Determination of α-, β-, and γ-Amanitin by High Performance Thin-Layer Chromatography in *Amanita phalloides* (Vaill. ex Fr.) Secr. from Various Origin

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A fast, sensitive high performance thin-layer chromatographic method for the determination of α-, β-, and γ-amanitin in crude, methanolic extracts of *Amanita phalloides* is described. The limit of detection is 50 ng of each amanitin.

With this method amanitin was determined in 24 pooled samples of *Amanita phalloides*, collected between 1970 and 1977 in Germany and Switzerland. The total amanitin content varied between 2010 and 7300 mg/kg dry weight and the average value was 4430 mg/kg of which 43% was α-amanitin, 49% β-amanitin and 8% γ-amanitin.

The origin of the fungi hardly influenced their amanitin content: in samples collected during the same year at different sites it fluctuated within a factor of 1.7.

The amanitin content of samples from the same site, but collected in different years, maximally varied within a factor of 3.7. The partial decomposition of amanitins during prolonged storage of the lyophilized samples undoubtedly contributed to this variation.

Phalloidin, which was determined by conventional thin-layer-chromatography, could not be detected in a sample from 1970, whereas its concentration in material collected during 1977 amounted to 2400 mg/kg dry weight.

The toxicity of the samples (LD_{50} of lyophilized defatted methanolic extracts intravenously for mice) varied within a factor of 2.5.

Introduction

In spite of considerable research in the chemistry and toxicology of the toxic peptides of *Amanita phalloides* [1, 2], little is known about the natural variation of the toxin content in the green death cap. This may be due to the fact that the determination of these toxins is not easy: the analytical methods used until now – adsorption chromatography on Sephadex LH-20, followed by thin-layer chromatography or amino acid analysis [3], inhibition of RNA polymerase [4], and radioimmunoassay [5] – are tedious, time consuming or not accessible to every laboratory.

For this reason the present authors developed a rapid and sensitive high performance thin-layer chromatographic method which enabled them to determine α-, β-, and γ-amanitin in crude methanolic extracts of 24 samples of *Amanita phalloides* of different origin. The results of this investigation and a detailed description of the analytical method are presented in this paper.

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Materials and Methods

The fungi were collected between 1970 and 1977 at several sites in Germany and Switzerland [6]. Each sample consisted of several more or less developed fruitbodies. The fungi were cleaned mechanically, lyophilized, ground to fine powder on a crushing mill and kept in tight glass bottles protected from light until use.

Extraction was performed by refluxing 0.5 g of the lyophilized sample twice with 2 × 50 ml of ethanol (Merck, No. 6009) during 1 h on a water bath. The combined extracts were evaporated under vacuum and adjusted to a volume of 2 ml.

Table I. Amanitin content (mg/kg dry weight) of *Amanita phalloides* collected in Germany and Switzerland during 1975.

Site of collection	Date	α-Amanitin	β-Amanitin	γ-Amanitin	Total amanitin
Gamburg (Nordbaden)	30. 7.	2300	3250	260	5810
Gramschatz (U'franken)	9. 9.	2300	1700	420	4420
Külsheim (Nordbaden)	15. 9.	2100	2250	260	4610
Würzburg (U'franken)	16. 9.	2500	2650	340	5490
Gamburg (Nordbaden)	21. 9.	*3250*	*3500*	640	*7390*
Geesdorf (U'franken)	23. 9.	2400	2500	*680*	5580
Monthey (Wallis)		2400	2800	510	5710
				$\bar{x} \pm s_{\bar{x}}$	5570 ± 370

The amanitin content of these extracts was determined by high performance thin-layer chromatography (HPTLC) on 10 × 10 cm silicagel 60 plates for nano-TLC, Merck, No. 5628, with pure α- [7], β- and γ-amanitin [8] as reference compounds. For this purpose, 100, 200, 300 and 400 nanoliter aliquots were spotted with the Camag Nanoplicator. These volumes corresponded to 25, 50, 75 and 100 µg of lyophilized material. For comparison we applied 100, 200, 300 and 400 nanoliter aliquots of amanitin standard solution containing 1.0 mg of each reference compound in 2 ml of methanol.

The chromatograms thus prepared were developed in the following mobile phases: Chloroform – methanol – acetic acid 100% – water 75 : 33 : 5 : 7.5 v/v [16]. 2-Butanol – ethylacetate – water 56 : 48 : 20 v/v [3].

In both systems the order of migration of the amatoxins was essentially the same, although with different R_f-values. The mobile phase containing acetic acid yielded more rounded spots for β-amanitin. After development by ascending migration in a presaturated chamber over a distance of about 8 cm (which took approximately 40 minutes), the plate was placed under a hood in a stream of air to allow complete evaporation of the adherent solvent (residual solvent in the layer was found to lower the sensitivity of amanitin detection).

Subsequently the chromatogram was sprayed with a freshly prepared solution of 1 ml cinnamaldehyde (trans-3-phenyl-2-propenal, Fluka, No. 96320) in 100 ml of methanol, after which it was placed in a glass tank containing a 100 ml flask filled with fuming hydrochloric acid (Merck, No. 317). Air was bubbled through the acid to saturate the tank with vapours. Amanitins appeared as bright reddish purple spots against a yellow background. After 5 – 10 minutes, when 0.05 µg of the individual amanitins could be clearly distinguished, the plate was removed from the tank and placed in a stream of air, whereupon the yellow background became white, thus improving the visibility of the spots.

The amanitin concentrations in the sample aliquots were determined by comparison with the series of standard spots either by visual estimation or by scanning the chromatogram with the Zeiss model PMQ II chromatogram spectro-photometer. These densitometric measurements had to be performed directly after optimal visibility was achieved, because the colour of the amanitin spots faded within 20 minutes.

Alternatively, we sometimes used 1,2-dihydroxycyclobutendion (Fluka 37670) as a chromogenic reagent under the same conditions as those described for cinnamaldehyde. This reagent revealed the amanitins as bluish green spots which were stable for more than 12 h.

Due to overload phenomena, the HPTLC method was not suitable for the determination of the phallotoxins. Thus, phalloidin [9] was determined by con-

Table II. Amanitin content (mg/kg dry weight) of *Amanita phalloides* from the same site of collection (Gamburg/Nordbaden) 1970 – 1977.

Date	α-Amanitin	β-Amanitin	γ-Amanitin	Total amanitin
20. 09. 1970	*650*	*1200*	*160*	*2010*
29. 08. 1974	1750	2100	520	4370
17. 09. 1974	1650	2100	330	4080
30. 07. 1975	2300	3250	260	5810
21. 09. 1975	*3250*	*3500*	*640*	*7390*
04. 09. 1977	2600	2250	390	5240

ventional TLC using 20 × 20 cm precoated silicagel/kieselgur plates (Merck, No. 5737) and the mobile phase chloroform- methanol- acetic acid-water as described above. In this TLC system phalloidin hat a R_f-value very close to that of γ-amanitin. Fortunately, interference from the latter compound could be minimized by evaluating the chromatogram at different stages of colour development: when exposing the plate to HCl vapour, phalloidin appeared as an orange brown spot before γ-amanitin could be clearly distinguished. At a later stage, phalloidin was visible as a bluish halo just beyond the purple amanitin spot, but the sensitivity of the colour reaction for phalloidin was low. For purposes of quantitation, it was necessary to subject 1 – 3 mg sample aliquots to TLC.

The toxicity of defatted lyophilized methanolic extracts was determined by intravenous administration to NMRI mice (Zentralinstitut für Versuchstiere, Hannover, FRG) as described elsewhere [10]. LD_{50} was statistically calculated by the method of Litchfield and Wilcoxon [11].

Results

The average amanitin content of the samples was 4430 ± 250 mg/kg dry weight ($\bar{x} \pm$ S. E.; $n = 24$); 43% of this amount was contributed by α-amanitin, 49% by β-, and 8% by γ-amanitin.

The total amanitin concentration of the individual samples varied from 2010 to 7390 mg/kg, *i. e.* within a factor of 3.7. The fluctuations for the individual amanitins amounted to factors of 5.0 for α-amanitin, of 2.9 for β-amanitin and of 5.7 for γ-amanitin.

Interestingly, there was not much variation in the amanitin content of samples collected during the same year at different sites. In seven samples from 1975 (Table I) it varied within a factor of 1.7 and in eight samples collected in 1970 the factor was 2.3. Thus, the sample richest in amanitin contained about twice as much of these toxins as did the poorest one.

In samples collected at the same site, but during different years, the differences in amanitin content were more pronounced: they varied within a factor of 3.7 (Table II). The oldest sample had been stored for 8 years and had the lowest amanitin content.

In a sample from 1977 the phalloidin concentration amounted to 2400 mg/kg dry weight. In material from 1970 it was well below the detection limit of 500 mg/kg.

The differences in toxicity (Table III) of the samples were far smaller than those measured between their amanitin contents: the sample poorest in amanitin was indeed the least toxic one, but the toxicity of all other samples did not differ significantly. Among the five samples examined, three caused amanitin poisoning and in the two others phalloto-

Table III. Amanitin content (mg/kg dry weight) and toxicity for mice *i. v.* (LD_{50} with confidence limits) of *Amanita phalloides*. Toxicity determined with defatted, lyophilized methanolic extracts. * = $p < 0.05$.

Site and date of collection		Amanitin content mg/kg	LD_{50} mg powdered sample per kg body weight	Cause of death
Gamburg	20. 09. 1970	2010	280 (187 – 420) *	Amatoxins
Gamburg	30. 07. 1975	5810	112 (91 – 138)	Amatoxins and Phallotoxins
Würzburg	16. 09. 1975	5490	162 (122 – 215)	Amatoxins
Gamburg	21. 09. 1975	7390	138 (90 – 211)	Amatoxins
Gamburg	04. 09. 1977	5240	140 (102 – 192)	Phallotoxins and Amatoxins

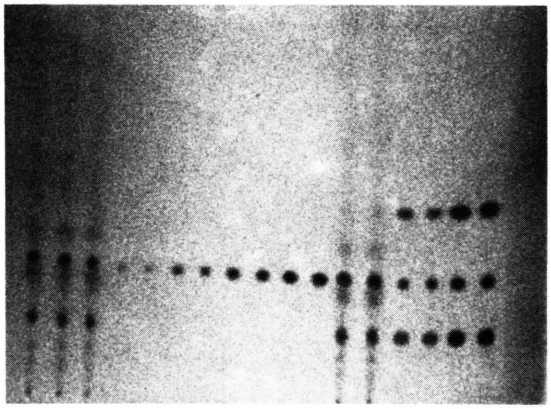

Fig. 1. HPTLC chromatogram. SiO_2 10 × 10 cm plate for nano-TLC. Mobile phase: chloroform-methanol-acetic acid-water (75 : 33 : 5 : 7.5 v/v). Chromogenic reagent: cinnamaldehyde – hydrochloric acid. From left to right: 0.1 mg of lyophilized *Amanita phalloides* (3 ×); series of α-amanitin concentrations ranging from 0.05 – 0.2 mcg, each aliquot spotted twice; 0.15 mg of *Amanita phalloides* (2 ×); reference compounds: R_f-values: $\gamma > \alpha > \beta$-amanitin.

xins contributed to the toxicity; in the most recently collected sample, containing 2400 mg phalloidin per kg, the effect of the phallotoxins was even predominating.

Discussion

During the last ten years, many workers tried to use TLC for the detection and determination of amanitins in crude or more or less purified extracts [3, 12 – 15]. The results of these attempts were often rather disappointing, because the quality of the layers is highly critical. Many commercially available ready-to-use plates simply do not give sufficient separation when used with the mobile phases described in the literature. Moreover, sensitive detection of the separated amanitins by spraying the chromatogram with cinnamaldehyde and subsequent exposure to HCl vapours also requires a certain know how: it is not for nothing that detection limits for α-amanitin as reported in the literature vary from 0.05 – 1.0 μg!

Not surprisingly, many research workers are suspicious of TLC for the detection of amatoxins and it is a communis opinio that the technique gives, at best, qualitative results, although Andary *et al.* reported a successful attempt to render it quantitative: they used diazotised sulphanilic acid as a chromogenic reagent and quantified the amanitins by direct spectrophotometry on the chromatogram [16]. In our hands this method yielded only mediocre results, because of incomplete resolution of the amanitins from impurities, and rapid background darkening after spraying with impaired sensitivity.

For this reason we applied the HPTLC technique using commercial precoated plates which proved to be most suitable for our purpose: under the conditions described above, the amanitins showed well rounded spots that were clearly separated from co-extracted impurities and from the phallotoxins (Fig. 1). Quantitation was greatly facilitated by the linear relationship that existed between concentration and spot intensity as determined by densitometry. In some cases a higher sample load (up to 0.2 mg) was necessary in order to quantify the minor component γ-amanitin.

The amanitin content of our *Amanita phalloides* samples is amazingly high. Other authors reported 50 – 3590 mg/kg on dry matter [3, 16 – 20], and 1350 – 3440 mg/kg in pooled material [16 – 19]. Obviously, all these values represent the yield of the isolation procedure which depends on the extraction method used and the number and extent of the purification steps, which invariably cause important losses. When chromatographic methods are used for the determination step, the results are also influenced by the separation characteristics of the system. It should be pointed out that the yield of our extraction method is also lower than the actual amanitin content of the fungi: when performing extraction with methanol containing 1% acetic acid, instead of using neutral methanol as in the present investigation, the amanitin content of the extracts was 15 – 20% higher. However, the amount of co-extractives also increased which impaired the resolution during HPTLC, rendering acid extraction less suitable for purposes of quantitation.

It is also worth noting that our material had a high concentration of β-amanitin, a compound that has not yet received much attention from the toxicologists. Considering the results reported in the literature [3, 16, 18 – 20] one was inclined to expect mainly α-amanitin in *Amanita phalloides* of European origin. Predominance of β-amanitin [3] seemed to be the exception rather than the rule. Yet in 19 of our 24 samples, β-amanitin predominated over the α-compound, even to a maximum of 1.85 : 1. No relation could be found between this ratio and the origin of

the fungi. In an earlier investigation [10] we had found also more β- than α-amanitin in 3 out of 11 pooled samples of *Amanita verna* Bull., which may be considered a subspecies of the green death cap. *Amanita phalloides* from the USA containing mostly β-amanitin [15, 17] has been considered a particular chemical race [11]. Our results would invalidate this assumption.

Apparently, the origin has relatively little influence on the amanitin content of the green death cap. This seems to be at least valid for pooled material such as analysed in the present investigation, because it corresponds to what is eaten in most cases of mushroom poisoning. However, in individual carpophores, one may expect wider variations or dwarf forms, respectively: Faulstich *et al.* [20] found about the same amanitin content in three out of four fruitbodies; the fourth one contained only 1/40 of this concentration.

The important differences in amanitin content among samples collected in different years are apparently due to a partial destruction of the toxins during prolonged storage. The trend to a decrease in amanitin content with time is fairly obvious (Table II). Phallotoxins are probably even less stable during storage: In the most recently collected sample, the toxic effects were mainly due to the phallotoxins (Table III), whereas in an 8 year old sample the phalloidin concentration was well below detection. However, the decomposition of the toxic compounds with time, as observed earlier in air-dried death caps [13], does not altogether explain the difference in amanitin content: despite longer storage, the autumn fungi from 1975 contained more amanitin than those gathered during 1977. Perhaps there are also seasonal variations: the autumn fungi of 1975 were somewhat richer in amanitin than the fungi gathered during the summer of the same year (Table II). However, when considering such small differences, it should also be taken into account that amanitins are not evenly distributed in the fruit-body, but accumulated in the cap [16], and it cannot be assumed that each pooled sample contained caps and stalks in the same ratio.

In an earlier investigation concerning the differences in toxicity among *Amanita phalloides* from different origin, the most poisonous sample was found to be three times as toxic as the least poisonous one [21], but, since the extracts also contained the labile phallolysin [22], these data seemed of limited value. However, the results of the present study, carried out with phallolysin-free methanolic extracts, show differences in the same order of magnitude, *i. e.* from 1 : 2.5.

There clearly is no correlation between amanitin content and toxicity; this may be partly due to the greater variability generally observed in the results of biological tests, but also to the fact that the phallotoxins contributed to the toxicity of several samples (Table III), *i. e.*, some of the animals died from phallotoxin poisoning. The often fatal outcome of *Amanita phalloides* poisoning in human beings is attributed to the amatoxins [1].

The relatively high constancy of the amanitin content in green death caps may be explained by the fact that under normal conditions these fungi do not grow saprophytically, but form mycorrhiza with live trees; consequently, there is probably little variation in the supply of nutrients. The constantly high toxin content also confirms the general experience that carpophores of *Amanita phalloides* are always poisonous.

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