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QUANTITATIVE DETARMINATION OF α - AMANITIN BY HPLC IN *AMANITA PHALLOIDES* GROWING IN İSTANBUL FORESTS

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SUMMARY

In Turkey, mushroom poisonings are frequent especially in autumn and spring. In recent years, a great number of mushroom poisoning cases have been reported in İstanbul. Excursions to forests in İstanbul and its surroundings evidenced the widespread growth of *Amanita phalloides* in large numbers. The amount of α-amanitin was determined by HPLC in samples collected in 1991 and 1992.The α-amanitin content is between 4.4-13.3 mg/100 g in fresh mushrooms. Humidity and temperature are the most important factors affecting the amatoxin content in mushrooms.

Key words: *Amanita phalloides,* α-amanitin, Mushroom poisoning, HPLC.

ÖZET

Türkiye'de mantar zehirlenmeleri, özellikle ilkbahar ve sonbaharda sık görülmektedir. Son yıllarda İstanbul'da çok sayıda mantar zehirlenmesi vakalarının meydana gelmesi üzerine İstanbul ve çevresindeki ormanlara yapılan gezilerde *Amanita phalloides* türünün yaygın olarak yetiştiği saptanmıştır. 1991 ve 1992 yıllarında toplanan *A.phalloides* örneklerinde HPLC ile α -amanitin miktar tayini yapılmış ve sonuç olarak 100g taze mantarda 4.4-13.3 mg arasında değiştiği belirlenmiştir. Havanın nemi ve ısısı mantardaki amatoksin miktarını etkileyen en önemli faktörlerdir.

Anahtar kelimeler: *Amanita phalloides,* α–amanitin, Mantar zehirlenmeleri, HPLC.

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INTRODUCTION

In Turkey, poisoning by mushrooms is the most frequent form of plant intoxications, especially in spring and autumn (l).

The edible and toxic mushrooms grow together and are very similar in appearance. These are the main reasons for confusion by the collectors.

About 90 % of the mushroom poisoning are caused by *Amanita phalloides* (Fig. 1). The main toxic substance of this species is α-amanitin (Fig. 2), a cyclopeptide which is hepatotoxic (2, 3). The α-amanitin content of *A.phalloides* growing in North America and Europe has previously been determined by

Fig. 1. *Amanita phalloides*, Belgrad Ormanı (Foto: A. Mat).

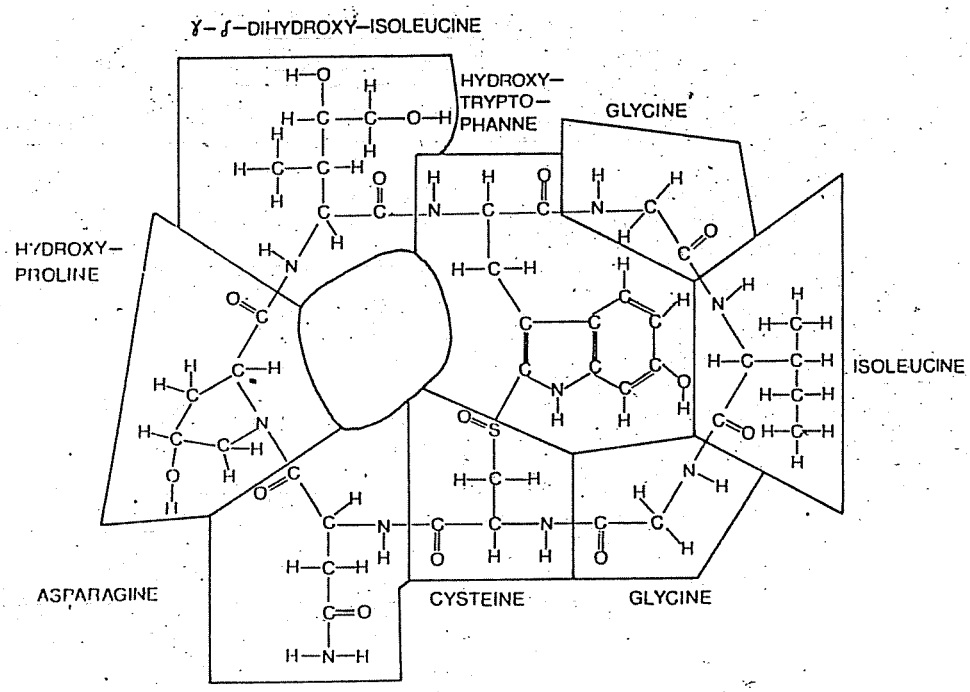

Fig. 2. α-Amanitin.

various investigators and has been found to vary depending on area and season (4-11).

In recent years, a great number of mushroom poisonings have been reported in İstanbul (12, 13). Excursions to forests in İstanbul and its surroundings evidenced the widespread growth of *A.phalloides* in large numbers. In this study, the α-amanitin content of samples collected in 1991 and 1992 was determined by HPLC.

RESULTS AND DISCUSSION

Two samples of *A.phalloides* collected in 1991 and 9 samples collected in 1992 were investigated for their α-amanitin content by HPLC.

The collection data of the samples and the amount of α-amanitin in all samples, given as mg/g dry weight and mg/100 g fresh weight, are shown in table 1. The chromatograms of α-amanitin standard solution and *A.phalloides*

Table 1: Amount of α-amanitin in *Amanita phalloides* specimens from Istanbul

Sample	Collection	Amount of α-amanitin mean ± SD (n=5)	
		mg/g dry weight	mg/100 g fresh weight
1	Belgrat Forest Büyükbent* 16.10.1991	1.704±0.051	a
2	Belgrad Forest Büyükbent* 16.10.1991	1.116±0.013	a
3	Belgrad Forest Büyükbent* 4.11.1992	b	12.23±1.252
4	Belgrad Forest Büyükbent* 4.11.1992	0.45±0.012	12.52±0.419
5	Belgrad Forest Büyükbent* 4.11.1992	b	12.07±0.636
6	Belgrad Forest Büyükbent* 4.11.1992	0.254±0.021	4.39±0.379
7	Beykoz, Göllü** 13.12.1992	0.563±0.009	8.23±0.139
8	Beykoz, Göllü** 13.12.1992	0.509±0.003	10.706±0.072
9	Beykoz, Göllü** 13.12.1992	0.49±0.011	12.508±0.3
10	Beykoz, Göllü** 13.12.1992	0.383±0.008	10.182±0.244
11	Beykoz, Göllü** 13.12.1992	0.693±0.008	13.256±0.184

a= Sample not weighed before drying
b= Sample extracted freshly

Mean: 1991 1.41 mg/g dry weight
1992 0.47 mg/g dry weight
10.79mg/100g fresh weight

* European part of Istanbul
** Asian part of Istanbul

extract are given in Fig.3. For comparison, two samples collected in 1992 were extracted freshly and the others after drying. All extracts showed the same chromatograms on TLC.

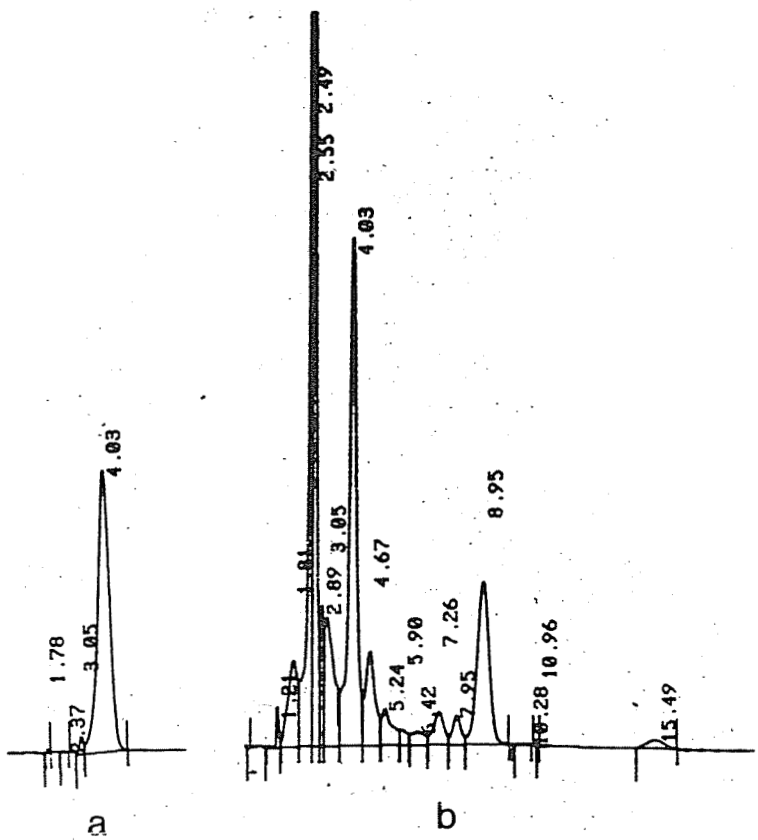

Fig. 3. Chromatograms :a) α-amanitin standard solution, b) *Amanita phalloides* extract, for chromatographic conditions: see text

The calibration curve of peak area of α-amanitin standard solution gave good linearity over a range of 40-200 ng (r=0.9967).

Andary et al. showed that the most important factors affecting the amatoxin content of *A.phalloides* are humidity and temperature (8).

The content of α-amanitin in the samples collected from the same area varies significantly depending on the year of collection. The α-amanitin content of the samples taken in 1991 from Belgrat Forest, Büyükbent, is 3 times as high as those taken from the same locality in 1992. According to information from Me-

teorology Station in İstanbul, the relative humidity between October and December 1991, was higher than the one measured in the same months of 1992. During the excursions in autumn 1993, when the relative humidity was very low no *A.phalloides* were found.

In samples collected from the Europen and Asian parts of İstanbul in the same year, no significant difference was found with respect to α-amanitin content.

The oral lethal dose of α-amanitin for humans is 0.1 mg/kg of body weight (9). Since the α-amanitin content in mushrooms varies from year to year, depending on relative humidity of the season, a definite number of mushrooms causing death in an individual cannot be stated accurately. The α-amanitin content in samples collected in 1991 and 1992 varies between 4.4-13.3 mg/l00 g in fresh mushrooms. According to these results, an amount of 50-150 g of fresh mushrooms ingested may cause fatal poisoning in an adult individual of an average body weight of 70 kg.

Poisonining by *Amanita phalloides* occurs also in European countries and in North America. Different methods of quantitation for amatoxins are used by various authors. For comparison,the α-amanitin contents of samples growing in Europe, North America and İstanbul (Turkey) and the methods used are summarized in Table 2.

Table 2: Amount of α-amanitin in *Amanita phalloides* specimens from different localities

Fungi from	α-amanitin (mg/g dry weight)	Method and References
Europe	1.0	Chromatography, spectrophotometry (4)
Europe	1.9	TLC, coupled with Pauly's reagent, scanning by spectrophotometry (6)
Europe	2.2	HPTLC, spectrophotometry or visual scanning after cinnamaldehyde/HCl (9)
North America	1-2.6	Estimation on TLC (3)
North America	1.2	Chromatography, spectrophotometry in fractions (5)
North America	1.6	TLC, coupled with by Pauly's reagent, scanning by spectrophotometry (10)
İstanbul 1991	1.41	HPLC
İstanbul 1992	0.47	HPLC

EXPERIMENTAL

Material: Fruiting bodies of *Amanita phalloides* were collected from Büyükbent/Belgrat Forest (European part of Istanbul) in October 1991 and November 1992, and from Göllü/Beykoz (Asian part of Istanbul)in December 1992. Fresh mushrooms were weighted and dried at 40°C in an oven under warm air flow. Dried bodies were weighted again, powdered and than extracted separately.

Standards and Solvents: α–Amanitin standard was purchased from Fluka, Buchs, Switzerland (Art.No.06422) and cinnamaldehyde from E.Merck, Darmstadt, Germany. All solvents were of HPLC grade. Water was obtained daily from Milli-Q 50 (Millipore) system.

TLC: For chromatographic separation on TLC the method of Stijve was adopted (14). Silica gel Si 60 F_{254} (Merck) plates were used. The mobile phase was Chloroform-Methanol- Acetic acid-Water (75:33:5:7.5 v/v) and detection was performed with a methanolic solution of cinnamaldehyde (1% v/v, and HCI vapors (12).

HPLC: The HPLC apparatus composed of the following units: a Model 501 solvent pump, a U 6 K Universal Injector, a Model 486 Tunable Absorbance Detector at 303 nm and a Model 746 Data Module (Waters, Milford, Massachussetts, USA). Among several methods of HPLC assayed, Beliardo's method (15) was adopted. The chromatographic conditions were as follows: Chart speed was 0.5 cm/min.; separations were performed at ambient temperature on a reversed phase μBondapak C_{18} column (30cmx3.9 mm ID, 10 μm particle size Waters); the mobile phase was methanol-water (40:60 v/v) run at a flow rate of 1 ml/min. The mobile phase was filtered through an HV-0.45 μm Millipore filter and degassed in an ultrasonic bath (Bandelin, Sonorex TK 52).The injection volume was 10 μl for samples and 20 μl for standard solutions.

Preparation of α-amanitin standard solutions: The stock solution of 0.1 mg/ml was prepared by dissolving 1 mg of α-amanitin in methanol and diluting to volume in a 10 ml volumetric flask (Solution 1). 1 ml of stock solution was quantitatively diluted to 10 ml with methanol (Solution 2). Use of 20μl of this solution led to acceptable peak heigh when UV detector attenuation was 32. A standard curve was obtained by using nine methanolic dilutions of solution 2. The standard solutions were stored at 4°C.

Preparation of *A.phalloides* extract: Different methods of extraction were assayed and the Stijve's method (14) was adopted. Dried mushrooms were powdered or fresh mushrooms were chopped and extracted with 100 ml of methanol under reflux on a water bath for 1 hour and allowed to cool. The extract was filtered through a plug of glass wool into 250 ml flask and the residue was re-extracted with 50 ml of methanol for 30 minutes. The second extract after filtration was added to the first one and evaporated to dryness under vacuum. The residue was disolved in 2 ml of methanol and transferred into 100 ml volumetric flask and adjusted to volume with methanol. 10 µl of this solution containing the toxins were injected to the apparatus.

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