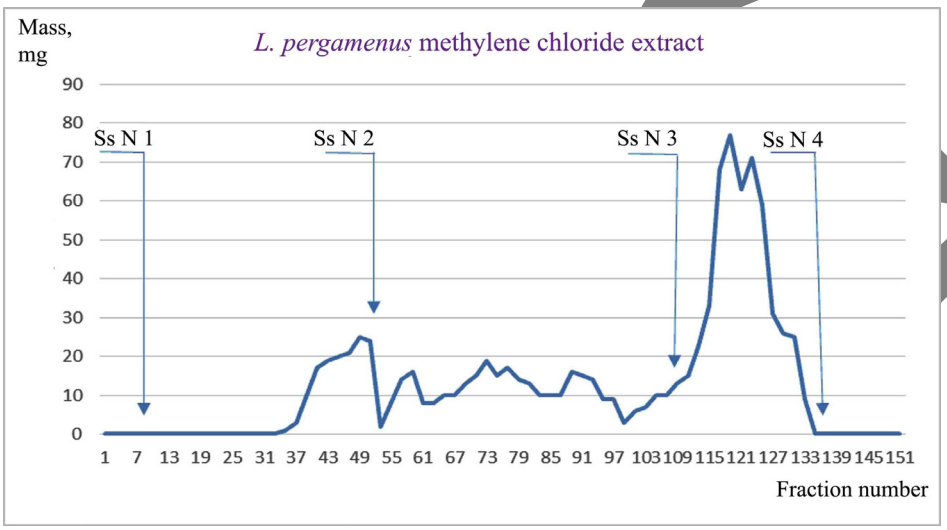


Fig. 2. Fractionation of methylene chloride extract *L. pergamenus* on silica gel column (Ss (Solvent system) N 1 - hexane: ethyl acetate – 8 : 1; Ss N 2 - hexane: ethyl acetate: methanol - 4: 2: 1; Ss N 3 - hexane: ethyl acetate: methanol - 2: 1: 2; Ss N 4 - methanol.

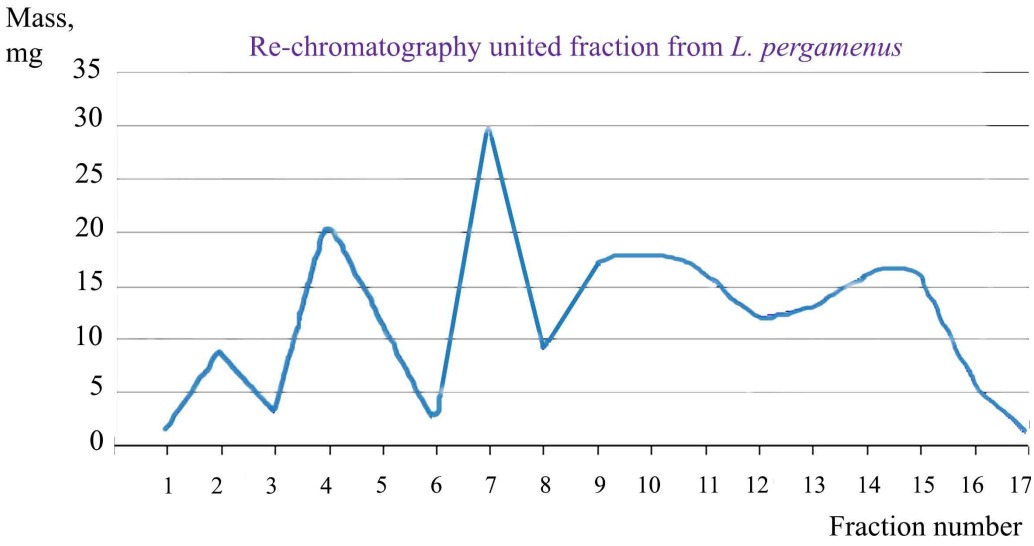


Fig. 3. Re-chromatography of causes united fraction from *L. pergamenus* on silica gel. Solvent system - hexane: ethyl acetate - 8: 1, volume fractions - 1.5 ml.

We proceeded from the assumption that if 1,4-phenylenediamine reacts with the compounds of methanol solution, it should lead to their reduction in the solution after the reaction. It can be fixed by gas chromatography with mass spectrometry detector.

So, No 7 fraction was dissolved in methanol to a concentration of 10 mg/ml and was divided into two parts. To the first portion (0.7 ml) 0.3 ml of 0.4% solution of 1,4-phenylenediamine was added, stirred and after 4 hours analyzed by GC-MS. To the second part, which was used for comparison, nothing was added and it also was analyzed by GC-MS. 0.4% solution of 1,4-phenylenediamine was used as control. The results of the analysis are presented in Table 1.

So, 2,2-dimethylocta-3,4-dienal is the most likely candidate to interact with 1,4-phenylenediamine to form colored in orange color substance. Taking into consideration that not enough degree of reliability of identification of this compound and the absence of such a substance in plants and fungi, it was decided to confirm its identity by means of TLC. In the catalogs of Western companies that produce chemical reagents 2,2-dimethylocta-3,4-dienal was missing.

In plants 2,2-dimethylocta-3,4-dienal occur extremely rare. We have found in the literature that such a compound is present in microquantity in the essential oil extracted from the African plant Cymbopogon citratus [11].

Table 1. Substances found in *L. pergamenus* methanol extract with and without the presence of 1,4-phenylenediamine.

Methanol extract of purified fraction			Methanol extract of purified fraction + 1,4-phenylenediamine		
Compound	**Precision (%)**	**Content (%)**	**Compound**	**Precision (%)**	**Content (%)**
Cyclodeca[b]furan-2(3H)-one	38	2.65	1,4-phenylenediamine	97	47.9
2-cyano-l-methylthio-2-(4-pyridiny1)vinylacetamidine	58	11.85	2-cyano-l-methylthio-2-(4-pyridiny1)vinylacetamidine	58	7.01
2,2-Dimethylocta-3,4-dienal	79	51.21	4-Cyano-3-methoxy-l,5-dimethyl-5,6–dihydroisoquinoline	72	25.18
Dibutyl phtalate	83	2.40	Dibutyl phtalate	83	1.40
Ethyl oleate	99	12.55	Ethyl oleate	99	5.61
Ethyl stearate	99	10.21	Ethyl stearate	99	4.63
Gibberellin A3	35	1.10	Gibberellin A3	47	0.41
Total:		91.97	Total:		92.14

Notes: Precision was determined as % resemblance to the substance of comparison. Certain substances with their content below 1% are not included in the table.

In plants quite common is 3,7-dimethylocta-2,6-dienal that exists in two isomers: E-isomer, known as geranial (citral A) and Z-isomer is known as neral (citral B). They are the main component of plant essential oils of citrus family (lemon, orange). In the essential oil of lemon balm drug present citronellal. All these substances could to react with 1,4-phenylenediamine to form colored compounds.

To test this hypothesis chromatography of these three substances on the silica plate with subsequent its detection by 0.3 % alcohol solution of 1,4-phenylenediamine and o-dianisidine was performed. After 30 minutes the appearance of colored spots was watched (Fig. 4).

In the B and C chromatogram numbers are marked: 1 – citral; 2 - citronellal; 3 - purified by chromatography aldehyde components of hexane fraction *Lactarius pergamenus* fruiting bodies*;* 4 - anise aldehyde; 5 – vaniline.

The chemical structures of the analyzed compounds are the following are shown in Fig. 5.

All the tested aldehydes react with 1,4-phenylenediamine and o-dianisidine to form colored compounds. The products of reaction exhibit different colors and R_f values in chromatograms. Citronellal gives a bright pink color with 1,4-phenylenediamine and green with o-dianisidine and citral and compound contained in fruiting bodies of *Lactarius pergamenus* gives a yellow-orange color, but are obviously different substances. They differ in R_f in chromatograms, color of stained compounds, resistance to air and odor. Unlike substances of *Lactarius pergamenus* fruiting bodies, citral has a strong odor and is much more resistant to oxidation in air.

A strong burning taste is characteristic only of fresh fruiting bodies of *Lactarius pergamenus*. Dried fruiting bodies of *Lactarius pergamenus* are completely devoid of burning taste. Frozen fruit bodies have a very low of burning taste. On the chromatograms of extracts from fresh mushrooms the substances with R_f = 0.3 and R_f = 0.4 had the greatest of burning taste (Fig.1, A-1).

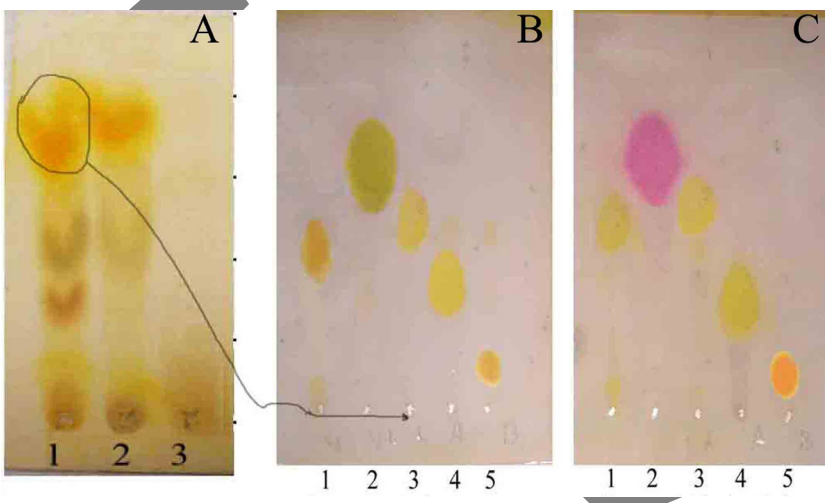


Fig. 4. TLC on Silufol plates of the methylene chloride extracts (A) isolated from *L. pergamenus* fruit bodies and purified fraction (B, C). B - detection of 0.3 % o-dianisidine solution, C - detection of 0.3 %1,4-phenylenediamine solution.

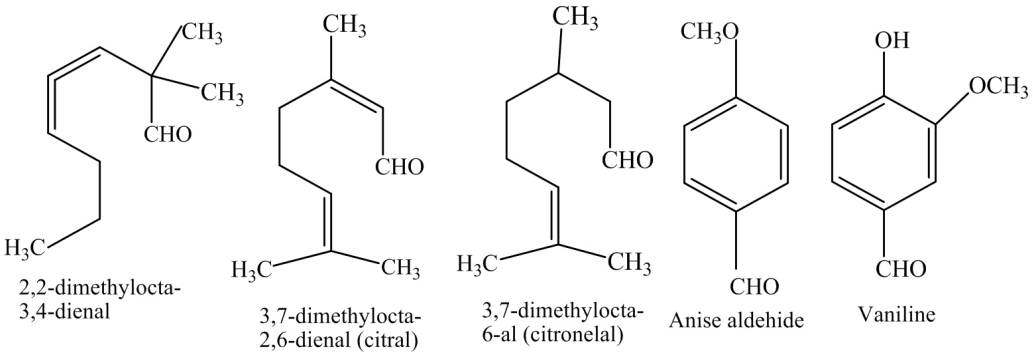


Fig. 5. The chemical structures of the analyzed compounds.

Conclusions

Fruiting bodies of *Lactarius pergamenus* contain several different aldehide structures able to interact with aromatic amines. These compounds are instabile in the air. Reaction with 1,4-phenylenediamine or o-dianisidine could be used for their detection. As these substances are present, detection and quantification of polyphenol oxydase, based on the oxidation of ortho-diphenoles should be done without the use of aromatic amines. Comparative study of the most common plant aldehydes and the substances derived from fruiting bodies of *Lactarius pergamenus* indicates that - the substances are different. The content of 2,2-dimethylocta-3,4-dienal (with a high degree of authenticity) was the highest among the aldehydes contained in the fruiting bodies of *Lactarius pergamenus*. Unlike citral and citronellal which are widely distributed in the plant world, 2,2-dimethyloc-ta-3,4-dienal occurs rarely, and among real mush-rooms hasn't been described.

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