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Toxicologically Significant Properties of Fly Agarics, and Chemicotoxicological Analysis in Poisoning Cases: a Review

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ABSTRACT

Every year the number of poisonings by *Amanita* (fly agaric), specifically red fly agaric (*A. muscaria*) and panther fly agaric (*A. pantherina*) increases. These species contain substances affecting the central nervous system, particularly muscimol, ibotenic acid, and muscarine. Ibotenic acid and muscimol are water-soluble isoxazole derivatives. They exert antagonistic effects on the central nervous system, stimulating and depressing it through inotropic glutamate receptors, which selectively bind N-methyl-D-aspartate, and gamma-aminobutyric acid receptors. The combined action of isoxazoles and other compounds of the fungus leads to the development of mycoatropin or pantherin syndromes. Muscimol is the most toxicologically significant, as it is capable of exerting a strong psychodysleptic effect, as well as causing disorder of consciousness up to the development of coma. Ibotenic acid is of equal importance in establishing the fact of fly agaric use, but in many cases it is almost completely converted to muscimol in the body. At this stage, active development of methods is underway to diagnose fly agaric poisoning: qualitative and quantitative determination of ibotenic acid derivatives in biological fluids (blood plasma and urine).

This review includes morphological features of red and panther fly agaric, their chemical composition and mechanisms of action of toxicologically significant compounds, types of qualitative and quantitative analysis, and symptoms of poisoning.

There are various methods to determine the etiology of poisoning, including polymerase chain reaction, micro- and macroscopy, but they do not allow determining the exact amount of toxicants correlating with the severity of poisoning. Precise physico-chemical methods such as chromatography and electrophoresis, which require multi-step sample preparation, are applicable for these purposes. Isolation from biological fluids or fruit bodies is accomplished by single-step or multi-step liquid-liquid or solid-phase extraction. The universal and most common extractant is 75% methanol. For qualitative analysis, thin layer chromatography with different solvent systems can be used. However, this analysis is non-specific and can be used in the preliminary phase of the study because the detectors used are group-wide. Gas and high-performance liquid chromatography are used for quantitative determination. These methods are highly precise; however, they require sample preparation. An alternative to chromatography is electrophoresis, an express method for the separation of muscimol and ibotenic acid.

Keywords: *Amanita*; mycoatropin syndrome; pantherin syndrome; muscimol; ibotenic acid; high-performance liquid chromatography; capillary electrophoresis; review.

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Токсикологически значимые свойства мухоморов и химико-токсикологический анализ при отравлениях: научный обзор

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АННОТАЦИЯ

С каждым годом возрастает число отравлений грибами рода *Amanita* (мухомор), особенно видами мухомор красный (*A. muscaria*) и мухомор пантерный (*A. pantherina*). Данные виды содержат вещества, влияющие на деятельность центральной нервной системы, в частности мусцимол, иботеновая кислота, мускарин. Иботеновая кислота и мусцимол — водорастворимые производные изоксазола. Они оказывают антагонистическое действие на центральную нервную систему, стимулируя и угнетая её через инотропные рецепторы глутамата, селективно связывающие N-метил-D-аспартат, и рецепторы γ-аминомасляной кислоты соответственно. Сочетанное действие изоксазолов и других соединений гриба приводит к развитию микоатропинового или пантериновового синдрома. Мусцимол является наиболее токсикологически значимым, поскольку способен оказывать сильный психодислептический эффект, а также вызывать угнетение сознания вплоть до развития комы. Иботеновая кислота имеет не меньшее значение при установлении факта употребления мухоморов, но во многих случаях она практически полностью преобразуется в мусцимол в организме. На данном этапе ведётся активная разработка методик, которые бы позволили диагностировать отравления мухоморами: качественно и количественно определять производные иботеновой кислоты в биологических жидкостях (плазме крови и моче).

В данном обзоре рассмотрены морфологические особенности мухомора красного и пантерного, их химический состав и механизмы действия токсикологически значимых соединений, варианты качественного и количественного анализа, а также клиническая картина отравления.

Существуют различные методы определения этиологии отравления — полимеразная цепная реакция, микро- и макроскопия, однако они не позволяют определить точное количество токсикантов, коррелирующее с тяжестью отравления. Для данных целей подходят точные физико-химические методы, такие как хроматография и электрофорез, требующие проведение многоэтапной пробоподготовки. Изолирование из биологических жидкостей или плодовых тел происходит с помощью одноэтапной или многоэтапной жидкость-жидкостной или твердофазной экстракции. Универсальным и самым распространённым экстрагентом является 75% метанол. Для качественного анализа возможно применение тонкослойной хроматографии с различными системами растворителей. Однако такой анализ неспецифичен и его можно применять на предварительном этапе исследования, поскольку используемые детекторы являются общегрупповыми. Для количественного определения применяют газовую и высокоэффективную жидкостную хроматографию. Это очень точные, но требующие пробоподготовки методы. Альтернативой хроматографии является электрофорез — экспрессный метод разделения мусцимола и иботеновой кислоты.

Ключевые слова: *Amanita*; микоатропиновый синдром; пантериновый синдром; мусцимол; иботеновая кислота; высокоэффективная жидкостная хроматография; капиллярный электрофорез; обзор.

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毒蝇伞毒理学相关特性和中毒时的化学毒理学分析：科学综述

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简介

*Amanita*菌（毒蝇伞）中毒，尤其是红色鹅膏菌（*A.muscaria*）和豹斑鹅膏（*A.pantherina*）中毒数量逐年增加。这些物种含有影响中枢神经系统活动的物质，特别是蝇蕈醇、鹅膏蕈氨酸和蝇蕈素。鹅膏蕈氨酸和蝇蕈醇是异恶唑的水溶性衍生物。它们对中枢神经系统具有拮抗作用，通过选择性绑定N-甲基-D-天冬氨酸的谷氨酸受体和γ受体（分别是氨基丁酸）刺激和抑制中枢神经系统。异恶唑和其他真菌化合物的联合作用导致毒蕈碱样症状或豹斑毒鹅膏菌中毒症状。蝇蕈醇在毒理学上最重要，因为它能够产生强烈影响，使精神错乱，并导致抑制意识直至昏迷。鹅膏蕈氨酸在确定毒蝇伞的摄入方面同样重要，但在很多情况下，它在体内几乎完全转化为蝇蕈醇。当前阶段，正在积极开发诊断毒蝇伞中毒的方法：定性和定量测定生物体液（血浆和尿液）中的鹅膏蕈氨酸衍生物。

本综述中介绍了红色鹅膏菌和豹斑鹅膏菌的形态特征、化学成分和毒理学重要化合物的作用机制、定性和定量分析的方案，以及中毒的临床情况。

确定中毒病因的方法有多种—聚合酶链反应、微观和宏观检查，但它们无法确定与中毒严重程度相关的毒物的确切数量。需要多级样品制备的精确物理化学方法，如色谱和电泳，适用于这些目的。通过单级或多级液-液或固相萃取，从生物液体或果实体中分离。通用且最常见的萃取剂是75%的甲醇。对于定性分析，可以使用不同溶剂系统的薄层色谱法。但是，因为所使用的探测器是通用型的，而这种分析属于非特异性的，只可以在研究的初步阶段使用。定量测定采用气相色谱法和高效液相色谱法。这是非常精确的，但需要样品制备的方法。替代色谱法的方法是电泳法，这是一种分离蝇蕈醇和鹅膏蕈氨酸的快速方法。

关键词： *Amanita*；毒蕈碱样症状；豹斑毒鹅膏菌中毒症状；蝇蕈醇；鹅膏蕈氨酸；高效液相色谱法；毛细管电泳；审查。

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INTRODUCTION

Every year, hallucinogenic mushroom poisoning attracts more and more attention from researchers and practitioners. *Amanita* poisoning is a particularly pressing issue. The Department of Acute Poisoning and Somatopsychiatric Disorders at the Sklifosovsky Institute for Emergency Medicine reports an increase in cases of *Amanita* poisoning over the past four years, reaching 260 cases, with 94 in 2023 and 55 in the first half of 2024 [1]. *A. muscaria* and *A. pantherina* are the two *Amanita* species responsible for the increase in the cases of poisoning.

Since ancient times, *Amanita* mushrooms have been used for their psychodysleptic properties to induce an altered state of consciousness for religious and shamanic purposes. *Amanita* mushrooms are used for various purposes, including their psychodysleptic effects, religious rituals, research, and therapy.

In addition, cases of accidental *Amanita* ingestion should be mentioned [2, 3]. Poisoning cases in pets are also reported [4]. This may be largely due to a lack of awareness about mushrooms and their effects, as well as the increasing popularity of microdosing [5].

Microdosing involves taking subthreshold doses of psychedelic substances that do not cause intoxication or altered consciousness, in order to enhance creativity and physical performance, promote emotional stability, and alleviate anxiety [6, 7].

The growing popularity of microdosing is evident from the increase in advertisements and online stores offering microdosing products. The number of search queries can indirectly suggest consumer demand for *Amanita*-based products. From 2022 to 2023, the number of Google searches for *A. muscaria* increased by 114%, and this trend persisted in 2024 [5].

Various *Amanita*-based products are available for microdosing, including packaged dried materials, powders, capsules, tinctures, extracts, and creams. The Russian market offers a variety of certified food additives. Manufacturers state that these products improve:

- Cognitive functions
- Sleep
- Anxiety
- Improved alertness
- Physical activity.

Some products are promoted as supplements for treating alcohol addiction and attention deficit hyperactivity disorder when antidepressants are not an option [5].

Therefore, it is crucial to develop and evaluate physical and chemical tests to confirm and assess the severity of *Amanita* poisoning in order to deliver prompt and appropriate medical treatment.

This article provides information on the morphology and toxicology of *Amanita*, as well as methods for identifying their toxins. This information will be used to develop a laboratory method for diagnosing mushroom poisoning.

AMANITA SPECIES

Fig. 1*a* illustrates the appearance of the fruiting bodies of *A. muscaria*.

- The cap ranges in size from 5 to 20 cm, sometimes reaching up to 50 cm.
- The cap begins as an almost spherical shape and then turns from convex to concave.
- The caps range in color from bright red to orange or nearly yellow.
- Warts may be white or yellowish, or they may also be absent.
- The cap has the gill-bearing underside.
- The stem has warts near its basal bulb and a white ring that hangs down as the fungus grows.
- It smells and tastes pleasant.

A. muscaria is widespread in forests throughout Russia [8–10]. This ectomycorrhizal fungus forms a symbiotic relationship with both coniferous and deciduous trees [4].

Fig. 1*b* illustrates the appearance of the fruiting bodies of *A. pantherina*.

- The cap is round with a flat depression in the center.
- The cap is brown, often with a greenish tint, but is never reddish or yellowish.

Fig. 1. Appearance of fruiting bodies of mushrooms: *a,* red fly agaric (Amanita muscaria); *b,* panther fly agaric (Amanita pantherina).

- Warts are white or grayish in color and arranged in a concentric pattern.
- The stem is white and has a hanging ring that usually disappears quickly.
- A special, sack-shaped structure called a volva is located at the base of the stem.
- The fungus is tasteless and smells like raw potatoes.

A. pantherina is widespread in the northern and temperate zones of Russia, as well as in mountain forests [8–10].

The circulation of *Amanita*-based products is legal in Russia, but some other countries regulate it, particularly its primary active ingredients: muscimol and ibotenic acid. The collection and ingestion of *A. muscaria* are illegal in Romania, the Netherlands, Taiwan and the US state of Louisiana. However, the collection and ingestion of *A. pantherina* are not regulated anywhere. Australia regulates the circulation of muscimol, but not mushrooms [5, 9].

CHEMICAL COMPOSITION

The quantity of active substances present in a fruiting body depends on the following factors:

- Geographic race
- Season
- Phases and conditions of growth
- Processing and storage options [9].

The two toxicologically significant components of *Amanita* mushrooms are ibotenic acid (Fig. 2*a*) and muscimol (Fig. 2*b*). Both are isoxazole derivatives. The highest concentrations of ibotenic acid and muscimol are found in the cap, followed by the volva, then the stem. These substances have relatively high boiling points of 152°C and 175°C, respectively. The acid dissociation constant (pKa) is 4.8 and 8.4 for muscimol [11] and 3.0, 5.0, and 8.2 for ibotenic acid [12], which indicates that ibotenic acid is more acidic. Pure substances are crystalline and freely soluble in water. In addition, they can pass through the blood-brain barrier by active transporters present in the brain [8]. In an aqueous medium, ibotenic acid forms a zwitterion,[1] hindering sample preparation and chromatographic analysis [11]. Ibotenic acid is unstable when exposed to chemicals (including in the gastrointestinal tract), light, or temperature changes because it converts into muscimol, a primary amine, or the decarboxylated form, when dehydrated (Fig. 2*b*) [8–10, 13].

Amanita mushrooms contain another derivative of isoxazole called muscazone. It forms when ultraviolet rays act on muscimol, thereby intensifying its hallucinogenic properties. This explains why *Amanita* mushrooms collected in sunny meadows have a stronger psychodysleptic effect than those grown in shaded areas [5].

In addition, *Amanita* mushrooms contain tricholomacridic acid, stizolobic scid, stizolobinic acid, and methyl-tetrahydro-carboline-carboxylic (MTC) acid, as well as various alkaloids [8, 9, 14–16]. Stizolobic and stizolobinic acids are amino acids of non-protein origin, oxidation products of L-dihydroxyphenylalanine (3,4-dihydroxyphenylalanine). They are most often found in the fruiting bodies of *Amanita pantherina*. These amino acids are found in trace amounts in *A. muscaria* mushrooms, especially those with yellowish caps. In addition, extracts from the fruiting bodies of *A. muscaria* contain MTC with a structure similar to that of harmaline compounds [16]. The main alkaloids found in them are muscarine and nightshade alkaloids such as scopolamine, atropine, and hyoscyamine. The poisonous substance bufotenine, found in the skin of toads, is less common [5]. *Amanita* mushrooms can bioaccumulate certain metals in the form of organometallic compounds. This process can sometimes increase their toxicity. For example, in *Amanita* muscarica, vanadium accumulates in the form of amavadin [3].

CLINICAL PICTURE OF POISONING

The effects of different species, or even populations of the same species, may vary depending on the substances they contain. Therefore, in Russia, the effects of *Amanita* species can be classified as either mycoatropin syndrome or pantherin syndrome [9, 10]. Table 1 shows the main clinical features of poisoning from various species of *Amanita*.

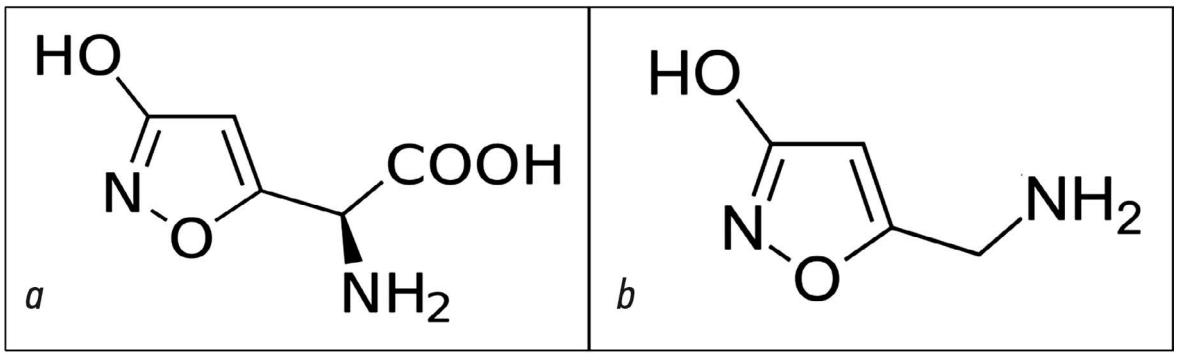

Fig. 2. Structural formulas of isoxazole derivatives: *a,* ibotenic acid; *b,* muscimol.

[1] A zwitterion is a molecule that is usually electrically neutral, yet contains functional groups that carry both positive and negative charges.

Table 1. Main syndromes appearing in case of poisoning by different species of genus *Amanita* mushrooms and their characteristics

Mycoatropin	Pantherin
No clear presentation of poisoning; it can easily be confused with other conditions, such as a stroke, alcohol intoxication, or, less often, gastroenteritis.	
Amanita species	
• *A. muscaria* • *A. regalis* • *A. gemmata* • *A. strobiliformis*	• *A. pantherina*
Main substances	
• Ibotenic acid • Muscimol • Muscazone	• Ibotenic acid • Muscimol • Muscazone • Nightshade alkaloids: scopolamine and atropine
Characteristics	
• Higher content of ibotenic acid • Predominance of CNS excitation over depression • Severe confusion, agitation	• A 3–4 times stronger effect • Higher content of muscimol • Higher content of nightshade alkaloids • Predominance of depression over excitation • Coma is common

Mycoatropin syndrome is a complex of symptoms that are similar to the effects of atropine and caused by ibotenic acid derivatives found in mushrooms. This syndrome is caused by consuming the following species:

- *A. muscaria*
- *A. regalis*
- *A. gemmata*
- *A. strobiliformis* [8, 9].

Pantherin syndrome is a similar condition caused by ingestion of *A. pantherina*. Both syndromes develop within 15–30 minutes of *Amanita* ingestion due to the action of isoxazole derivatives and small amounts of alkaloids. This type of poisoning has no specific clinical presentation or typical symptoms [17]. In contrast to acute intoxication with other poisonous mushrooms, signs of toxic gastroenteropathy syndrome and gastroenteritis are not consistently observed in cases of *Amanita* poisoning, even during autopsy [16, 18]. Therefore, clinics often misdiagnose conditions such as alcohol intoxication and cerebrovascular disease [17]. However, pantherin syndrome is characterized by the predominance of central nervous system (CNS) depression, whereas mycoatropin syndrome is characterized by the predominance of CNS excitation. This is due to the high levels of ibotenic acid found in *A. muscaria*, *A. regalis*, *A. gemmata* and *A. strobiliformis*, and the high levels of muscimol found in *A. pantherina* [14, 17]. There is no specific antidote. In case of poisoning, only supportive and symptomatic therapy can be provided [3].

Amanita species that cause mycoatropin syndrome, contain the main psychodysleptic component called ibotenic acid. Ibotenic acid is a glutamate analog that can stimulate various types of glutamate receptor. Its primary target is ionotropic glutamate receptors that bind selectively to N-methyl-D-aspartate (NMDA). Their stimulation activates the nervous system [8, 15]. This is why ibotenic acid is used in rat models of Alzheimer disease [3, 12].

The first stage of the excitation phase is characterized by relatively harmless symptoms:

- Strong desire to be active
- Excitement
- Assertiveness
- Vitality
- Improved alertness [2, 17]

All of these manifestations are associated with the ability of ibotenic acid to increase serotonin levels. This is similar to the effects of lysergic acid diethylamide (LSD) [3].

The second phase manifests as follows:

- Severe psychomotor agitation with convulsions, which often develop in children [19], and ataxia
- Emotional arousal up to the point of hysteria
- Illusions such as micropsia and macropsia, as well as blurred vision and blurred outlines of objects (as in a migraine aura)
- Hallucinations involving visual, auditory, and gustatory [2, 17]

The excitement ends in the third phase, when hallucinations worsen and loss of orientation and coordination develop. The hallucinogenic effect is also caused by the effects of MTC on the CNS [16]. A patient becomes disoriented in space, time, and person. CNS depression eventually develops [2, 17].

CNS depression is mainly caused by the effects of muscimol. Muscimol is structurally similar to gamma-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the CNS. Therefore, muscimol interacts with $GABA_A$ receptors, and partially with $GABA_C$ receptors [8, 15]. At the same time, it provides relatively long-lasting suppression because, unlike GABA, it is not affected by GABA transaminases or their reuptake systems. In addition, CNS depression is largely the result of muscimol's ability to reduce catecholamine levels in the CNS. In contrast, ibotenic acid increases catecholamine levels [3]. This phase is characterized by:

- Depression of consciousness
- Lethargy
- Exhaustion
- Apathy
- Somnolence

The depression phase usually culminates in sopor, which is characterized by higher awakening thresholds and vivid, memorable dreams. Awakening is typically accompanied by retrograde amnesia [16]. In the case of pantherin syndrome, CNS depression prevails over excitation, and the depression phase is longer, often culminating in coma [2, 14, 17]. Ingesting a large amount of *A. pantherina* at once results in a rapid transition (within 30–60 min) from the initial stages to a coma. These phases may repeat and alternate many times [16].

These syndromes are also associated with:

- Mydriasis (less commonly miosis [16])
- Dyspnea
- Tachycardia (in the excitation phase) and bradycardia (in the depression phase)
- Salivation and lacrimation
- Excessive sweating
- Attacks of abdominal pain

Abdominal pain is associated with the effects of muscarine, which is present in very low amounts in both species of *Amanita*. Due to its poor oral absorption, however, this alkaloid plays a minimal role in toxicity [17]. The anticholinergic effects are primarily caused by stizolobic and stizolobinic acids [16]. Gastrointestinal spasms are primarily associated with the increased serotonin levels caused by muscimol [3]. In cases of severe poisoning, vomiting and diarrhea are possible [16].

Therefore, the clinical presentation varies greatly due to significant fluctuations in levels of ibotenic acid, muscimol, and other intoxicating substances. It is important to identify the most common symptoms of this syndrome when diagnosing [9, 16].

A single 50–70 gram fruiting body is sufficient to initiate nervous disorders. It may contain 0.015%–0.1% isoxazole derivatives (such as muscimol and ibotenic acid, at concentrations of approximately 6 mg and 30–60 mg, respectively) [3, 12, 16]. Notably, hallucinations occur with exposure to 10–15 mg of muscimol and 50–100 mg of ibotenic acid, respectively [11]. The most severe cases of poisoning are reported in children, older adults, and people with pre-existing health conditions [19, 20]. In most cases, however, poisoning from these *Amanita* species resolves favorably within 24 to 49 hours after the onset of symptoms [16]. The risk of death increases significantly when ingesting other *Amanita* species:

- *A. phalloides*
- *A. verna*
- *A. virosa* [21]

Fatal cases of poisoning from *A. muscaria* and *A. pantherina* are extremely rare. Most cases occur from acute poisoning after ingesting large quantities of the mushrooms [12, 16, 20], or after ingesting the mushrooms with tranquilizers, such as benzodiazepines [20]. Death occurs as a result of cardiac and respiratory arrest [22]. An autopsy does not reveal any typical pathological signs of *Amanita* poisoning [16]. In rats, the lethal dose (LD_{50}) of muscimol was found to be 4.5 mg/kg intravenously and 45 mg/kg orally.

Isoxazole derivatives are highly soluble in water, which results in the relatively rapid excretion of some unchanged compounds. Both ibotenic acid and muscimol can be detected in urine within one hour of ingestion. However, approximately 10%–20% of ibotenic acid is converted into muscimol when the mushrooms are digested in the acidic environment of the stomach [3, 12]. Ibotenic acid is also metabolized in the liver and brain [3].

TOXICOLOGICAL ANALYSIS

Currently, there are many ways to detect *Amanita* poisoning. Previously, one way to confirm ingestion of these mushrooms was to perform macro- and microscopic examination of stomach or intestinal contents for mushroom pieces and spores. However, microscopy is a labor-intensive method that is more appropriate for postmortem identification [4, 12]. These methods are prone to error because crushed mushroom tissues lose their specific morphological characteristics when exposed to gastric and intestinal secretions. This makes visual identification impossible. Activated carbon and other sorbents are often used for self-treatment when dyspeptic symptoms of poisoning appear. This complicates identification because the chyme is stained black.

In some cases, the etiology of poisoning was established using polymerase chain reaction. However, this only identified the species of mushroom and not the amount of toxicants that entered the body. Even when gene databases (e.g., GenBank[2]) and amino acid sequence search algorithms (e.g., BLAST[3]) are used, inaccurate species identification is possible because many fungi grow together (for example, molds can be found in chyme) [4].

It is necessary to identify the toxicants involved in order to determine the severity of the poisoning and monitor the effectiveness of the therapy [11]. Advanced physical and chemical techniques, such as chromatography and electrophoresis, are the most reasonable methods for the qualitative and quantitative determination of ibotenic acid and muscimol. A sample of the biological object should be prepared by purifying the analytes[4] from related substances in the biological matrix.

SAMPLE PREPARATION

Samples are prepared using extraction. Fresh fruiting bodies are crushed and dried samples are ground into a powder. Single-stage, single-phase extraction is primarily used to prepare samples for qualitative identification. Because of the high hydrophilicity of ibotenic acid and muscimol, various polar solvent systems are used for extraction, such as a mixture of methanol and water at 7:3 or a solution of 50% ethanol and 0.1% formic acid [15]. The use of 75% methanol

[2] GenBank® [Internet]. Bethesda (MD): National Library of Medicine (US); 2013–2024. Available at: https://www.ncbi.nlm.nih.gov/genbank/

[3] Basic local alignment search tool® [Internet]. (MD): National Library of Medicine (US); 1990–2024. Available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi Accessed on January 9, 2025.

[4] An analyte is a target chemical substance for analysis.

as a universal extractant is considered optimal because it inhibits decarboxylase activity, which stabilizes ibotenic acid and prevents its conversion to muscimol. For the same reason, samples of fresh mushrooms are stored in methanol at a ratio of 4 mL per gram of raw material. The resulting sample is then stored at 4°C [23].

Highly purified extracts are obtained for the quantitative analysis of biological objects, such as urine and plasma. This is necessary for the optimal use of chromatographic columns. In addition, dansyl chloride, a common derivatization agent[5] in high-performance liquid chromatography (HPLC), reacts not only with ibotenic acid and muscimol but also with the amino acid residues of proteins when analyzing fruiting bodies or plasma, and this decreases the sensitivity of the method [24]. Multi-stage liquid-liquid or solid-phase extraction is typically used (Table 2).

Table 2. Isolation schemes of ibotenic acid and muscimol out of bioassays

Sample Preparation	Source
Plasma	
• Extract with a mixture of formic acid and acetonitrile. • Centrifugate. • Dansylation. • Extract with acidic dichloromethane. • Dry with nitrogen. • Redissolve in a mixture of acetonitrile and water.	[24]
Urine	
• Perform an ion exchange. • Wash with acidified ethanol (HCl), then remove the aqueous layer. • Perform ethylation. • Purify with pyridine. • Dry with nitrogen. • Redissolve in ethyl acetate.	[27]
• Dilute at 1:10 or 1:100 with a mobile phase containing high concentrations of isoxazoles, then concentrate using solid-phase extraction at low concentrations. • Dry with nitrogen. • Re-dissolve in a mixture of mobile phase A and mobile phase B at 1:1.	[28]
Dried mushroom fruiting bodies	
• Extract with 50% ethanol. • Filter. • Perform multi-stage cleaning with organic solvents. • Re-extract with methanol.	[29]
• Extract with 50% ethanol. • Centrifugate. • Perform solid-phase extraction using formic acid in methanol as an eluent. • Evaporate. • Redissolve in a mixture of formic acid and methanol.	[30]
• Perform double extraction using a mixture of methanol and water at 7:3. • Centrifugate.	[26]
• Extract in 70% methanol using an ultrasonic bath. • Centrifugate. • Perform trimethylsilylation.	[25]

QUALITATIVE ANALYSIS

The most common techniques for qualitative identification include thin-layer chromatography (TLC), gas-liquid chromatography (GLC), and gas-liquid chromatography-mass spectrometry (GLC-MS). TLC uses a methanol extract obtained from fresh, frozen, and dried fruiting bodies. The mobile phase can be represented by various solvent systems, for example:

- N-propanol : 10% ammonia (95:5)
- N-butanol : acetic acid : water (60:20:20) etc.

Silica gel is the most common stationary phase [23]. Ninhydrin and fluorescamine can be used as detectors, followed by ultraviolet treatment [25]. Pentacyanoferrate reagents, which produce purple spots, are less common. Note that ninhydrin interacts with not only MTC but also with stizolobic and stizolobinic acids, both of which are non-protein amino acids. However, they produce orange and yellow-brown coloration, respectively [16]. Preparative TLC can be used for purification and preparation for further GLC and HPLC. Elution is performed using n-butanol, ethanol, and aqueous acetic acid [26].

Various instrumental chromatographic techniques are available (Table 3 for general information).

GLC-MS is only performed after the derivatization stage[5] because the substances have a fairly high boiling point and ibotenic acid is thermally unstable [25]. In addition, when analyzed directly without derivatization,[5] ibotenic acid and muscimol typically exhibit only one characteristic transition in the mass spectrum:

- ibotenic acid: 159 → 113
- muscimol: 115 → 98

However, this is insufficient to accurately identify toxicants [24]. Trimethylsilyl and ethyl derivatives are obtained [25, 27]. Ethylation is used to identify compounds in urine, but methods for trimethylsilylated compounds are also being developed [1]. The stationary phase is usually represented by semi-polar silicon dioxide sorbents with cross-linked phenolic groups, and helium serves as the carrier gas [25, 27].

HPLC is considered the most effective technique. If the levels of ibotenic acid and/or muscimol in the biofluids are considered high, the samples are diluted 10- or 100-fold. If the levels are low, the samples are concentrated using solid-phase extraction. Acetate or formate buffers mixed with acetonitrile are most often used as mobile phases. Gradient mode is preferred due to the differences in acid-base properties between the substances being analyzed and the ability of ibotenic acid to form a zwitterion[1] in an aqueous medium [13]. More polar sorbents are the most preferable because the high polarity of substances being analyzed reduces the efficiency of non-polar C18 columns [24, 26–30]. Levels are calculated using both internal and external standards, as well as the spike test, though the latter is significantly less common (see Table 3).

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Table 3. Schemes of chemical and toxicological analysis of blood plasma and urine for the presence of ibotenic acid and muscimol and chemical analysis of mushroom fruiting bodies via chromatographic methods

Techniques	Mobile phase	Stationary phase	Detector	Conditions	Calculation method	Source
			Plasma			
RP-HPLC	• A: 10 mM formic acid and 6 mM ammonium formate • B: acetonitrile	• A non-polar C18 column with heptafluorobutyric acid	• Time-of-flight mass spectrometric	• Dansylation • Gradient mode • 30°C	• Internal standard: • Ibotenic acid: L-tyrosine-$^{13}C_9$,^{15}N; • Muscimol: tyramine-d_4	[24]
			Urine			
GLC-MS	• Helium	• Semi-polar column • HP–5MS UI® (Agilent Technologies, USA)	• Mass spectrometric	• Ethylation	• Internal standard: cycloserine	[27]
HPLC-MS/MS	• A: 10 mM ammonium acetate in a mixture of water : acetonitrile at 1:9 • B: 0.1% aqueous formic acid	• Semi-polar column: ZORBAX StableBond SB-CN® (Agilent Technologies, USA)	• Quadrupole Orbitrap mass spectrometer	• Gradient mode • 40°C • Positive ionization	• External standard: • ibotenic acid; • muscimol	[28]
			Dried mushroom fruiting bodies			
HPLC, UHPLC	• Acetonitrile : ammonium acetate 10 mM: 80:20 pH 6.8	• Hydrophilic interaction liquid chromatography: silica gel with cross-linked triazole	• Ultraviolet, 255 nm	• Isocratic mode	• Spike test	[29]
HPLC-MS/MS	• A: 0.5% aqueous formic acid • B: a mixture of 0.5% formic acid and acetonitrile	• Silica gel with cross-linked carbamoyl groups: TSK-GEL Amide-80® (Tosoh Bioscience, Japan)	• Mass spectrometric	• Gradient mode	• Internal standard: • Acivicin	[30]
RP-HPLC-MS	• A: 10 mM ammonium acetate • B: acetonitrile	• Non-polar C18 column	• Mass spectrometric • Diode array (256 nm)	• Gradient mode • Dansylation with ethylation • 40°C	• External standard: • muscimol; • ibotenic acid	[26]
GLC-MS	• Helium	• Semi-polar column: DB–5MS® (Agilent Technologies, USA)	• Mass spectrometric	• Trimethylsilylation	• External standars: • muscimol; • ibotenic acid • Internal standard: • n-pentadecane	[25]

Note. RP-HPLC, reversed-phase high-performance liquid chromatography; UHLPC, ultra-high performance liquid chromatography; GLC, gas liquid chromatography; MS, mass-spectrometry.

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ELECTROPHORESIS

Chromatographic techniques are complex, time-consuming, and relatively expensive, especially in cases of multi-stage sample preparation with derivatization.[5] These factors limit their use when a quick result is necessary. Therefore, special attention is given to capillary electrophoresis using a diode-array or mass spectrometric detector (Table 4). The main advantages of electrophoresis include:

- Rapid test
- Easy sample preparation
- High sensitivity to ibotenic acid and muscimol

This technique, when used with tandem mass spectrometry, allows for the identification of isoxazole derivatives in urine at a relatively low detection threshold. For example, the threshold is 0.15 ng/mL and 0.05 ng/mL for ibotenic acid and muscimol, respectively [5]. The following characteristic transitions are detected:

- Ibotenic acid: 159 → 113 and 159 → 99
- Muscimol: 115 → 98 and 115 → 86 [12, 24]

A faster, more efficient technique has been developed to determine muscimol. This technique uses microextraction for sample preparation. A urine sample diluted with a phosphate buffer solution at pH of 4 serves as the donor phase. It contains a large number of dihydrogen phosphate anions, which form neutral ion pairs with the positively charged muscimol molecule. Ion pairs are extracted into octanol. Due to a concentration gradient, they then move into a more acidic acceptor phase (pH 3) which contains fewer dihydrogen phosphate ions and more muscimol cations. Electrophoresis is performed on the acceptor phase. This technique requires a minimal amount of chemical reagents, can be performed quickly (in 7 minutes), and allows for the analysis of only 200 µL of sample [11].

CONCLUSION

A review of publications reveals a global trend of increased use of *Amanita* species, particularly *A. muscaria* and *A. pantherina* via microdosing to enhance creativity, physical performance, and emotional well-being. This trend is associated with an increase in mushroom poisoning rates. This is due to the low awareness of the pharmacological effects of *Amanita* and the wide availability of various *Amanita*-based products on the market. This is why it is important to develop and implement toxicological techniques that can rapidly and accurately detect the active substances, muscimol and ibotenic acid, in the *A. muscaria* and *A. pantherina*.

GLC and HPLC are the most common chromatographic techniques. Among rapid tests, electrophoresis with mass

Table 4. Schemes of chemical and toxicological analysis of urine for the presence of ibotenic acid derivates and chemical analysis of mushroom fruiting bodies via capillary electrophoresis

Sample preparation	Conditions				Detection	Testing time, min	Detection limit		Source
	Injection parameters	pH	T, °C	Special conditions			Ibotenic acid	Muscimol	
Urine									
• Dilute five times with deionized water. • Filter through a 0.22 µm membrane microfilter.	• 0.1 atm • 5 s	2.7	25	• Create the environment using 0.2 M acetic acid. • Perform electrospray ionization in the isocratic mode.	Mass spectrometric	24	0.15 ng/mL	0.05 ng/mL	[12]
• Perform single-drop microextraction. • Extract from the donor phase into octanol at pH 4. • Re-extract into the aqueous phase (pH 3).	• 0.0068 atm • 1 s	3	18	• The creatinine appears on the electropherogram.	Diode array (214 nm)	7	—	16 µg/L	[11]
Dry fruiting bodies									
• Extract into a mixture of methanol and buffer solution with ultrasound. • Filter and evaporate the extract. • Dissolve in a mixture of methanol and buffer at 2:1.	• 0.034 atm • 5 s	3	22	• The mobile phase contains 5% acetonitrile.	Diode array (214 nm)	20	1.5 µg/mL	1.8 µg/mL	[24]

[5] In chemistry, derivatization is an analytical technique that converts a chemical compound into a derivative, which is a product with a similar chemical structure.

spectrometric or diode-matrix detection stands out due to its high sensitivity. This makes it a promising approach for developing techniques in chemical toxicology to detect *Amanita* poisoning.

ADDITIONAL INFORMATION

Authors' contributions: V.A. Zelenshchikova: conceptualization, collection and analysis of published data, writing—original draft; M.V. Belova, E.V. Melnik: conceptualization, collection and analysis of published data, writing—review & editing. Thereby, all authors provided approval of the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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