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Review

Methods for chromatographic determination of amanitins and related toxins in biological samples

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ABSTRACT

Methods for the chromatographic determination of amanitins, toxins of *Amanita phalloides* (Fr.), Link mushrooms and related toxins are reviewed; particular emphasis is given to high-performance liquid chromatographic methods. The main chemical and toxicological aspects are discussed, but the focus of the present review is on the analytical problems arising in a laboratory charged with the setting up of a procedure which can direct the appropriate clinical management of an intoxicated patient or solve a forensic case.

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LIST OF ABBREVIATIONS

AP	*Amanita phalloides*
EC	Electrochemistry
EIA	Enzyme immunoassay
FAB–MS	Fast atom bombardment–mass spectrometry
GC–MS	Gas chromatography–mass spectrometry
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
IgG	Immunoglobulin G
LOD	Limit of detection
RIA	Radioimmunoassay
RP	Reversed-phase
TLC	Thin-layer chromatography

1. INTRODUCTION

Statistics on mushroom poisonings are not readily available; differences in the weather cause large changes in the amateur mycologists' harvest and, furthermore, intoxications are often not properly reported [1]. However, *Amanita phalloides* (AP), *Amanita verna* (Bull.:Fr.) Lamk and *Amanita virosa* (Fr.) Bertillon are the most frequently implicated mushrooms. AP causes the majority of the intoxications [2,3]; it has been estimated that 10–40% (several hundreds every year) of these intoxications, and higher percentages in children, lead to death [4,5].

Amanita toadstools contain amatoxins (α-, β-, γ- and ε-amanitins, amanin and amanullin) together with phallotoxins, phallolysins and virotoxins; however, the lethal pathology is mainly attributable to the amatoxins, potent inhibitors of RNA polymerase type II.

Since the rapid adoption of an efficient treatment (not always free from risks to the patient) is crucial, fast and reliable assays for *Amanita* toxins in biological fluids are essential. In fact, Homann *et al.* [6] were able to measure amanitoxins in the gastric fluid and urine of an intoxicated boy collected as soon as 8 h after ingestion.

In addition, since any case of mushroom intoxication may have legal consequences, the accurate determination of mushroom toxins is also of primary importance for forensic pathologists and toxicologists.

2. STRUCTURE OF THE MAIN TOXINS OF *AMANITA PHALLOIDES*

The structures of the active substances of AP have been thoroughly studied and elucidated by Wieland [7]. Amatoxins and phallotoxins are bridged cyclopeptides, with some of the constituent amino acids in a modified form (for example, aspartic acid, proline, leucine, isoleucine and tryptophan in the 6′-position are hydroxylated).

The tryptophan residue (numbered 4 in amatoxins and 3 in phallotoxins) in linked at the 2-position of the indole ring to the sulphur atom of a cysteine residue orginally present in the cyclo-

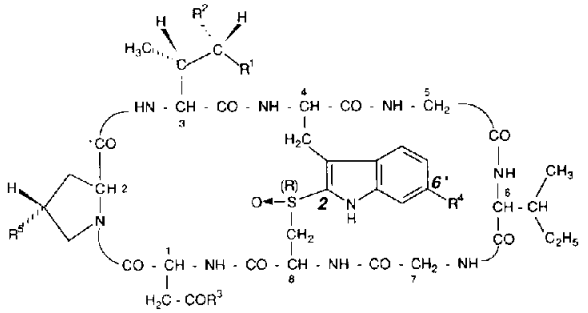


Fig. 1. General formula of amatoxins.

peptide (numbered 8 in amatoxins and 7 phallotoxins): this bridging part is named tryptathionine.

2.1. Amatoxins

The amatoxins are bicyclic octapeptides derived from one parent molecule and differ only in the number of hydroxyl groups (R^1, R^2, R^4 and R^5) and by the amide group (R^3) of the aspartic acid residue (numbered 1 in Fig. 1). The heteroatom of the tryptathionine occurs as (*R*)-sulphoxide. The concentration of amatoxins can reach values as high as 5 mg/g dry mass in AP; the toxins are found in some other *Amanita* species (mainly *A. verna*, *A. virosa*) but also in other genera which are not taxonomically related [*Lepiota brunneoincarnata* Chod. et Mart., *L. cristata* (Bolt.:Fr.) Kummer, *Galera marginata* (Batsch) Kuhn, etc.]; this last feature explains the occurrence of some fatal poisonings which are not caused by the consumption of *Amanita* mushrooms.

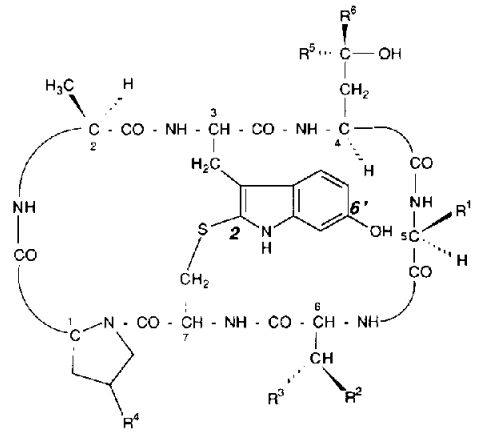


Fig. 2. General formula of phallotoxins.

2.2. Phallotoxins

Similarly, all phallotoxins are derived from the same cyclic peptide backbone (2, Fig. 1) composed of seven amino acids. In this class of compounds the sulphur heteroatom occurs as a thioether (Fig. 2). In the same way, they differ from one another by the number of hydroxyl or carboxyl groups (R^2, R^3, R^4, R^5 and R^6) or by a substituent (R^1). *Amanita* species containing amatoxins have been shown to contain phallotoxins as well, but these substances have not been detected in *Lepiota* and *Galerina* species.

The tryptathionine moiety and, more specifically, the spatial structure inducing the conformation of the peptide seems to be critical for the biological activity of amatoxins and phallotoxins [8].

3. TOXICOLOGY

After a 6–12 h latent period, the patient presents with nausea, vomiting, abdominal pain and profuse diarrhoea; jaundice follows after two to three day of remission. In serious cases, severe liver and kidney failure and convulsions lead to irreversible coma and death. These symptoms are the result of cellular destruction and liver, kidney and gastrointestinal tract necrosis [9].

The toxicity of amatoxins results from a strong inhibition of DNA-dependent RNA polymerase type II in the nuclei of the liver cells with consequent block of protein synthesis. Phallotoxins seem to exacerbate the action of amatoxins; they are reported to displace the equilibrium G-actin $\rightleftarrows$ F-actin toward F-actin filaments, thereby disrupting the normal stability [7]. Effective therapy of AP poisoning does not represent an easy task since it is difficult to remove the amatoxins from their receptor enzymes in the cell nuclei. Removal of gastric and intestinal contents, intake of charcoal, haemoperfusion, haemodialysis and plas-

mapheresis [7] can remove only the toxins not yet bound. Heroic measures such as liver transplantation have been reported in children [10,11].

4. DETERMINATION OF AMATOXINS AND PHALLOTOXINS

Several papers and a few monographs have been published in recent decades concerning the chemical, toxicological and clinical aspects of toadstools [7–9,12], but the subject of detection, recognition and quantitative analysis of peptide toxins in biological fluids, quite surprisingly, has aroused little interest in researchers. Only a few pages (out of almost 300) of the authoritative monograph of Wieland [7] discuss these problems.

4.1. Non-chromatographic methods

Radioimmunoassay (RIA) is the most used non-chromatographic technique for the assay of mushrooms in biological fluids; it was arduous to develop these assays because the scarce antigenicity of the peptides and their intrinsic toxicity hampered the raising of suitable antibodies.

The conjugation of β-amanitin with serum albumin, attempted in 1959, yielded a toxic derivative unsuitable for the production of antibodies [12]. Similarly, the conjugate of β-amanitin with proteins through a carbodiimide bond, very toxic for the protein-consuming cells, rapidly killed the rabbits and the mice into which the complex was injected [13]. The binding of α-amanitin to macromolecules through a spacer arm proposed by Faulstich and Trischmann [14] was unsuccessful too, owing to the low antigenicity of the compound. In 1975, Fiume *et al.* [16] were able to raise antibodies by injecting amanitin–albumin conjugate into rats, which are much more resistant to amanitins and amanitin conjugates than rabbits and mice. The assay, which employed antiserum diluted 1:400, tritiated amanitins and saturated ammonium sulphate solution for the precipitation step, required a 12 h incubation and reached the sensitivity of 500 pg/ml amatoxins in serum. Using this method it was possible to detect amatoxins in the serum, urine an duodenal juice of patients poisoned by AP [17].

The same year, Faulstich *et al.* [18] obtained another antiserum from rabbits. The animals were injected with a conjugate of serum albumin and the N-hydroxysuccinimide ester of β-amanitin, less toxic than the conjugate with the thiophenyl ester. The toxicity was reduced by linking with polylysine. Notwithstanding the death of many animals and the low titre of antiserum, the authors were able to develop an assay capable of detecting as little as 50 pg of toxin. While the procedure initially required 15 h of incubation at 4°C, it could be shortened to 45 min at 25°C in order to meet the requirements of clinical emergencies. More recently, Faulstich *et al.* [19] have proposed a solid-phase method. Antibodies were raised against α-amanitin, or better still against the 6′-hydroxindole moiety of α-amanitin (six molecules were introduced into one molecule of fetuin). In fact, these fetuin derivatives showed the same antigenicity as albumin derivatives but were much less toxic and were employed for the immunization of rabbits. The obtained antiserum underwent a purification step using a Sephacryl S-300 column (Pharmacia, Freiburg, Germany) and the immunoglobulin G (IgG) fraction was coupled to nylon-activated nets. After coupling, the sheets were equilibrated with tritiated α-amanitin, washed and cut into small pieces (0.75 × 0.75 cm). The execution of the competitive assay was quite simple: 1.1 ml of serum was incubated with a piece of nylon net for 24 h at 4°C or 2 h at 22°C and then 1 ml of solution was counted. Although this method was certainly simple, it showed a limit of detection (LOD) of about 3 ng/ml and an optimum operation range of 10–100 ng/ml amatoxins. The low sensitivity of this technique (which is widely used for urine samples) hampers its use in assays of serum samples for the diagnosis of AP poisoning and for monitoring the effect of detoxication procedures such as haemoperfusion and haemodialysis [19].

A significant improvement of the assay of amatoxins in blood was described in the articles of Andres *et al.* [20] and Adres and Frei [21]. The authors prepared a low-toxicity, oxidized form of

α-amanitin, named aldoamanitin. The resulting antiserum, raised in rabbit, exhibited the same cross-reactivity for α- and γ-amanitin and a 44% cross-reactivity for β-amanitin. An iodinated tracer was produced by using the Bolton Hunter labelling technique. The assay required only a 90-min procedure, reaching an LOD in serum of 100 pg/ml. Because of its good sensitivity and speed, the kit based on this procedure was a commercial success. The method was compared with the assay developed by Faulstich *et al.* [19] on urine samples, showing major differences in quantitation but a good agreement in the clinical assessment.

In recent years there have been no further reports of new immunometric methods, but the development of specific antisera has again attracted the interest of investigators. Kirchner and Faulstich [22] prepared antibodies against β-amanitin purified by affinity chromatography. Zhelev *et al.* [23] used β-amanitin–concanavalin A conjugate to produce amanitin-specific antibodies.

However, the validity of immunological methods, as is well known, is hampered by some problems of specific and non-specific interferences from the matrix, which require the confirmation of the results by alternative techniques.

4.2. Chromatographic methods

The intrinsic difficulties and limits of immunoassays prompted the development of chromatographic methods for the detection and measurement of these toxins. While paper chromatography and thin-layer chromatography (TLC) were extensively used for evaluating toxin concentration in mushroom extracts, they were soon found to be unsuitable for assays on biological fluids because of their inherently limited sensitivity.

4.2.1. Thin-layer chromatography

Although useful, paper chromatography lacks recent applications [6,24,25], while TLC has generally been adopted by several authors for the identification of *Amanita* toxins.

According to Palyza [26], who used commercially prepared plates (Silufol UV, Kavalier, Czechoslovakia), the best solvent system was Butyl Cellsolve–25% aqueous ammonia with 0.2% cinnamaldehyde, which also acted as a component of the developing system. The detection method with cinnamaldehyde–hydrochloric acid assured a good sensitivity (50 ng). Wieland [7] used silica gel plates and two solvent systems: butanol–ethyl acetate–water (14:12:4, v/v) and chloroform methanol–water (65:5:4, v/v). Visualization was obtained with cinnamaldehyde and vapours of hydrochloric acid. He listed the R_F of several AP toxins obtained under standardized conditions (*e.g.* freshly prepared solvents and constant temperatures).

Another procedure for the determination of amatoxins, including a purification step, used a 120-min migration with a solvent mixture of chloroform–methanol–acetic acid–water (75:33:5:7.5, v/v) and staining with diazotate sulphanilic acid solution. Quantitation was obtained by simultaneous reflectance and transmittance spectrometry at 480 nm [27].

As is obvious, the direct chromatography of crude extracts allowed more rapid, but generally poorer, results than methods requiring a preliminary fractionation by chromatography (*e.g.* on Sephadex LH-20) [28].

High-performance thin-layer chromatography (HPTLC) reportedly reduces the needs for tedious sample preparation steps. Stijve and Seeger [29] evaluated the toxin content of AP collected between 1970 and 1977 from different locations in Germany and Switzerland. The samples were lyophilized and then extracted with refluxing methanol. Aliquots of samples were assayed by HPTLC with mobile phases of chloroform–methanol–acetic acid–water (75:33:5:7.5, v/v) or 2-butanol ethyl acetate–water (56:48:20, v/v). The migration lasted about 40 min; the staining was by cinnamaldehyde–hydrochloric acid; amanitins appeared as bright reddish purple spots against a yellow background. The LOD of this method was reportedly 50 ng for α-, β- and γ-amanitin [30].

4.2.2. High-performance liquid chromatography (HPLC)

Early results, dating back to 40 years ago, concerned column chromatographic separation of amatoxins and phallotoxins. The authors used Sephadex G-25 with organic–aqueous solvent mixtures and then Sephadex LH-20 with water as eluent, collecting about 200 fractions for further investigations [7].

The drawbacks of low-pressure chromatographic techniques, such as low sensitivity and difficulty in achieving consistent quantitation, induced the authors to investigate the development of HPLC methods, especially in clinical environments. However, it must be mentioned that, because of inherent characteristics of reproducibility, efficiency and speed, HPLC was also successfully used to measure toxins in the fungal matrix.

With this aim, Caccialanza *et al.* [31] proposed an HPLC method requiring only an easy sample pretreatment; 2 g of dried mushrooms were soaked with a mixture of ethanol and water (50:50, v/v), the supernatant was taken to dryness, the residue resuspended with 5 ml of a water–ethanol mixture and 2 ml of the suspension were diluted to 20 ml with water and filtered; finally 20 μl of sample were injected into the chromatograph. The analysis was carried out using an isocratic reversed-phase separation with UV detection.

Quite recently, Enjalbert *et al.* [32] proposed a very interesting procedure for the simultaneous assay of eight amatoxins and phallotoxins in mushrooms by HPLC. Ten aliquots of 2 g of AP were extracted with 3 ml of methanol–water–0.01 *M* hydrochloric acid (5:4:1, v/v). After an overnight incubation at 4°C, the whole extract was centrifuged. The pellet was resuspended with 8 ml of the extractive mixture, incubated overnight at 4°C and finally centrifuged; 20 μl of the pooled supernatants were injected. Chromatographic separation was carried out by gradient elution. Solvent A was 0.02 *M* aqueous ammonium acetate and acetonitrile (90:10, v/v) and solvent B was 0.02 *M* aqueous ammonium acetate and acetonitrile (76:24, v/v). The gradient was: 0–4 min, 0% B; 4.1–16 min, 57% B; 16.1–26 min, 100% B. Absorbance was simultaneously monitored at 214 and 295 nm using a UV diode-array detector.

As previously discussed, HPLC is particularly suitable for measuring amanitins in biological fluids; therefore the proposed methods will be described in detail.

4.2.2.1. Sample pretreatment. First, Pastorello *et al.* [33] proposed a very simple procedure for the measurement of α-amanitin: the sample was added to methanol (1:2, v/v) and centrifuged; the supernatant was concentrated to 100 μl and 20 μl were injected. This sample treatment procedure was adopted also by Belliardo and Massano [34] with the minor modification of an increased methanol-to-sample ratio. Unfortunately, these simple but very rough procedures gave very dirty chromatograms in our hands, especially when the detection technique was rather aspecific, such as UV absorption at about 300 nm wavelength.

A procedure yielding cleaner samples was developed by Caccialanza *et al.* [31] for the measurement of α- and β-amanitins in serum and urine. It was based on the extraction of 1 ml of serum with 2 ml of a mixture of ethanol–chloroform (50:50, v/v); the mixture was shaken for 3 min and then centrifuged. The supernatant was filtered and concentrated under a stream of nitrogen to 100 μl and 10 μl were injected.

A much more elaborate and consequently selective sample treatment was proposed by Jehl *et al.* [35]. A 5-ml aliquot of serum was deproteinized with acetonitrile, which was then removed using dichloromethane and discarded. Seven 500-μl aliquots of the aqueous phase were applied at the top of a Sep-Pak C_{18} cartridge (Millipore, Milford, MA, USA) previously rinsed and equilibrated with distilled water. A 6-ml aliquot of a water–acetonitrile mixture (95:5, v/v) was applied; the first 1 ml and the final 3.5 ml of the eluate were discarded while the middle 1.5 ml was collected in a tube. Then, 6 ml of a water–acetonitrile mixture (80:20, v/v) were applied; the first 1 ml and the final 3.5 ml of the eluate were discarded while the middle 1.5 ml was collected in a second tube. At the end of the iterative process, 10.5 ml of eluate were collected in the two tubes containing β- and α-amanitin; they were evap-

orated and lyophilized; 20 μl were finally injected.

This method, very selective but obviously hardly applicable to clinical diagnosis, was simplified by Fenoil *et al.* [36]: 5 ml of serum were applied on the top of an RP C_{18} cartridge (Baker, Deventer, Netherlands), washed with 3 ml of distilled water and eluted with 1.2 ml of a mixture of methanol and water (40:60, v/v), which was finally evaporated under nitrogen. The purified amanitins, redissolved in the mobile phase, were injected into the HPLC system.

Urine samples required a second purification on a bare silica cartridge. The residue from the first cartridge was dissolved with 200 μl of chloroform–methanol–acetic acid (80:50:4.5, v/v), loaded onto the silica column and washed with 2 ml of the same solvent mixture followed by 0.5 ml of dichloromethane–methanol (70:30, v/v). The compound of interest was eluted with 2 × 1.2 ml of dichloromethane–methanol (50:50, v/v); the pooled eluates were evaporated. The residue, reconstituted with the HPLC mobile phase, was injected.

The complexity of the sample treatment proce-

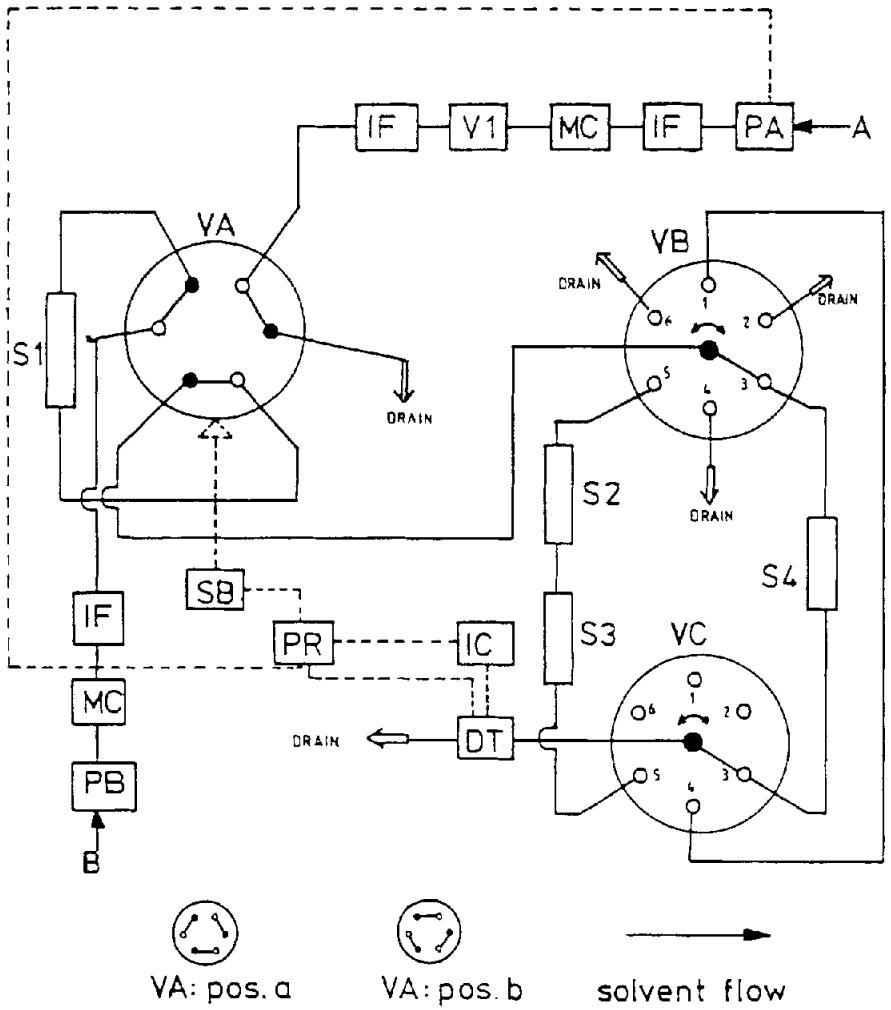


Fig. 3. Scheme of a column-switching system for the analysis of α-amanitin and phalloidin. A = eluent A; PA = pump A; IF = inlet filter; MC = mixing chamber; V1 = injection valve 1; VA, VB and VC = automatic valves; S1 = precolumn; S2, S3 and S4 = analytical columns; B = eluent B; PB = pump B; DT = detector; IC = integrator; SB = switchbox; PR = programmer (from ref. 37 with permission).

dures required to achieve extracts suitable for injection induced Rieck and Platt [37] to develop a column-switching technique. It allowed, after deproteinization, α-amanitin and phalloidin enrichment onto a first RP-8 column and then a second eluent transported the enriched α-amanitin and phalloidin to an RP-8 analytical column (or, alternatively, onto a combination of columns RP-8 and RP-18). The analysis required 30 min. The complete set-up is quite complex and is summarized in Fig. 3.

The need for a very selective and consequently complex extraction procedure can be reduced somewhat by increasing the selectivity of the further analytical steps (*i.e.* separation and detection). This approach allowed Tagliaro *et al.* [38], using an electrochemical detector, to simplify the procedure proposed by Fenoil *et al.* [36]. A 2-ml aliquot of serum was loaded onto a Sep-Pak C_{18} cartridge (Millipore) and amanitins were eluted with 2 ml of methanol–water (40:60, v/v), which was then extracted with 4 ml of methanol–chloroform (50:50, v/v). The organic phase was dried under a stream of air and the residue was redissolved in 400 μl of chloroform–methanol–acetic acid (80:50:4.5, v/v) and applied to a Sep-Pak silica cartridge (Millipore). The analytes, after a washing step with 500 μl of dichloromethane–methanol (70:30, v/v) were eluted with 1.2 × 2 ml of dichloromethane–methanol (50:50, v/v). The eluate was dried, redissolved in the mobile phase and injected. In the same paper an immunoextraction of amanitins from urine, based on anti-amatoxin antiserum bound to nylon nets furnished as a component of a commercial RIA kit (Max Planck Institute, Heidelberg, Germany), is described. The nets were washed with 20 ml of 0.05 *M* phosphate buffer pH 5 containing 30% methanol. After equilibration with 0.05 *M* phosphate buffer pH 7.5, the nets were incubated overnight, under mixing, with 1 ml of urine, buffered at pH 7.5. The nets were washed with 2 × 3 ml of saline and then amatoxins were eluted by incubating for 5 min with 200 μl of 0.05 *M* phosphate buffer pH 5 containing 30% methanol. The eluate was injected directly into the chromatograph.

The recent optimization of detection selectivity allowed the same group to simplify the extraction further using a single Separon SGX C_{18} RP cartridge (Tessek, Prague, Czechoslovakia); amanitin was eluted with methanol, which was then taken to dryness. The residue was redissolved in water–acetonitrile (91:9, v/v), passed through a Bio-Rex anion-exchange filter (Bio-Rad, Richmond, CA, USA), and finally injected [39].

4.2.2.2. HPLC separation. The chromatographic conditions for amanitin separation used by different authors are very similar indeed.

Reversed-phase HPLC under isocratic conditions has generally been employed except that Caccialanza *et al.* [31] adopted a gradient separation. The mobile phases used were acetonitrile–water (15:85 or 17:83, v/v) [33,37], acetonitrile 0.02 *M* acetate buffer (12:88 or 8:92, v/v) [31,35,38] or methanol water (40:60 or 25:75, v/v) [34,36]. Tagliaro *et al.* [39] employed 0.05 *M* phosphate buffer acetonitrile (91:9, v/v) at pH 9.5 in order to improve the electrochemical response of amanitins; for this reason, they adopted a pH-stable polystyrene–divinylbenzene column (PLRP-S 100 A, Polymer Labs., Church Stretton, UK). Other authors generally used reversed-phase silica-based columns such as Spherisorb ODS 5 μ (250 mm × 4.6 mm I.D.; Phase Separations, Queensferry, UK) [33,38], Hibar-Li-Chrosorb RP-18, 10 μm particle size (250 mm × 4 mm I.D.; Merck, Darmstadt, Germany) or Polygosil 60-10 C_{18} (250 mm × 4 mm I.D.; Machery-Nagel, Düren, Germany) [34], Lichrosorb RP-18, 5 μm particle size (125 mm × 4 mm I.D.; Merck) [31], Ultrasphere ODS, 5 μm particle size (250 mm × 4.6 mm I.D.; Beckman, Berkeley, CA, USA) [35], MPLC cartridge RP-8 Spheri-5, 5 μm particle size (100 mm × 4.6 mm I.D., Kontron, Milan, Italy), Hypersil WP300Butyl, 5 μm particle size (25 mm × 4.6 mm I.D., Shandon, Sewickley, PA, USA) [38].

4.2.2.3. Detection and quantitation. The preferred detection technique is usually UV absorption at 302 nm [33], 303 nm [34–36], 270 nm [31,36] or 313 nm [34]. In order to obtain a greater selectivity and sensitivity, Tagliaro *et al.* [39] adopted electrochemical detection, based on the

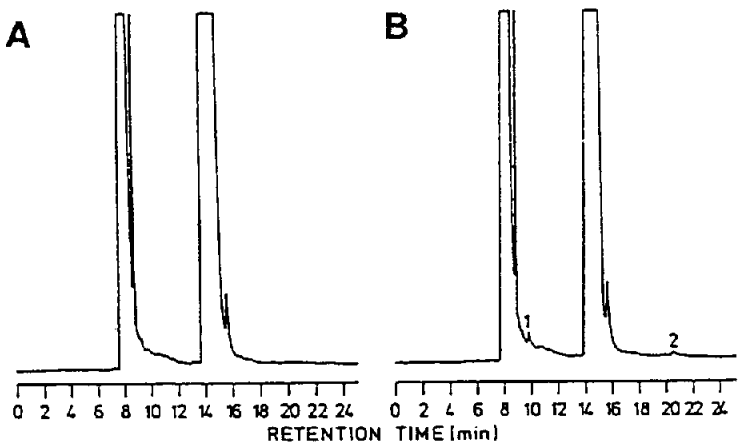


Fig. 4. (A) HPLC of blank plasma. (B) HPLC of plasma spiked with 10 ng/ml α-amanitin and phalloidin (from ref. 37 with permission).

presence of a 6-hydroxytryptophan residue in α-, β- and γ-amanitins containing a phenolic hydroxyl. Since the hydrodynamic voltammograms of the three amanitins proved to be rather different, the authors used a potential of + 0.6 V (*vs.* an Ag/AgCl reference electrode) giving the optimum response for α-amanitin with an acceptable background current (in the range 1.8–2.5 nA) [38].

Quite recently the same group set up a different detection procedure based on an alkaline eluent which enhanced the electro-oxidation character of the phenolic group, promoted by its ionization, so allowing the use of a potential as low as +350 mV (*vs.* an Ag/AgCl reference electrode) [39].

The sensitivity, which is a crucial parameter for clinical diagnosis, achieved by the HPLC methods has increased in the last ten years. The LOD reported by Pastorello *et al.* [33] for α-amanitin was 500 ng/ml; Belliardo and Massano [34] reported a sensitivity for α-amanitin around 1.3 μg/ml, while Caccialanza *et al.* [31] reported a sensitivity of 0.5–1 μg/ml for α- and β-amanitins and phalloidin. The more complex and selective sample pretreatment procedures used by Jehl *et al.* [35] and Fenoil *et al.* [36] allowed a substantial increase in sensitivity, essentially as a result of a reduction in the matrix-related noise; α- and β-amanitins could be detected down to levels of 10 ng/ml. The column switching proposed by Rieck and Platt [37] achieved a similar sensitivity of 10 ng/ml in the measurement of α-amanitin and phalloidin with automated sample pretreatment (Fig. 4). Electrochemical detection allowed Tagliaro *et al.* [38] to achieve a sensitivity first of 10–20 ng/ml and then, after further refinements, of 2 ng/ml (Fig. 5) [39].

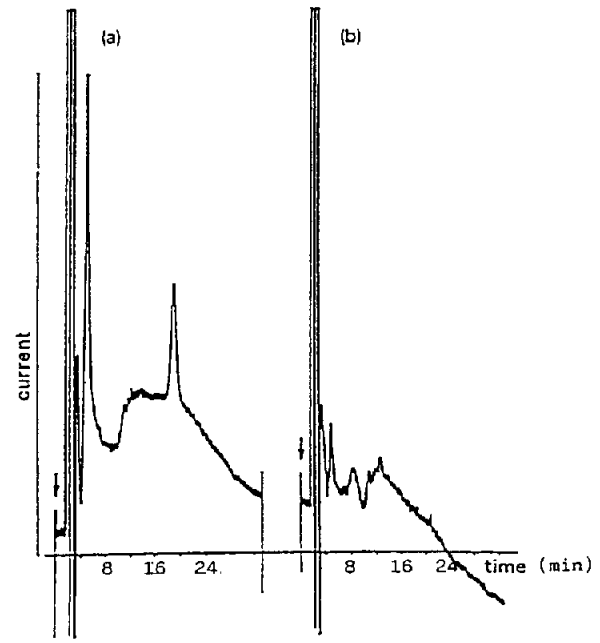


Fig. 5. (a) HPLC of plasma spiked with 12.5 ng/ml α-amanitin. (b) HPLC of blank plasma (from ref. 39 with permission).

5. DETECTION AND QUANTITATION OF OTHER LETHAL MUSHROOM TOXINS

Besides AP, many other mushroom species are highly toxic, and the detection of the toxins or their metabolites in biological fluids could help the management of the poisonings, since the patient very often does not associate his condition with a prior consumption of mushrooms and he is not able to describe the responsible fungi. Only a few methods of detecting non-AP toxins have been described in literature.

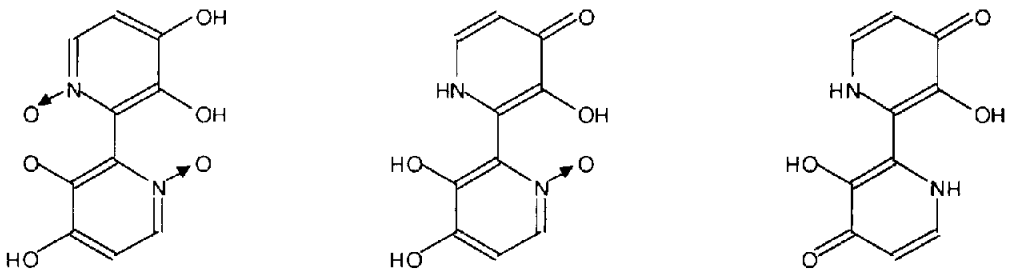
Poisonings by *Cortinarius orellanus* (Fr.) and *Cortinarius speciosissimus* Kuhn and Romagn, both members of the genus *Cortinarius* (Moser) section Orellani, occur every year and cause acute and chronic kidney failure after an unusually long latency period (two to three weeks) [40]. Orellanine and its breakdown products, orelline and orellinine, are the main toxins (Fig. 6). Orellanine has been reported to be the principal toxin of *Cortinarius orellanus*. This toxin has been quantitated in extracts by TLC methods and more recently by HPLC methods using UV and electrochemical detectors. However, TLC, because of the low stability, poor solubility and high polarity of the toxin, yielded unsatisfactory results.

The best TLC results were reportedly obtained using a cellulose layer and *n*-butanol–acetic acid–water (3:1:1, v/v) as the eluent.

Rapior *et al.* [41] reported a procedure for the assay of orellanine in biological fluids. Plasma samples were loaded onto a column containing Amberlite XAD-4 resin (Rohm & Haas, Darmstadt, Germany) adjusted to pH 2 and the elution was carried out by water brought to pH 2 with hydrochloric acid, methanol–water (1:1, v/v) and methanol. Two-dimensional TLC on cellulose allowed the specific separation of orellanine, which was quantified by fluorescence of its first breakdown product, orelline. This method surprisingly demonstrated very large quantities of toxin (6 mg/l) still present in the systemic circulation even ten days after the consumption of mushrooms.

HPLC methods have also been developed using Si-CN and Si-C_{18} columns with phosphoric acid at pH 1 as the mobile phase and UV detection at 260–290 nm. Ion pairing chromatography has also been employed with a mixture of phosphoric acid 1–6%, acetonitrile and 2.5 m*M* 1-octanesulphonic acid as mobile phase [42,43].

Some of these experiments were performed at the borderline of the use specifications of the equipment (*e.g.* very low pH, short retention time, high concentration of the samples, wide peaks) and their immediate application to the detection of toxins in body fluids seems difficult. However, the procedure could be improved by using acid-resistant stationary phases such as polystyrene–divinylbenzene (Biogel PRP 170-5, 150 mm × 4.6 mm I.D., Bio-Rad) [44]. A further improvement was given by the derivatization of orellanine or orellinine with silylating reagents; this chemical modification led to the use of gas chromatography–mass spectrometry (GC–MS),



1 2 3

Fig. 6. Formulas of orellanine (1), orellinine (2) and orelline (3).

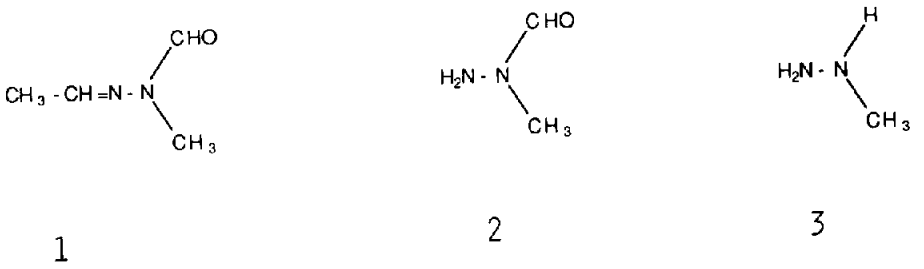


Fig. 7. Formulas of gyromitrin (1), methylformylhydrazine (2) and methylhydrazine (3).

which could unambiguously demonstrate the persistence of the toxin in the systemic circulation [45].

Reports have been published on TLC and HPLC methods for the analysis of cortinarin A, a toxin from *Cortinarius speciosissimus*. The separation was carried out using an ODS column (250 mm × 4.5 mm I.D., Joncs Chromatography, Littleton, CO, USA) and a mobile phase of acetonitrile–water (25:75, v/v) [46].

Gyromitra esculenta (Pers.:Fr.) Fr. mushrooms have caused severe poisoning and even death in man [47]. Clinical data are primarily characterized by vomiting and diarrhoea followed by jaundice, convulsions and coma. No method for the determination of the toxin gyromitrin (acetaldehyde methylformylhydrazone (1, Fig. 7) or its hydrolysis by-products, N-methylformylhydrazine (2, Fig. 7) and methylhydrazine (3, Fig. 7), in human body fluids has ever been published. However, these substances have been detected in the peritoneal fluid of mice [48] and in the urine of rabbits after *per os* administration [49,50]. Gyromitrin has also been detected by IR and UV spectroscopy and TLC in the viscera during a post-mortem examination of a poisoned patient [51].

Poisoning by *Amanita pantherina* (DC.:Fr.) Krombh. is characterized by dizziness, incoordination, ataxia, muscular jerking, hyperkinetic activity, stupor and hallucinations. The responsible toxin is ibotenic acid (1, Fig. 8), which readily decarboxylates *in vivo* yielding muscimol [52,53] (Fig. 8). Muscimol has been detected by GC MS in the urine of poisoned victims as its trifluoroacetylated derivative [54].

6. CONCLUSIONS AND PERSPECTIVES

In the 1990s HPLC, because of its sensitivity and ease of automation, will be the technique of choice for measuring amatoxins in clinical chemistry laboratories. This technique allows an accurate and sensitive (down to a few ng/ml) quantitation in a few hours and represents the only

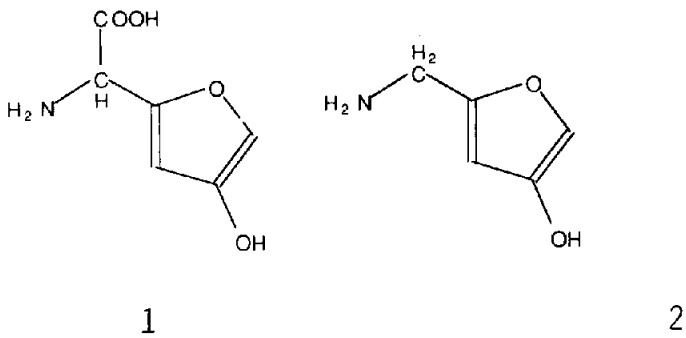


Fig. 8. Formulas of ibotenic acid (1) and muscimol (2).

chromatographic alternative to the RIA methods. Recently, Pudill [55] suggested a quite innovative approach to the assay of α-amatoxins and phallotoxins involving fast atom bombardment mass spectrometry coupled to HPLC separation. Although interesting, the paper did not give clear data on sensitivity and reproducibility and therefore this technique is not yet to be considered mature.

As a matter of fact, the radioactive reagents used in sensitive and rapid RIA methods require authorized personnel and laboratories and exhibit shelf-lives too short to organize an efficient mushroom toxicology service. In this regard, we think that the setting up of enzyme immunoassay methods could be extremely useful.

Moreover, in forensic laboratories, immunoassays require confirmation by alternative techniques when legal validation is necessary [15]. In this respect, HPLC is, at present, the only practicable means of confirmation. On the other hand, the chromatographic alternative to immunometric methods for emergency toxicological purposes will be, perhaps, a "fire and forget" dedicated, fully automated instrumentation, which will couple toxicologists' requirements of sensitivity, accuracy and specificity with those of rapidity and operative simplicity needed by workers in emergency departments.

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