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QUANTITATIVE CHROMATOGRAPHIC ANALYSIS OF TOXINS IN SINGLE MUSHROOMS OF *AMANITA PHALLOIDES**

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SUMMARY

By a simple combination of column and thin-layer chromatography, all of the toxic compounds in 1–2 g of air-dried mushrooms of *Amanita phalloides* (25 g of fresh material) were isolated and quantitatively determined. In pure, non-resolved, fractions of toxins, *e.g.*, mixtures of phalloin, phalloidin and phallisin, the amount of each toxin was determined after acid hydrolysis of the peptides by ion-exchange chromatography of the corresponding γ-hydroxyleucine and γ-hydroxyisoleucine lactones, which in ion-exchange amino acid analysis behave like basic amino acids. For γ-lactones with chiral γ-carbon atoms, epimerisation during hydrolysis was observed. By this direct analysis of crude extracts, it was observed that the portion of acidic toxins is much larger than has been determined so far, namely up to 70% of all toxic peptides. It was further discovered that phallacidin is accompanied by an acidic, higher hydroxylated analogue. It contains, like phallisin, γ,δ,δ'-trihydroxyleucine and will be called phallisacin.

INTRODUCTION

Since the first isolation and identification of different *Amanita* toxins by WIELAND and co-workers[1–6] by paper chromatography and paper electrophoresis, many efforts were made to devise simple chromatographic assays to detect the toxins in extracts of the mushroom, avoiding laborious purification steps. Thus BLOCK *et al.*[7,8] succeeded in characterising the violet and blue spots of the toxins after chromatography of methanolic extracts on paper strips, after reaction with cinnamic aldehyde and exposure to hydrochloric acid, according to the method of WIELAND and co-workers[4]. SULLIVAN *et al.*[9] made use of thin-layer chromatography. BENEDICT *et al.*[10] and TYLER *et al.*[11] used this method to demonstrate the presence of amanitins in species different from *Amanita phalloides*. RAAEN[12] introduced striated thin-layer plates, which were also used together with new solvent systems by PALYZA and co-workers[13,14].

All of these tests were qualitative, or only semi-quantitative, with respect

* Dedicated to Professor TH. WIELAND on the occasion of his sixtieth birthday.

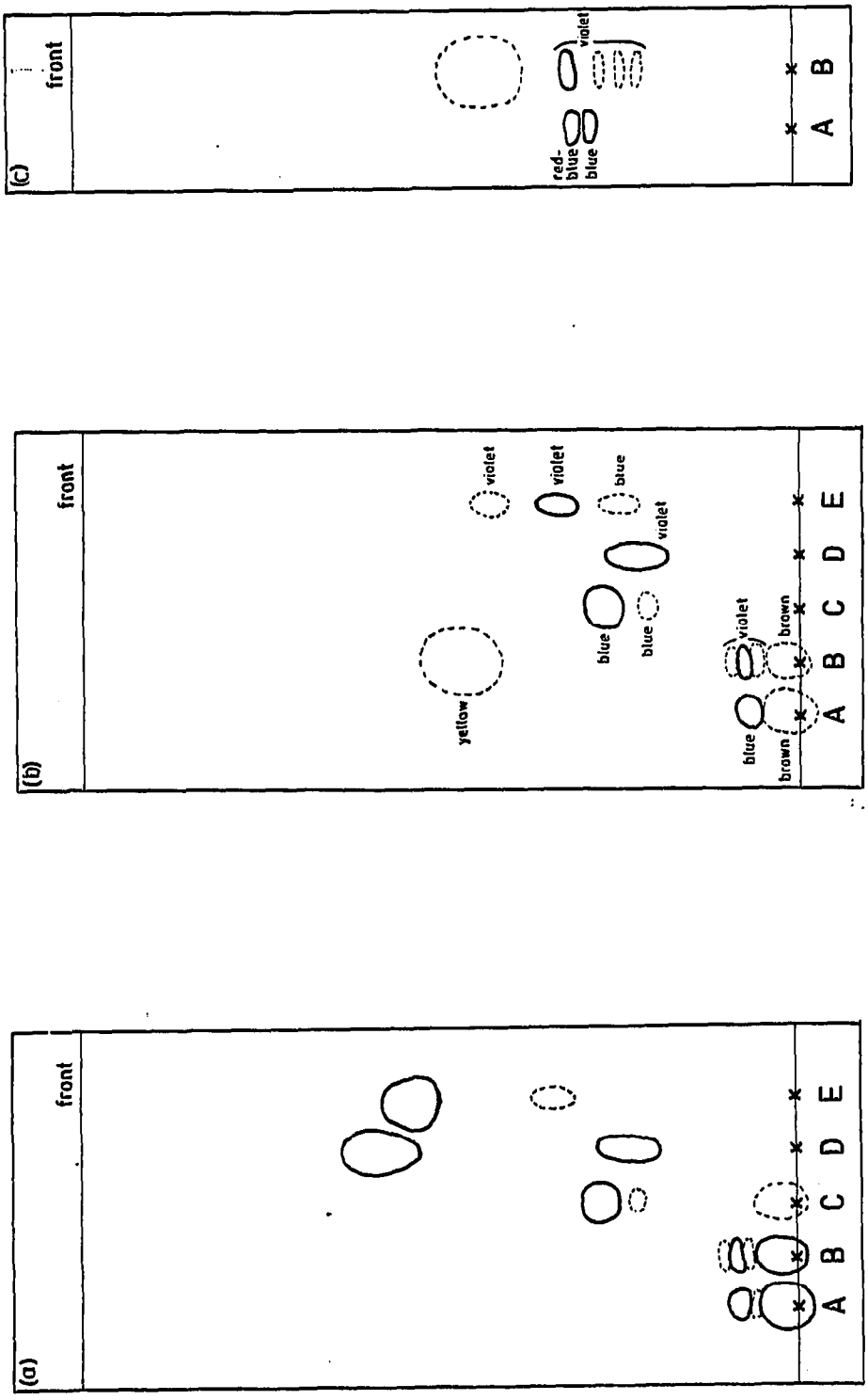

Fig. 2. Thin-layer chromatograms of column fractions A, B, C, D and E (Charge II): (a) in *sec.*-butanol–ethyl acetate–water (14:12:5) observed in UV light; (b) in the same solvent, detected with cinnamic aldehyde–HCl; (c) in *sec.*-butanol–3% ammonia solution (100:44) detected with cinnamic aldehyde–HCl.

to the amanitins. Moreover, some of them required a great deal of preparatory work in order to obtain reproducibility. Therefore, we preferred a simple column chromatographic procedure, which yielded toxins[15] in milligram amounts from crude extracts of mushrooms. These fractions could be identified easily and, above all, determined quantitatively by thin-layer chromatography and amino acid analysis.

EXPERIMENTAL

Usually 2 g of dried *Amanita phalloides* mushrooms were ground in a Star mixer and soaked with 100 ml of water for 1 h. After addition of 100 ml of methanol, the suspension was stirred on a magnetic stirrer for 20 h, then centrifuged at 2000 × *g*. The tissue pellet was extracted once in the same way with 100 ml of methanol–concentrated ammonia solution at pH 10 for a further 2 h. Corrrespondingly, 25 g of preserved mushrooms, which had been kept under methanol for several months, were minced and treated likewise.

The extracts were evaporated *in vacuo*, combined, and thoroughly dried in a desiccator over P_4O_{10}, followed by treatment with dry peroxide-free ether with magnetic stirring for 2 h. The dried residue was again extracted with small amounts of boiling methanol, until no more coloured material was extracted. The combined extracts were then evaporated to dryness, re-dissolved in about 10 ml of water, centrifuged at high speed if necessary, and applied to a Sephadex LH-20 column (250 × 2 cm), equilibrated with a 0.02 % solution of sodium azide. The flow-rate should be at least 25 ml/h. The effluent was monitored at 283 nm (Uvicord II, LKB). In the elution diagram, the toxins were identified by their UV absorption spectra[16] in fractions A, B, C, D and E (Fig. 1).

These fractions were evaporated, re-dissolved in known amounts of methanol (about 1 ml), centrifuged in small Eppendorf vessels and stored tightly closed. Aliquots of these solutions were used for further purification by thin-layer chromatography, for which we used 20 × 20 cm analytical plates pre-coated with a 0.25-mm layer of silica gel with fluorescent dye (Merck 60 F_{254}, Darmstadt). The samples were applied as spots with glass micropipettes (5 × 5 µl per spot), and developed in *sec.*-butanol–ethyl acetate–water (14:12:5), preferably in the dark. Better purification of the acidic toxins was achieved when *sec.*-butanol–3 % ammonia solution (100:44) was used. A second plate from the same batch was run in parallel, sprayed with cinnamic aldehyde (1 % in methanol), air-dried and exposed to an HCl atmosphere, to serve for identification by UV light of the toxin spots on the preparative plate. The spots were scraped off and eluted with methanol, with re-

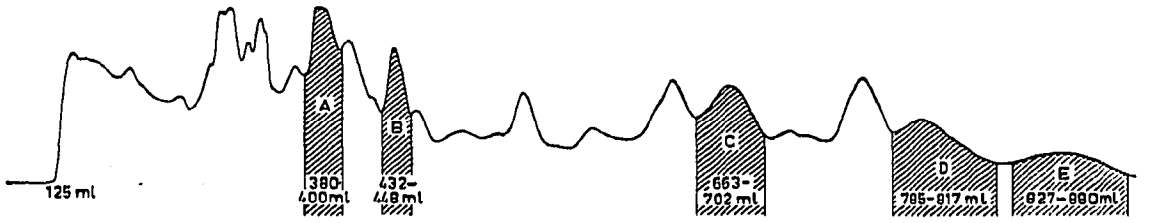

Fig. 1. Characteristic elution diagram of extracts of *A. phalloides* from a Sephadex LH-20 column, developed with water.

coveries of 65 % for the acidic phallotoxins in fraction A and 85 % for β-amanitin in fraction B and for all the neutral toxins in fractions C, D and E. The extent of recovery was first determined by using crystalline samples of the different toxins. In the solutions obtained, the concentrations of the toxins were determined spectrophotometrically, using the 310-nm absorption band of the amatoxins (ε = 14,500) and the 300-nm absorption band of the phallotoxins (ε = 11,000). Thin-layer chromatograms are shown in Fig. 2.

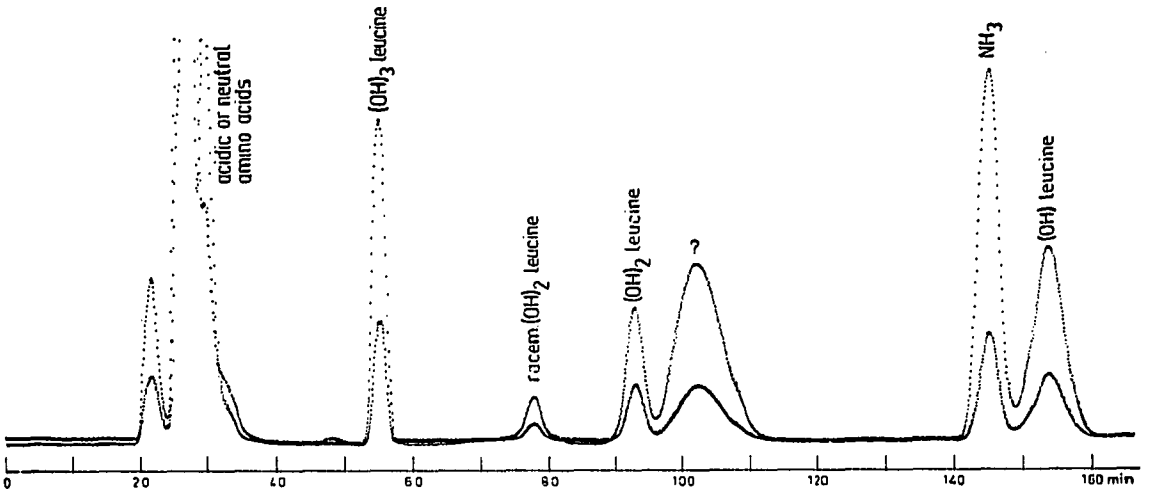

Fig. 3. Elution diagram of γ-hydroxyleucine lactones of phallotoxins.

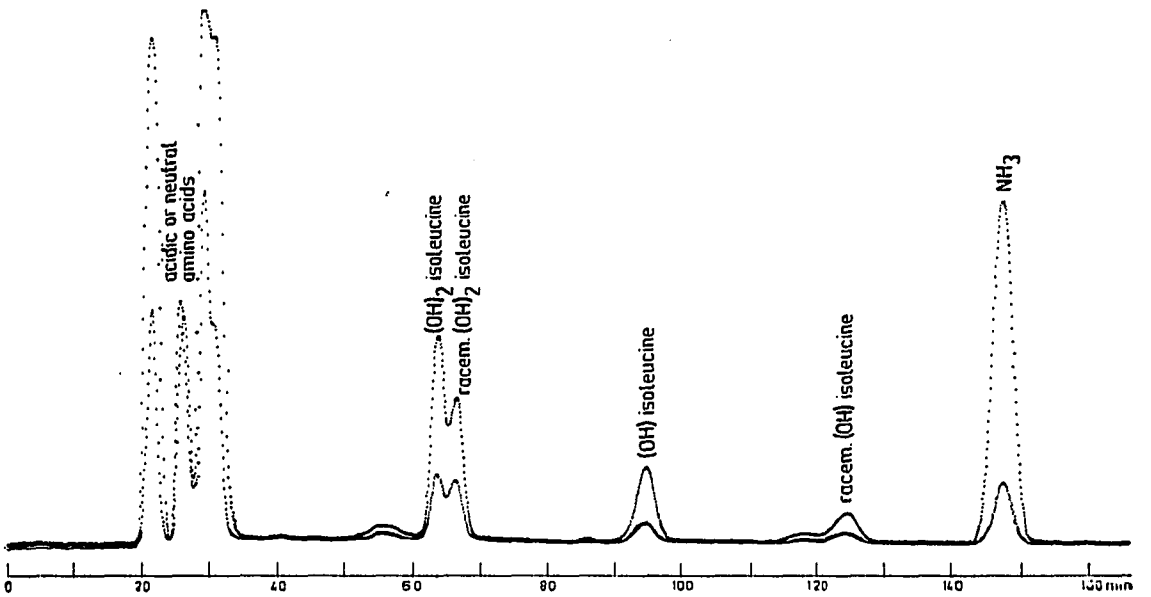

Fig. 4. Elution diagram of γ-hydroxyisoleucine lactones of amatoxins.

The eluates of fractions A and C, which contained mixtures of toxins, were evaporated *in vacuo* and hydrolysed with 6*N* HCl at 110° for 24 h in order to determine the ratio of the different toxins in each fraction by amino acid analysis of their γ-lactones. This analysis was performed on a Beckman Unichrom analyzer. The column, 55 cm × 0.9 cm I.D., was filled with resin M82 (Beckman, Munich). The temperature of the column was 55°, the elution rate 60 ml/h, the ninhydrin flow-rate 30 ml/h and the temperature in the colour reaction 100°. The γ-lactones were eluted with 0.2 *N* citrate buffer, 0.5 *M* in NaCl and at pH 6.2, prepared as follows. To 196 g of sodium citrate dihydrate were added 8 ml of concentrated HCl, 292 g of NaCl, 1 ml of *n*-caprylic acid, 40 ml of detergent solution (50 g of BRIJ from Serva, Heidelberg, in 200 ml of water), and the mixture was made up to 10 litres with water. Elution diagrams for γ-hydroxyleucine and γ-hydroxyisoleucine lactones are shown in Figs. 3 and 4.

An estimation of the amount of each toxin was obtained empirically by using $C_{HW(tox)}$ values for the corresponding γ-lactones on amino acid analysis of the pure toxins.

$$\text{Values of } C_{HW(tox)} = \frac{\text{extinction units} \times \text{time units (at half value)}}{\text{micromoles of toxin}}$$

and the retention (*R*) values of the lactones are given in Table I.

TABLE I

R AND $C_{HW(tox)}$ VALUES OF NATURALLY OCCURRING γ-LACTONES IN AMINO ACID ANALYSIS UNDER THE CONDITIONS DESCRIBED IN THE TEXT

γ-Lactone	*Natural source*	*R* (*min*)	$C_{HW(tox)}$
γ,δ,δ'-Trihydroxyleucine	Phallisin or phallisacin	55	12.3
γ,δ-Dihydroxyleucine	Phalloidin or phallacidin	93	5.2
Epimeric γ,δ-dihydroxyleucine	Phalloidin or phallacidin	78	1.2
γ-Hydroxyleucine	Phalloin	154	27.0
γ,δ-Dihydroxyisoleucine	α- or β-Amanitin	63	15.3
Epimeric γ,δ-dihydroxyisoleucine	α- or β-Amanitin	66	7.7
γ-Hydroxyisoleucine	γ-Amanitin	94	7.1
Epimeric γ-hydroxyisoleucine	γ-Amanitin	(118), 124	2.1
Ammonia	—	140	

RESULTS AND DISCUSSION

This simple technique of fractionating crude extracts of *Amanita phalloides* proved successful in providing a measure of all of the toxins in single mushrooms of that species. It was observed that acidic toxins are present in a larger amount than the neutral toxins, accounting for up to 70 % of the total toxins present. The sensitivity of this technique enabled us to determine very small amounts of every toxin known so far, and, in addition, to discover some new ones that had not been previously detected even in large-scale preparations[17]. The amount of lipid material, which is detrimental to chromatographic procedures, was reduced by extraction with dry ether. A second extraction with hot methanol left most of the inorganic

salts and proteinaceous residue undissolved and proved superior to treatment with dry pyridine. By this extraction, however, a small amount of acidic phallotoxin ammonium salts remained in the insoluble material.

In the column chromatography which followed, we made use of the procedure of WIELAND AND BUKU[18], who isolated toxins on a preparative scale by this method. Due to adsorption effects of the aromatic parts of the peptides on polysaccharides, the toxins could be eluted by water well separated from each other and from most of the impurities (Fig. 1). In fraction A we found phallacidin (PHCD) and a toxic component not previously described, for which we propose the name phallisacin (PHSC). It probably represents the trihydroxyleucine analogue of phallacidin, because the amino acid analysis yielded the lactone of phallisin besides valine and alanine in a ratio of 1:1[15]. In fraction A amanin (AMN) was also detected. Fraction B consisted mainly of β-amanitin together with three compounds which gave amanitin-like reactions with cinnamic aldehyde–hydrochloric acid and which were not further investigated, but did not include ε-amanitin. It also probably contained urea[17], which gave a yellow colour reaction with cinnamic aldehyde–hydrochloric acid. In fraction C we found phalloidin (PHD), phallisin (PHS) and in one instance trace amounts of phalloin (PHN) as determined by its γ-lactone. Fraction D contained nearly all of the α-amanitin, together with a more lipophilic substance, which absorbs at 254 nm but gives a negative test with cinnamic aldehyde–hydrochloric acid. Fraction E consisted mainly of γ-amanitin together with a small amount of α-amanitin, perhaps trace amounts of amanullin (AMO), and an unknown substance similar to that in fraction D. As all fractions contained some brown polar material, they were subjected to thin-layer chromatography as described above.

Nevertheless, fraction C remained nearly unresolved with respect to the toxins even on thin-layer chromatography. Likewise, the resolution of fraction A was not complete, even in *sec.*-butanol–ammonia solution; although we obtained phallisacin as a single compound, phallacidin was always associated with phallisacin. Fractions A and C were hydrolysed and the ratios of the different toxins were calculated by an amino acid analysis of their γ-hydroxylated leucines. This was easy to perform, because the γ-lactones of the hydroxylated leucines (and isoleucines) are eluted separately from the columns like basic amino acids, which are absent in *Amanita* toxins (Figs. 3 and 4). As the γ-hydroxyamino acids, however, are not eluted quantitatively as γ-lactones, but part of them also as neutral γ-hydroxyamino acids, and as most of the colour coefficients for the ninhydrin reaction of the lactones have not been determined, we related the areas of the different lactones obtained on amino acid analysis empirically to the lactone yield of known amounts of pure toxins, thus avoiding these uncertainties. In each hydrolysate, both of amanitins and phallotoxins, a remarkable amount of ammonia was found, which could be used as an internal standard for the R values of the various γ-lactones. Additionally, on hydrolysis of all phallotoxins, a basic compound was produced, which was not further investigated and which probably originates from the decomposition of the indole nucleus of the tryptophylthioether moiety of the phallotoxins.

During hydrolysis of the toxins, lactones with more than one chiral carbon atom undergo epimerisation[19], which could be followed on the amino acid analyzer. This epimerisation appears to take place at the chiral γ-carbon atom, as deduced from the fact that no racemisation at the α-carbon atom was observed for γ-hydroxy-

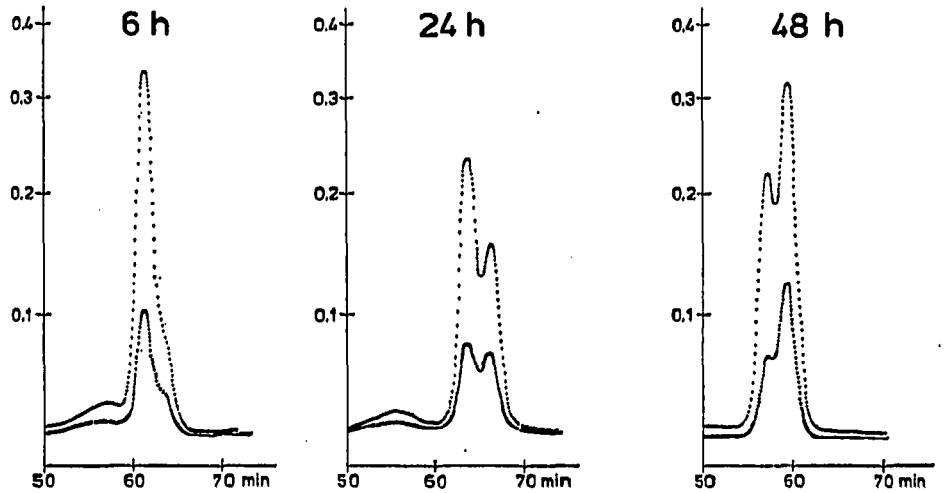

Fig. 5. Elution diagram of γ,δ-dihydroxyisoleucine lactones after 6, 24 and 48 h of hydrolysis of α-amanitin in 6 *N* HCl at 110°.

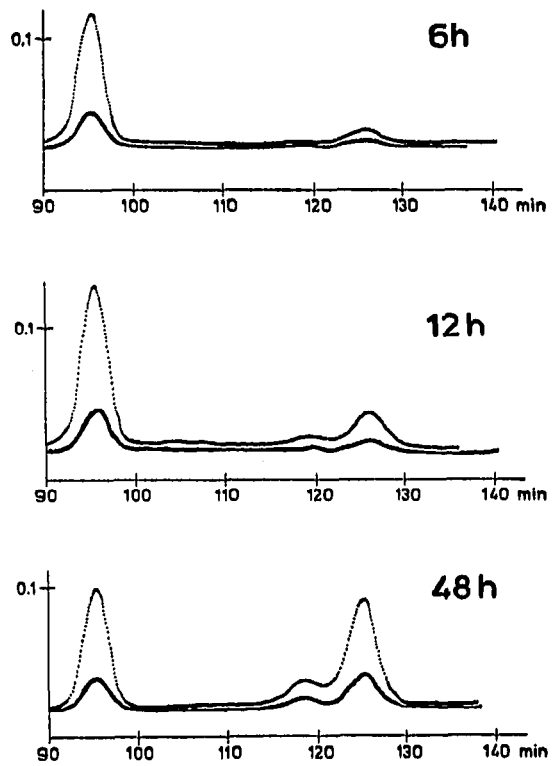

Fig. 6. Elution diagram of γ-hydroxyisoleucine lactones after 6, 12 and 48 h of hydrolysis of γ-amanitin in 6 *N* HCl at 110°.

D-leucine lactone[20] under the same conditions of acidic hydrolysis. Hydrolysis for different time intervals revealed the naturally occurring lactones from the amanitins, and also indicated the rates of epimerisation of these lactones. The equilibrium for γ,δ-dihydroxyisoleucine clearly does not favour the naturally occurring isomer, which possesses the most sterically hindered conformation[19]. Elution diagrams are shown in Figs. 5 and 6. During the epimerisation of γ-hydroxyisoleucine, a minute amount of a third compound was formed, possibly a member of the *allo*-isoleucine series formed by epimerisation at the chiral β-carbon atom.

Under the conditions commonly used (24 h in 6 *N* hydrochloric acid at 110°), the rates of epimerisation were found to be reproducible to within 16% for the dihydroxylated leucine, obtained either from phalloidin or phallacidin, and to within 37% for the dihydroxylated isoleucine obtained either from α- or β-amanitin. The corresponding epimerisation for the γ-hydroxylated isoleucine of γ-amanitin was found to be about 50%.

Comparison of the toxin values obtained by the lactone analysis showed that the amounts were consistently lower than those found by the spectrophotometric method. To ensure that the spectrophotometric measurements of the various toxins were correct, we checked these values by determination of the lethal doses of all the fractions when applied parenterally to white mice. The toxicities found in this test were in good agreement with the spectrophotometric data. The lactone analysis was therefore used only to determine the ratio of toxins in fractions which could not be resolved by chromatographic methods. The average values for the various toxins found in 2-g samples of air-dried material (I) and in 25 g of fresh mushrooms (II) are given in Table II. Sample I differs distinctly from sample II in having suffered higher hydroxylation of the side-chains with respect to the phallotoxins, for it contains much more phallisacin than phallacidin and about as much phallisin as phalloidin. With respect to the amatoxins, there are only small differences between the two samples.

TABLE II

AVERAGE AMOUNTS OF VARIOUS TOXINS FOUND IN MUSHROOMS OF *Amanita phalloides* FROM DIFFERENT SOURCES

I: 2 g of air-dried material, collected near Trento, Italy; II: 25 g of fresh mushrooms collected near Heidelberg, G.F.R.

Toxin	*Abbrevation*	*I* (*mg*)	*II* (*mg*)
Phallisacin	PHSC	6	2.6
Phallacidin	PHCD	2.4	3.7
Amanin	AMN	traces	0.6
β-Amanitin	β-AMA	2.3	1.2[a]
Phallisin	PHS	0.8	0.2
Phalloidin	PHD	0.9	2.0
Phalloin	PHN	traces	—
α-Amanitin	αAMA	2.0	1.8
γ-Amanitin	γ-AMA	0.2	0.2[b]

[a] Plus 0.5 mg of three unknown acidic amatoxins.
[b] Together with trace amounts of a more lipophilic amatoxin, probably amanullin (AMO).

The procedure described was also successfully applied to 1 g of dried *Amanita phalloides* mushrooms, and may serve to detect qualitatively the main toxins in much smaller amounts of plant tissue. Additionally, the column procedure may be used to prepare amatoxins and mixtures of phallotoxins from single specimens of *A. phalloides*.

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