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Simultaneous assay for amatoxins and phallotoxins in *Amanita phalloides* Fr. by high-performance liquid chromatography

F. Enjalbert*

Laboratoire de Botanique, Phytochimie et Mycologie, Faculté de Pharmacie, Avenue Charles Flahaut, 34060 Montpellier (France)

C. Gallion, F. Jehl and H. Monteil

Institut de Bactériologie, 3 Rue Koeberlé, 67000 Strasbourg (France)

H. Faulstich

Max-Planck-Institut für Medizinische Forschung, Jahnstrasse 29, W-6900 Heidelberg (Germany)

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method is described that allows the simultaneous determination of up to eight amatoxins and phallotoxins. The method identifies both neutral toxins (α- and γ-amanitin, phalloidin, phallisin and phalloin) and acidic toxins (β-amanitin, phallacidin and phallisacin). Toxins were separated, identified and determined by gradient elution with 0.02 *M* aqueous ammonium acetate–acetonitrile and simultaneous monitoring of the absorbances at 214 and 295 nm. The assay was successfully applied to the analysis of the toxins in a crude extract of *Amanita phalloides*. The limit of detection for each toxin was 10 ng/ml of extraction medium. The assay was further validated by analysing the toxin content in *Galerina marginata*, a species containing only amatoxins. This relatively simple method should be suitable for the detection of amatoxins and phallotoxins in almost any species of mushrooms.

INTRODUCTION

The isolation and complete chemical characterization of the bicyclic peptide toxins from *Amanita phalloides* Fr. has led to the identification of two major classes of peptides, known as amatoxins and phallotoxins [1]. The amatoxins are a family of bicyclic octapeptides which bind and inhibit eukaryotic RNA polymerase II and are considered to be the principal agents responsible for human poisoning by this mushroom. The phallotoxins, bicyclic heptapeptides, block depolymerization of filamentous F-actin to globular G-actin [2,3].

Various chromatographic methods have been used to determine the toxic peptides in mushrooms. The most reliable of these approaches is column chromatography, followed by the identification of single toxic compounds using spectrophotometric or colorimetric methods [4–6]. Although this technique allows the determination of single components, it is time consuming and laborious. Other analytical attempts are based on thin-layer chromatography (TLC) or high-performance TLC (HPLTC) [7–11]. In these procedures, chromatographic spots on thin-layer plates, coloured for amatoxins and fluorescent for phallotoxins, were measured spectrophotometrically. Most of these investigations have been reviewed [1].

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Small amounts of biologically active toxic peptides can be determined by bioassays and binding assays using either the target proteins or antibodies. A binding assay employing the affinity of muscle rabbit actin for a labelled phallotoxin derivative, [^{3}H]demethylphalloin, has been reported [12]. A more sensitive assay is based on the protective effect of phalloidin on the inhibition of a pancreatic desoxyribonuclease by actin [13]. For the determination of amatoxins, a technique using the specific inhibition of the DNA-dependent RNA polymerase II has been developed [14,15]. It is obvious that only the sum of all phallotoxins or amatoxins can be determined using bioassays; the same is true for radioimmunoassays (RIA). First, Fiume *et al.* [16] succeeded in raising antibodies against β-amanitin in rats. In rabbits also, amatoxin-specific antibodies could be obtained when conjugates of α-amanitin with fetuin [17,18], or detoxified amanitin derivatives [19] are used as antigens. RIA methods, permitting the detection of amanitins in the range of nanograms per millilitre, have mostly been applied to the detection of amatoxins in biological fluids from patients [18,19].

For the same purpose, high-performance liquid chromatographic (HPLC) methods for amatoxins have been developed by several laboratories [20–25]. These methods are fairly sensitive and can therefore quickly provide information on the severity of an intoxication from *A. phalloides* or from other amatoxins containing fungi or on the effectiveness of the detoxication treatment. Although HPLC can be extremely useful for the determination of toxic components in mushroom extracts [10,23], so far only a few papers have been published reporting applications of this technique. Moreover, no detailed quantification of amatoxins and phallotoxins has been reported.

Here we present a method based on reversed-phase HPLC for the simultaneous determination of up to eight amatoxins and phallotoxins in *A. phalloides* and other mushroom extracts.

EXPERIMENTAL

Standard solutions and chemicals

α-Amanitin (α-Ama), β-amanitin (β-Ama), phalloidin (PHD) and phallacidin (PCD) were provided by Sigma (St. Louis, MO, USA). γ-amanitin (γ-Ama), phalloin (PHN), phallisin (PHS) and a mixture of phallacidin (PCD) and phallisacin (PSC) were prepared according to previously published procedures [26–29] and in our laboratory [5].

Stock solutions of 500 μg/ml of each toxin were prepared in doubly distilled water and stored at −20°C. Methanol, hydrochloric acid, acetonitrile, ammonium acetate and acetic acid were of analytical-reagent grade (Merck, Darmstadt, Germany). Water was obtained daily from a Milli-Ro–Milli-Q system (Millipore, Molsheim, France).

Extraction procedure

A fresh, fruiting body (18.4 g) of *A. phalloides* consisting of three organs (cap, stipe and volva) was chopped into small pieces. Each fragment of about 2 g was introduced in a cartridge of a Model 6700 freezer-mill (SPEX Industries, Edison, NJ, USA), crushed for 1–2 min and extracted with 3 ml of extraction medium [methanol–water–0.01 *M* hydrochloric acid (5:4:1, v/v/v)]. Overall, 30 ml of extraction medium were utilized. The mixed extracts were incubated overnight at 4°C, then centrifuged at 1000 *g* for 10 min. The supernatant was collected and preserved at 4°C and the pellet was mixed again with 8 ml of the extraction medium and incubated for other 12 h at 4°C. The mixture was centrifuged again at 1000 *g* for 10 min and the supernatant was pooled with the other. A 20-μl aliquot of the combined supernatants was used for the separation and determination of the main toxins. The same procedure was used to prepare extracts from *Galerina marginata* (Batsh) Kühn. and *Amanita pantherina* (D.C.: Fr.) Krombh.

Apparatus and chromatographic conditions

Chromatographic separation of different toxins was carried out by gradient elution. The HPLC apparatus was composed of the following units: a Model 114 M solvent-delivery module, a Model 210 A sample injection valve with a 20-μl loop and a Model 168 variable-wavelength UV diode-array detector (all from Beckman, Fullerton, CA, USA). This detector allows the monitoring of the eluate at two wavelengths simultaneously and the recording of absorbance spectra at definite time intervals (from 1 spectrum per 2 s to 16 spectra/s). In this work, the system was set at 1 spectrum/s. Chromatograms were processed with a System GOLD chromatographic data system (Beckman).

Separations were performed at ambient temperature on a reversed-phase 5-μm Ultrasphere ODS column (250 × 4.6 mm I.D.) (Beckman). The mobile phase was a mixture of two solvents: solvent A was 0.02 *M* aqueous ammonium acetate–acetonitrile (90:10, v/v) and solvent B was 0.02 *M* aqueous ammonium acetate–acetonitrile (76:24, v/v). The pH of mixtures A and B was adjusted to 5 with filtered glacial acetic acid. The gradient profile was as follows: 100% A for 4 min, then 57% B for 16 min, then 100% B for 10 min and finally 100% A. The mobile phase flow-rate was 1 ml/min. The absorbance of the eluate was monitored simultaneously at 214 and 295 nm.

Quality control parameters of the method

A calibration graph was prepared in the extraction medium with increasing amounts of each of the eight toxins yielding concentrations of 1, 50 and 100 μg/ml (except for PCD: 1, 5 and 10 μg/ml). The limit of detection was defined as the lowest concentration of each toxin resulting in a signal-to-noise ratio of 4. The accuracy of the method was investigated for each toxin at three concentrations (1, 50 and 100 μg/ml) by comparing the amount of toxin added to the extraction medium with that actually measured. The precision was determined by calculating the within- and between-day relative standard deviations (R.S.D.) (n = 10) at the same levels.

RESULTS

Chromatographic parameters of toxins

The chromatographic parameters of the toxins are shown in Table I. The values of the capacity factors are in agreement with the currently accepted values. The selectivity factors for two neighbouring peaks, defined as the ratio of their capacity factors, k'_2/k'_1, are 1.15, 1.21, 1.06, 1.18, 1.20, 1.34 and 1.59. A good resolution of each toxin was obtained (Fig. 1).

Amatoxins eluted earlier than phallotoxins. Within each of the two families the toxins eluted as a function of lipophilicity: acidic toxins eluted earlier than neutral toxins (β-amanitin before α- and γ-amanitin; phallisacin and phallacidin before phallisin, phalloidin and phalloin), and toxins having a higher number of hydroxy groups eluted earlier than those with a smaller number (α-amanitin before γ-amanitin; phallisacin before phallacidin; phallisin before phalloidin before phalloin).

TABLE I

CHROMATOGRAPHIC CHARACTERISTICS OF TOXINS

Results are means ± S.E.M.; n = 4.

Toxin	Retention time (min)	Capacity factor (k')	Absorbance ratio (214/295 nm)
β-Ama	8.12 ± 0.09	1.86 ± 0.03	2.68 ± 0.03
α-Ama	8.87 ± 0.02	2.14 ± 0.03	2.81 ± 0.05
PSC	10.18 ± 0.04	2.61 ± 0.03	2.30 ± 0.06
γ-Ama	10.64 ± 0.06	2.77 ± 0.04	2.65 ± 0.01
PCD	12.04 ± 0.18	3.27 ± 0.1	2.52 ± 0.15
PHS	13.89 ± 0.18	3.94 ± 0.09	2.73 ± 0.05
PHD	17.66 ± 0.31	5.28 ± 0.14	2.72 ± 0.18
PHN	26.57 ± 0.16	8.44 ± 0.13	2.63 ± 0.04

The 214/295 nm absorbance ratios were established for each toxin with pure standards and are reported in Table I.

Quality control

The limit of detection for both groups of toxins was 10 ng/ml of extraction medium, corresponding to a detection limit of 0.5 ng/g of fungal matrix. Quantification of toxins, based on peak areas, was linear between 1 and 100 μg/ml (correlation coefficient 0.9987). This concentration range includes the concentration of toxins usually found in fresh mushroom extracts. The accuracy of the measurements of α-amanitin and phalloidin is shown in Table II. Similar values were obtained for other toxins. The precision was evaluated by calculating intra- and inter-day R.S.D. values which ranged from 1.1 tot 4.2% (n = 10).

Assay

The chromatogram illustrated in Fig. 2 shows the analysis of the toxins in an *A. phalloides* extract. The eight toxins are completely resolved from endogenous peaks. The purity of each peak was monitored by comparison of its 214/295 nm absorbance ratio and the superimpostion of its UV absorption spectrum with those of the standards. The maxima for the phallotoxins and the amatoxins are located

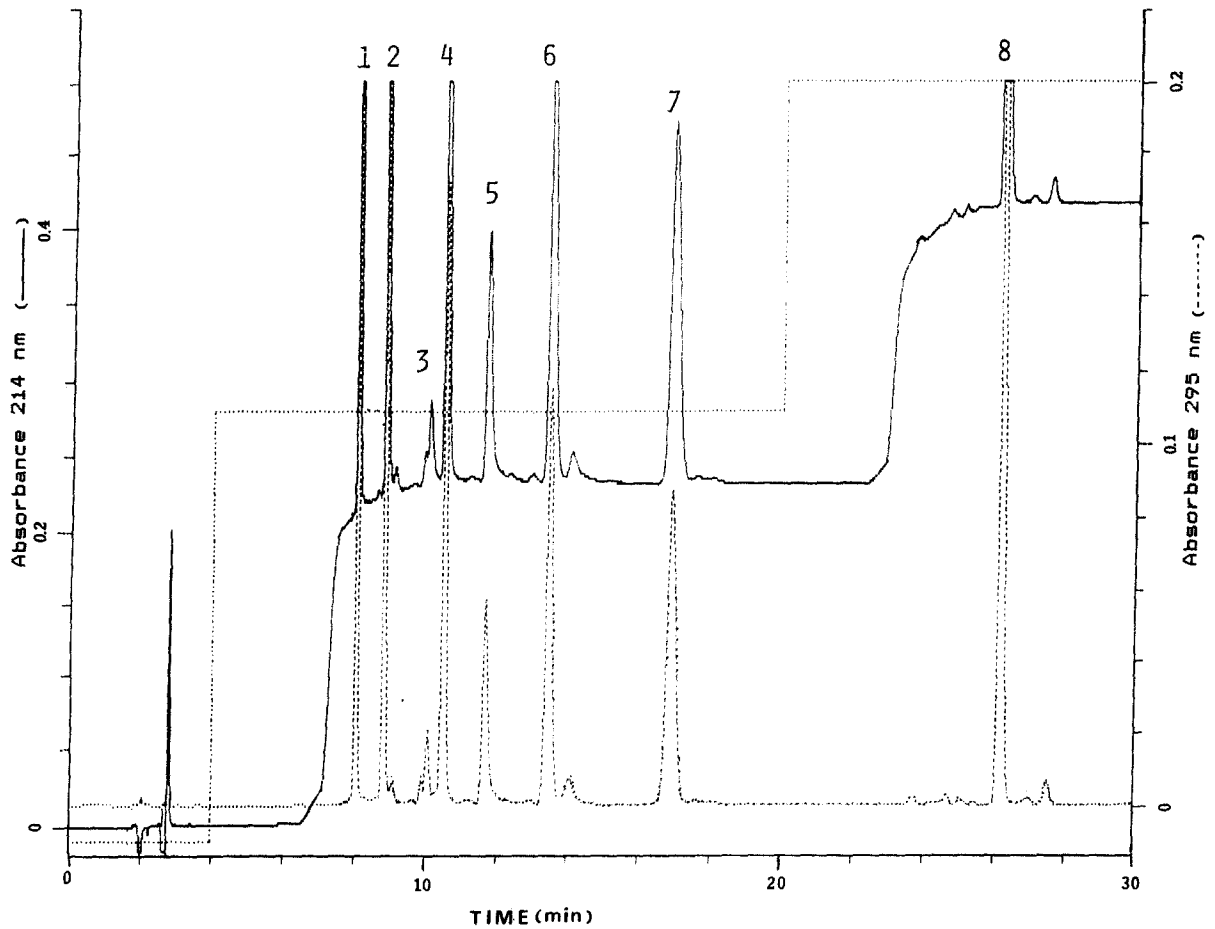

Fig. 1. Chromatogram resulting from the analysis of the standard mixture of the eight toxins. Concentrations were 100 μg/ml (except for phallisacin, 10 μg/ml). Peaks: 1 = β-Ama; 2 = α-Ama; 3 = PSC; 4 = γ-Ama; 5 = PCD; 6 = PHS; 7 = PHD; 8 = PHN.

TABLE II

RECOVERY OF α-AMANITIN AND PHALLOIDIN IN THE EXTRACTION MEDIUM

Compound	Toxin added (μg/ml)	Toxin measured (μg/ml)[a]	R.S.D. (%)
α-Amanitin	1	0.95 ± 0.05	5.0
	50	50.90 ± 0.91	1.8
	100	98.00 ± 1.96	2.0
Phalloidin	1	1.05 ± 0.05	5.0
	50	51.20 ± 1.22	2.4
	100	103.00 ± 3.10	3.0

[a] Mean ± S.D. (n = 3).

at wavelengths 285 and 305 nm, respectively (Fig. 3).

The content of toxic peptides in a single sample of *A. phalloides* was 0.07% of the fresh tissue and is reported in Table III. The amatoxin content was found to be 291 μg/g of fresh tissue and is in good agreement with the amount of amatoxins assayed by RIA (200–400 μg/g of fresh tissue [30]; acidic amatoxins represented 16% and neutral amatoxins represented 25.4%. The phallotoxin content was found to be 412.2 μg/g of fresh tissue, which corresponds to 5.15 mg/g dry weight. This value is very close to those obtained by spectrophotometry, *i.e.*, 3.95–5.1 mg/g dry weight [6]. Acidic phallotoxins

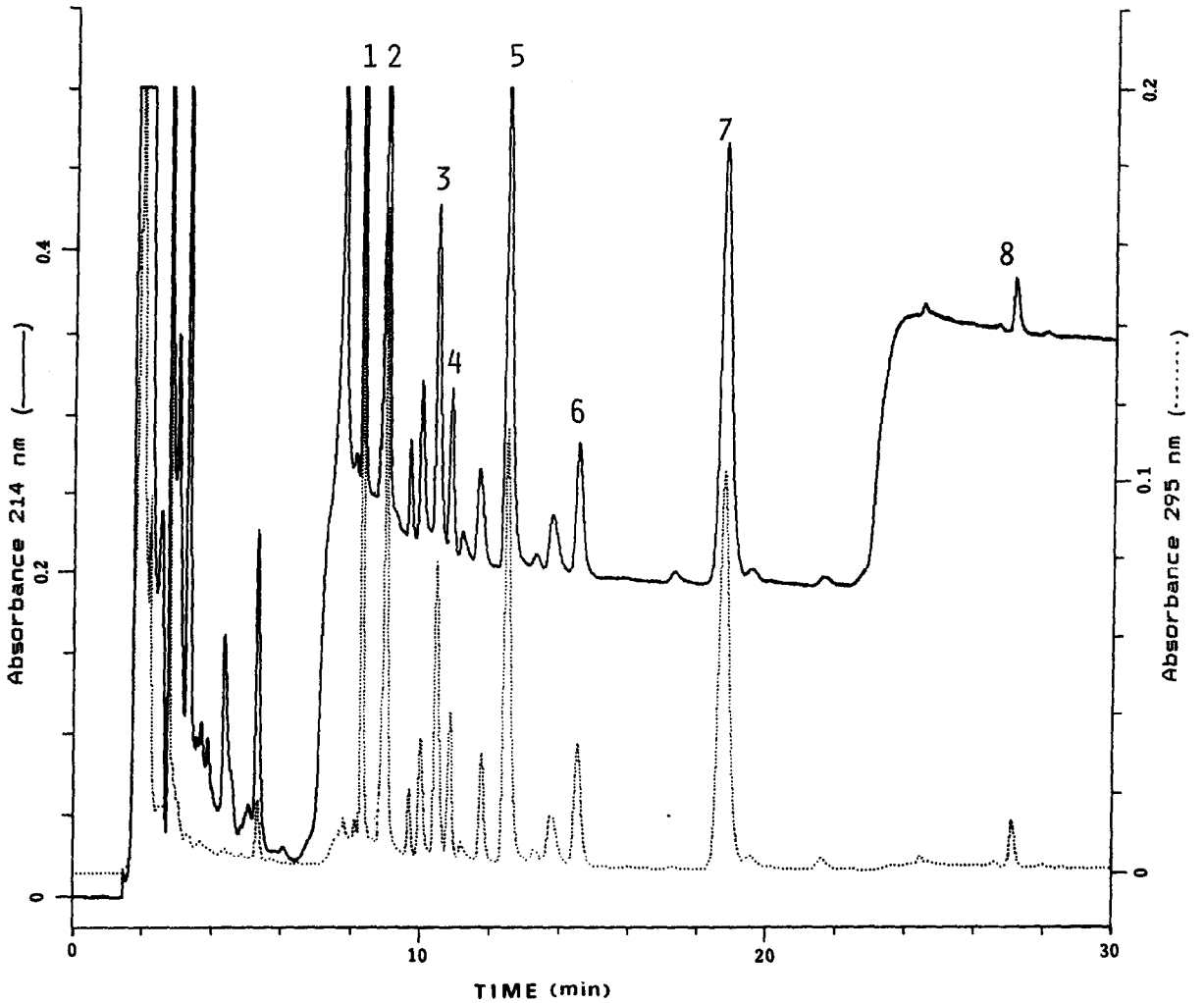

Fig. 2. Chromatogram of an *Amanita phalloides* extract. Peaks as in Fig. 1.

represented 41.3% of the toxin content and neutral phallotoxins 17.3%.

This method was successfully applied to the assay of amatoxins in *Galerina marginata*. This species does not belong to the genus *Amanita*, but it contains amatoxins. Good resolution of the three amatoxins (β-, α- and γ-Ama) was observed (Fig. 4). The amatoxin in this mushroom was 54.3 μg/g of fresh tissue; α-Ama represented 46%, β-Ama 45.3% and γ-Ama 8.7%. Phallotoxins were not detected in this species; these results confirm those reported in the literature [31].

Lastly, this procedure was applied to an *A. pantherina* extract. The chromatogram displayed in Fig. 5 shows the analysis of this specimen. The retention time of the major peak (8.5 min) was very close to that of β-Ama, but its scan analysis revealed that it was different from this toxin. The comparison of its 214/295 nm absorbance ratio with that of β-Ama confirms this result. In an attempt to detect amatoxins and phallotoxins, we prepared a concentrated extract. Even under these conditions, neither of the toxic cyclopeptides could be found.

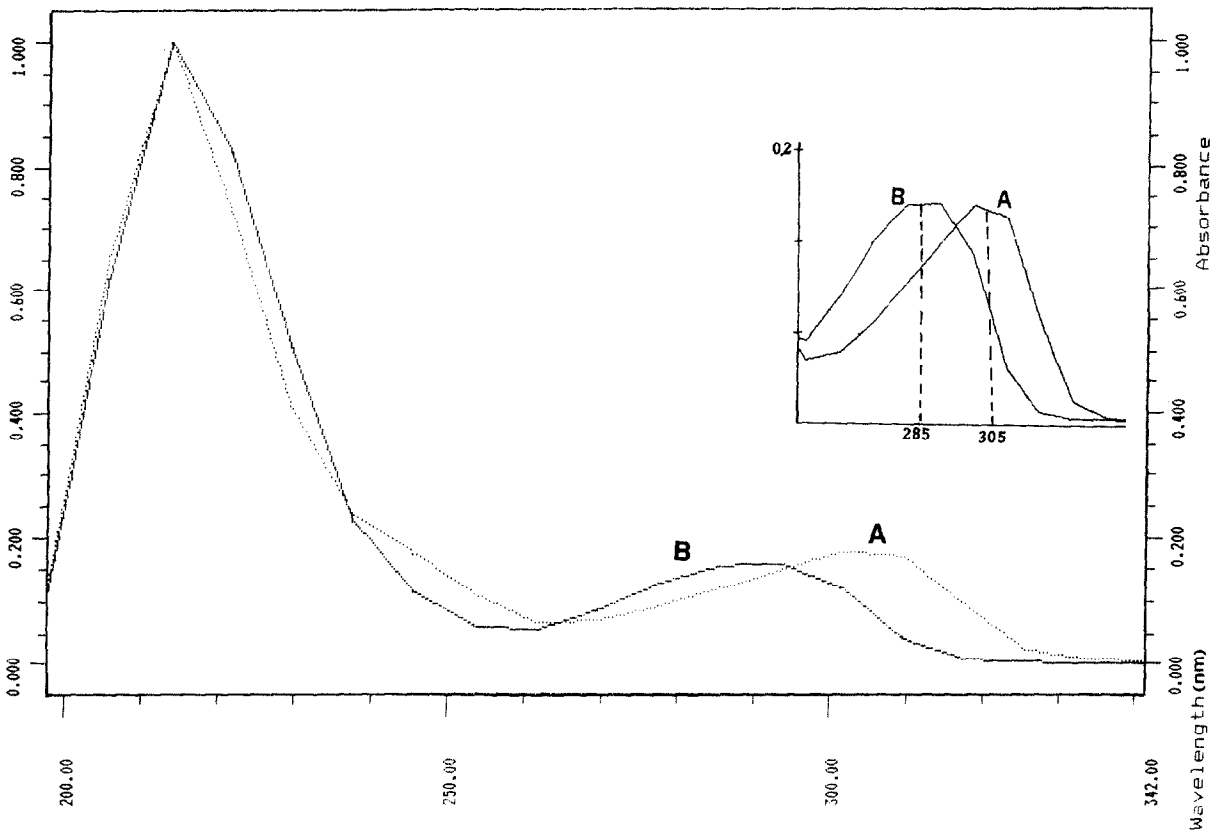

Fig. 3. Absorbance spectra (200–342 nm) of (A) amatoxins and (B) phallotoxins, measured on-line with the diode-array detector. Expanded: comparison between spectra of amatoxins and phallotoxins at 285 and 305 nm.

TABLE III

TOXIN CONCENTRATIONS (μg/g OF FRESH TISSUE) IN A SINGLE SPECIMEN OF *AMANITA PHALLOIDES*. TOTAL CONTENT (mg) OF THE WHOLE MUSHROOM (FRESH WEIGHT 18.4 g) AND DISTRIBUTION OF TOXINS

Toxin	Concentration (μg/g)	Total amount (mg)	Distribution (%)
β-Ama	112.5	2.07	16
Total acidic amatoxin	112.5	2.07	16
α-Ama	123	2.26	17.5
γ-Ama	55.6	1.02	7.9
Total neutral amatoxins	178.6	3.28	25.4
Total amatoxins	291.1	5.36	41.4
PSC	104.8	1.93	14.9
PCD	185.7	3.41	26.4
Total acidic phallotoxins	290.5	5.34	41.3
PHS	11.3	0.20	1.6
PHD	105.5	1.94	15
PHN	4.9	0.09	0.7
Total neutral phallotoxins	121.7	2.23	17.3
Total phallotoxins	412.2	7.59	58.6
Total toxins	703.3	12.94	

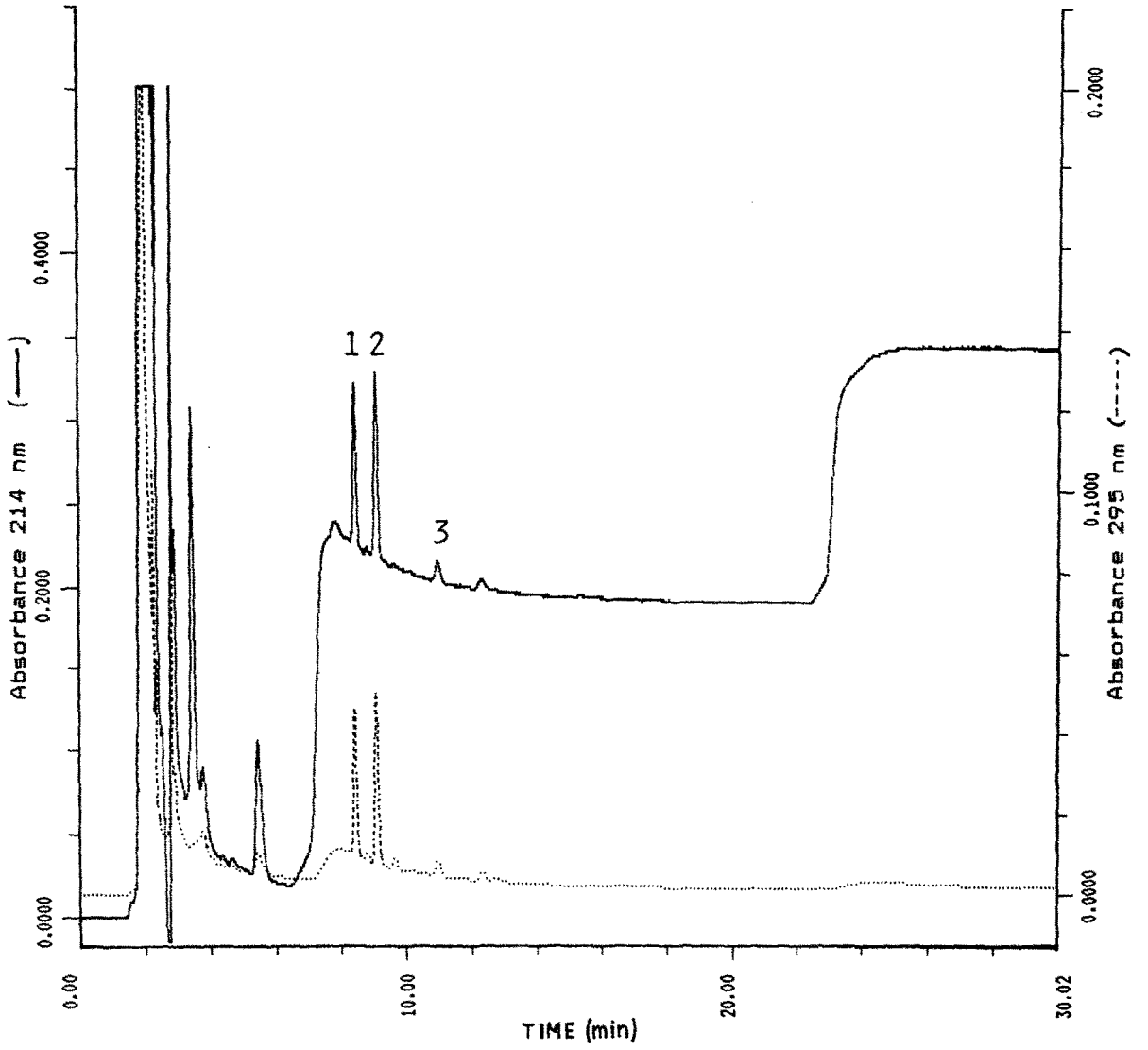

Fig. 4. Chromatogram of a *Galerina marginata* extract. Peaks: 1 = β-Ama; 2 = α-Ama; 3 = γ-Ama.

DISCUSSION

Sample preparation procedure

Significant variations in the amounts of toxins present in *A. phalloides* have been reported [8,32]. For this reason, we analysed an individual carpophore instead of pooled material. The advantage of carrying out these assays on fresh material has been reported [8,11], as drying causes degradation of toxins and chiefly phallotoxins. The extraction medium methanol–water–0.01 *M* hydrochloric acid (5:4:1, v/v/v) was preferred to methanol–water (1:1, v/v), as a slightly acidic pH appears to enhance the stability of molecules [6] and to increase the recovery of toxins. Most of the extraction procedures reported in the literature were carried out using a Soxhlet apparatus [8,13,15], by stirring with a magnetic stirrer [4,6,7,10] or by ultrasonication [11]. The tissues

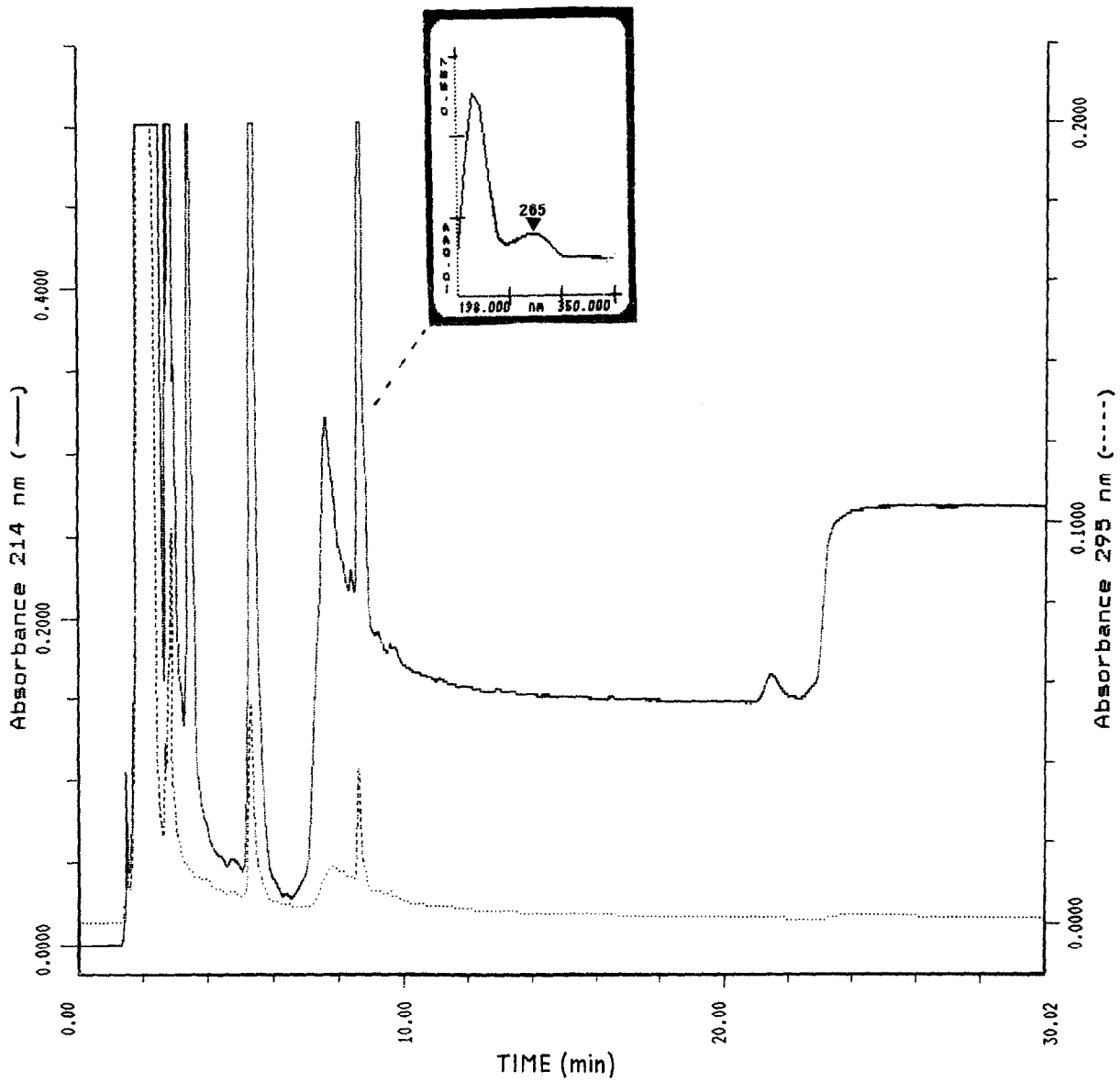

Fig. 5. Chromatogram of an *Amanita pantherina* extract. Expanded: spectrum of the peak (retention time = 8.5 min) located at 265 nm.

were crushed with a freezer-mill to avoid heating of the samples [11]. A rapid and almost complete extraction was achieved by this procedure, as additional operations with the same extraction medium did not lead to the recovery of detectable amounts of residual toxins. Moreover, when small volumes of extraction medium are used no concentration step is needed.

HPLC method

The HPLC technique described here proved to be highly efficient for assaying toxins in mushroom extracts. To our knowledge, this is the first description of an assay for the simultaneous determination of eight toxins. Comparison of this method with existing methods reveals two advantages: first, peak identification is carried out by means of the super-

imposition of its scan analysis with that of the standard, followed by comparison of its 214/295 nm absorbance ratio with the reference value previously established with the pure toxin (Table I). As shown in Fig. 3, the maximum wavelengths of the UV absorption spectra for phallotoxins (285 nm) and amatoxins (305 nm) are sufficiently different to allow their spectral discrimination. The second advantage is the limit of detection, down to the nanogram range. In fact, TLC and HPTLC methods with staining of toxins by cinnamaldehyde/HCl, diazotized sulfanilic acid or p-dimethylaminobenzaldehyde/H_2SO_4 are of limited sensitivity [7,8,11]. Bioassays, such as protein-binding assay, RIA or enzyme inhibition assay are able to measure small amounts of toxins in the nanogram range [12–14,18,19]. But they cannot be used to determine the amounts of the individual toxins in each family because of varying degrees of crossreactivity of the binding protein [12] and the antibodies [18]. Moreover, a crude mushroom extract may contain compounds that interfere with bioassays [31].

The HPLC method was able to resolve simultaneously the main amatoxins and phallotoxins with acceptable sensitivity. In fact, an acidic and two neutral amatoxins (β-, α- and γ-amanitin) together with three neutral phallotoxins (PHS, PHD and PHN) and two acidic phallotoxins (PCD and PSC) could be assayed. Phallacin (PCN), another acidic phallotoxin, could not be analysed because the toxin standard was not available. However, in our *A. phalloides* sample, examination of the UV spectra of all peaks did not reveal any molecule having a maximum UV absorption around 285 nm that might be attributed to PCN; either the PCN content was very low or this toxin was not present in the mushroom.

In papers on the determination of cyclopeptides of *Amanita* species from the northeastern USA, by means of UV absorption or direct spectrophotometry [6,10], only the amounts of three phallotoxins (PHN, PHD and PCD) were reported. A more detailed analysis has been described, using a combination of chromatographic separation with spectrophotometric determination. In a single *A. phalloides* mushroom, up to eleven toxic compounds can be identified, but this method is time consuming and unsuitable for application to large numbers of specimens [5]. On the other hand, the advantages of the present procedure are selectivity, sensitivity and rapidity.

The determination of the toxin content in *Galerina marginata* shows that the proposed method can be used to measure the amounts of amatoxins in species other than *A. phalloides*. Overall, the results indicate that this novel HPLC method can be used to determine amatoxins and phallotoxins in many mushroom species. Furthermore, it should be possible to scale it up to a preparative level. Finally, this approach has proved its effectiveness for diagnostic purposes in clinical toxicology [20–25].

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