CHAPTER 3

Amanitin Contents of *Amanita* Mushrooms in Chiang Mai Community Forests

3.1 Introduction

In Thailand, wild mushrooms are common only in rainy season, which occurs from June to October. Although the edible wild species command higher prices than cultivated mushrooms, people prefer to consume them due to their flavour and texture (Dell *et al*., 2000). Some poisonous wild mushrooms are often indistinguishable from edible species. The high risks of illness and death rate from consuming the poisonous wild mushrooms were reported to increase every year (Table 1.4 Chapter 1).

Amatoxins interfere with DNA transcription by inhibiting RNA polymerase II (RNA polymerase B), synthesis of messenger RNA and subsequent protein synthesis is interrupted (Wieland, 1986). Cells with high rates of protein synthesis (e.g. those of the gastrointestinal tract, the liver and the kidney) are particularly sensitive to injury. Depending on the development stage, the concentration of amatoxin is higher in old than in young mushrooms. (Jaeger *et al*., 1989). α - Amanitin (a member of amatoxin) are frequently lethal, and are responsible for 90 % of fatal human mushroom poisonings worldwide (Benjamin, 1995). The human LD_{50} is 0.1 mg / kg body weight. This approximately 7 mg toxin for an adult male.

The toxin content and composition of *Amanita phalloides* tissues were determined in three specimens at two carpophore development stages. The carpophore was subdivided into six parts: the cap, gills, ring, stipe, volva and bulb. Substantial differences in the tissue toxin content were revealed. The ring displayed a very high amount of toxins, whereas the bulb had the lowest toxin content. Compositional differences in relation to the nature of the tissue were also noted. The highest amatoxin content was found in the ring, gills and cap, whereas the bulb and volva were the richest in phallotoxins. The differences in the distribution of individual toxin in the tissues might be related to the carpophore developmental stage. The characteristics of the soil at the collection site also affected the toxin concentrations;

however, this effect differed from one site to another and was not similar for all the tissues(Enjalbert *et al*., 1993; Enjalbert *et al*., 1999).

In Thailand, Chaiear *et al*. (1999) reported a case study relating to the death of Thai people who consumed the poisonous mushrooms which mainly belong to genus *Amanita* . However, there is little information available about the α - amanitin in *Amanita* spp. in Thailand. In this study, *Amanita* mushrooms were subsequently examined for the presence of α - amanitin by Thin Layer Chromatography and confirmed by High Performance Liquid Chromatography that has been proven sensitve enough to detect toxins (Enjalbert *et al*., 1992). In addition, a screening test kit based on the Weiland or Meixner method for rapid and efficient detection of α - amanitin was modified, developed for finding the suitable paper to detect α - amanitin.

3.2 MATERIALS AND METHODS

3.2.1 Collection of mushrooms

Fruiting bodies of *Amanita* spp. were collected from June 2003 to August 2005 in 5 areas of Chiang Mai community forests in Doi Saket, Mae Orn, Mae Rim, Mae Wang, and San Kamphaeng districts. The harvested fruiting bodies were dried at 80 °C. for 2 days and powdered in a Moulinex blender.

3.2.2 Standard, reference compounds

Standards of α-amanitin were purchased from Sigma. Stock solutions of 100 ng/µl were prepared in methanol (HPLC grade) and stored, protected from light, at – 20 °C. Methanol and acetonitrile (HPLC grade) were purchased from Merck. The HPLC buffers were filtered through 0.45 micrometre membrane (Sartorius, Gottingen, Germany).

3.2.3 Amanitin extraction

The powdered sample (2.0 g) of single fruiting bodies was defatted with 30 ml of light petroleum for 3 h in a Soxhlet extractor. The insoluble residue were dried

in an oven and extracted with 30 ml of methanol for 3 h in a Soxhlet extractor. The resulting extracts were evaporated to dryness using a rotary evaporator and redissolved in 2 ml of methanol and centrifuged for 30 min using a bench centrifuge. The supernatant were used as a methanolic extract.

3.2.4 Qualitative test for the presence of α-amanitin

The methods was modified from the methods of Block *et al*. (1955), Wieland (1968) and Yocum and Simons (1977). α -Amanitin standard solutions was prepared by dissolving 1 mg of α-amanitin (Sigma, Singapore) in 10 ml of methanol. Portions (30 μl) of the methanolic extracts from the samples, amanitin standard solution, was loaded on to a Whatman No.1 chromatographic paper, which was then developed for 40 min , using butanone-acetone-water (30:3:5, v/v) as a running a solvent. The dried chromatogram was sprayed with a 1 % solution of *trans*-cinnamaldehyde in methanol and immediately exposed to the fume of concentrated hydrochloric acid (violet spots indicate α-amanitin ; while orange, yellow, brown and other spots are of no significance).

3.2.5 High-performance liquid chromatography (HPLC) analysis of α-amanitin

HPLC analysis was carried out by gradient elution. The HPLC apparatus was composed of the following units: a Waters Automated Gradient Controller Model 680 solvent – deliver module, a Waters HPLC pump Model 510 sample injection valve with a 10 μl loop and a Waters Programmable Multiwavelength UV Detector Model 490 (all from Waters). 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) In this work, the system was set at 1 spectrum/s. Chromatograms were processed with a System Chromatography Station for Windows (Waters).

Separations was performed at ambient temperature on a reversed-phase 5-μm ultrasphere ODS – 80TS column (150 x 4.6 mm I.D.)(TOSOH / TSK-GEL). 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 207 and 305 nm.

A calibration graph was prepared in the medium with increasing amounts of the α-amanitin yielding concentrations of 1, 5, 10, 25, 50 and 100 ng/μl. The limit of detection was defined as the lowest concentration of α-amanitin. The accuracy of the method was investigated for α-amanitin at six concentrations (1, 5, 10, 25, 50 and 100 ng/μl) by comparing the amount of α-amanitin added to the extraction medium with that actually measured.

Dried samples from qualitative test of α-amanitin producing species was evaluated for the types and quantity of the toxin using a modification of the method of Enjalbert *et al*. (1992). About 2 g of chopped sample was placed in a cartridge of a freezer-mill, crushed for 1-2 minutes 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 was used. The mixed extracts was incubated overnight at 4 ^{o}C, then centrifuged at 6,000 g for 10 minutes. The supernatant was collected and preserved at 4 ^{o} C and the pellet was mixed again with 8 ml of the extraction medium and incubated for other 12 h at 4 ^{o} C. The mixture was centrifuged again at 6,000 g for 10 minutes and the supernatant was pooled with the others. A 10 μl aliquot of the combined supernatants was used for the separation and determination of the α-amanitin.

3.2.6 Rapid screening test of α-amanitin

This test was done according to the method of Wieland and Faulstich (1978) which based on the reaction of lignin (found in cheap newsprint paper) with the amatoxins in the presence of concentrated acid to produce a blue color (Beutler and Vergeer, 1980). High-quality, glossy paper has had almost all the lignins removed and should not be used. Filter paper, if available, is ideal. The aims of rapid screening

test was to find the suitable paper which has high lignin content and that can be found in Thailand.

A 30 µl portion of the α-amanitin standard solution (25, 50, and 100 ng/µl) were dropped on newsprint paper or the other suitable paper, leaving the spot to dry at room temperature or dry gently with a hair dryer (protect from direct sunlight). One drop of 8 to 12 N (concentrated) hydrochloric acid was dropped adjecently to the dried spot on the tested newsprint paper. The development of blue colour (Benjamin, 1995) was appear within 20-30 min (up to an hour) that indicated to presence of α-amanitin. However, this event should be compare with the control spot that was negative (not blue). The Wieland or Meixner test were modified as the treatment design in Table 3.1 for comparing the effects of drop ordering of concentrated acid to the development of reaction.

Table 3.1 Experimental design in the Wieland or Meixner test.

Treatment1*	Treatment2+	Remark
8-12 N HCl	Distilled water	
8-12 N HCl	MeOH	
8-12 N HCl	Standard solution of α-amanitin	
Distilled water	8-12 N HCl	
MeOH	8-12 N HCl	
Standard solution of α-amanitin	8-12 N HCl	

Note: 1. Treatment1 * = 1 drop of 8-12 N HCl and following with 1 drop of standard solution of α-amanitin.

2.Treatment2 + = 1 drop of standard solution of α-amanitin and allowing the spot to dry at room temperature. After that add one drop of 8-12 N HCl adjacent the dried spot on the tested paper.

3.3 Results

3.3.1 Qualitative test for the presence of α-amanitin

The presene of α-amanitin in *Amanita* mushrooms in Chiang Mai community forests were found in dried samples of *Amanita cokeri*, *Amanita* sp.1, *Amanita* sp. 2, *Amanita* sp. 3, *A. verna,* and *A. virosa.* (Figure 3.1, Table 3.2). All of them gave violet spots on a dried chromatogram, which indicates the presence of α-amanitin. The other species gave negative results. A dried samples confirmed the presence of α-amanitin by using high performance liquid chromatography (HPLC) analysis of α-amanitin.

(A) (B) (C) (D) (E) (F) (G) (H)

Figure 3.1 Paper Chromatogram of extracted solutions of (A) *Amanita princeps* (B) standard α-amanitin (C) *Amanita cokeri* (D) *Amanita* sp.1 (E) *Amanita* sp.2 (F) *Amanita* sp.3 (G) *Amanita verna* and (H) *Amanita virosa*

3.3.2 High performance liquid chromatography (HPLC) analysis of α-amanitin

Quality control parameters, high-performance liquid chromatography (HPLC) analysis of α-amanitin, were investigated by preparing the extraction medium with increasing amounts of α-amanitin yielding concentrations of 1, 5, 10, 25, 50 and 100 ng/μl. The limit of detection is defined as the lowest concentration of α-amanitin standard. Quantification of α-amanitin, based on peak areas, was linear between 1 to 100 ng/μl (correlation coefficient 0.99). This concentration ranges included the concentration of α-amanitin usually found in dry tissue of mushroom extracts.

A 10 μl aliquot solution of the sample was used for determination of the α-amanitin contents of each *Amanita* sample. The results of the analysis are shown in Table 3.2. It was found that *Amanita* sp. 1, *Amantia* sp. 2, *Amanita* sp.3, *A. verna,* and *A. virosa* in section *Phalloidae* contained α-amanitin. Only *Amanita cokeri* in section *Lepidella* contained α-amanitin. The other species of sections *Amidella*, *Validae*, *Amanita*, and *Vaginatae* had no α-amanitin.

The quantity of α-amanitin in *Amanita cokeri, Amanita* sp.1*, Amanita* sp.2*, Amanita* sp.3*, A. verna,* and *A. virosa* were found to be 0.162, 0.137, 0.178, 0.160, 1.144 and 0.237 mg/g of dry tissue, respectively. Each of these species showed HPLC peaks that agreed with the standard of α - amanitin (Figure 3.3). Other amanitins or other toxins, for which standards were not available, may be present.

The HPLC chromatograms illustrated in Figures 3.4-3.9 shows the analysis of the toxins in *Amanita verna*, *A. virosa*, *Amanita* sp.1, *Amanita* sp.2, *Amanita* sp.3 and *A. cokeri* extracts. The α-amanitin is completely resolved from endogenous peaks. The maxima for α-amanitin is located at wavelengths 305 nm (Figure 3.2).

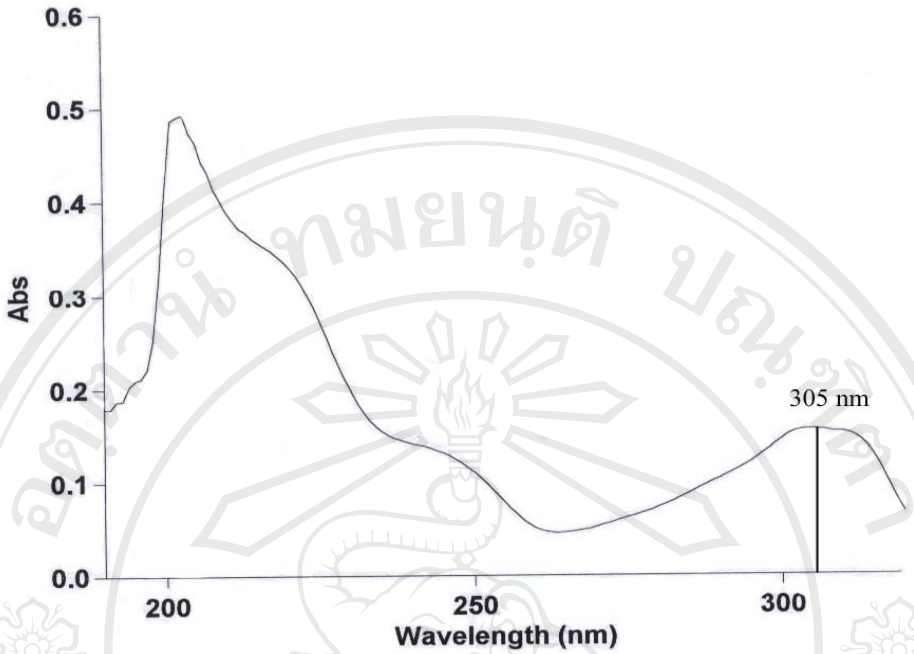


Figure 3.2 Absorbance spectra (200-342 nm) of α - amanitin

Table 3.2 Qualitative test for the presence and HPLC analysis of α-amanitin content of *Amanita* mushroom 42 species in Chiang Mai community forest (Mae Awn, Doi Saket, Mae Rim, Mae Wang and San Kamphaeng districts)

section	species	Qualitative test [1]	α-amanitin content	
			µg/ml	mg/ g.dry wt.[2]
Amidella	*Amanita avellaneosquamosa*	-	-	-
Lepidella	*Amanita castanopsis*	-	-	-
	Amanita cokeri	+	161.76	0.162
	Amanita gymnopus	-	-	-
	Amanita hongoi	-	-	-
	Amanita sp.	-	-	-
	Amanita thiersii	-	-	-
	Amanita virginea	-	-	-
	Amanita virgineoides	-	-	-

Table 3.2 (continued)

section	species	Qualitative test [1]	α-amanitin content	
			µg/ml	mg/ g.dry wt.[2]
Phalloideae	*Amanita arocheae*	-	-	
	Amanita phalloides	-	-	-
	Amanita pseudoporphyria	-	-	-
	Amanita subjunquillea	-	-	-
	Amanita sp. 1	+	136.70	0.137
	Amanita sp. 2	+	178.25	0.178
	Amanita sp. 3	+	159.70	0.160
	Amanita verna	+	1144.00	1.144
	Amanita virosa	+	236.86	0.237
Validae	*Amanita xanthella*	-	-	-
	Amanita brunnescens	-	-	-
	Amanita fritillaria	-	-	-
	Amanita spissacea	-	-	-
Amanita	*Amanita obsita*	-	-	-
	Amanita sychnopyramis	-	-	-
	Amanita farinosa	-	-	-
	Amanita siamensis	-	-	-
	Amanita cecilliae	-	-	-
	Amanita concentrica	-	-	-

Table 3.2 (continued)

section	species	Qualitative test [1]	α-amanitin content	
			µg/ml	mg/ g.dry wt.[2]
Vaginatae	*Amanita angustilamellata*	-	-	-
	Amanita battarae	-	-	-
	Amanita caesarea	-	-	-
	Amanita calopus	-	-	-
	Amanita chepangiana	-	-	-
	Amanita fuligineodisca	-	-	-
	Amanita fulva	-	-	-
	Amanita griseofolia	-	-	-
	Amanita hemibapha	-	-	-
	Amanita huijsmanii	-	-	-
Vaginatae	*Amanita longistriata*	-	-	-
	Amanita ovalispora	-	-	-
	Amanita princeps	-	-	-
	Amanita spreta	-	-	-
	Amanita sp.1	-	-	-
	Amanita vaginata	-	-	-

Note 1 = Qualitative test for α-amanitin

2 = weight (g) of dry tissue

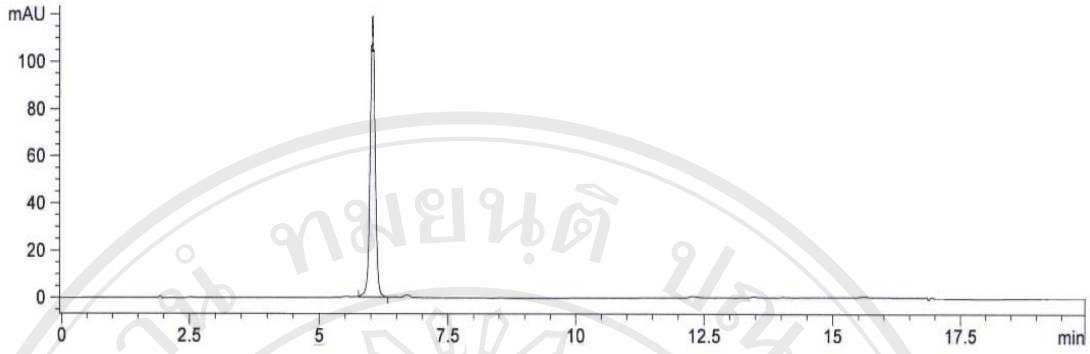


Figure 3.3 HPLC chromatogram of α - amanitin standard solotion (100 ng/µl) (RT=6.033min, 305 nm).

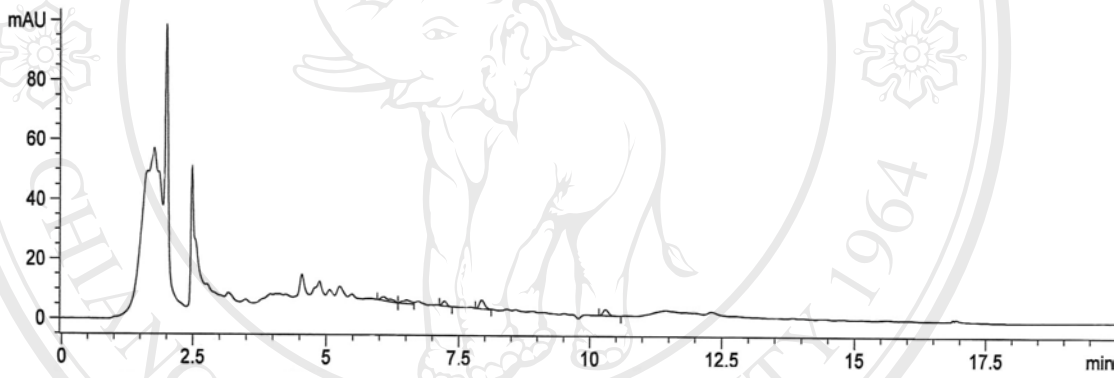


Figure 3.4 HPLC chromatogram of *Amanita cokeri* in α - amanitin detection

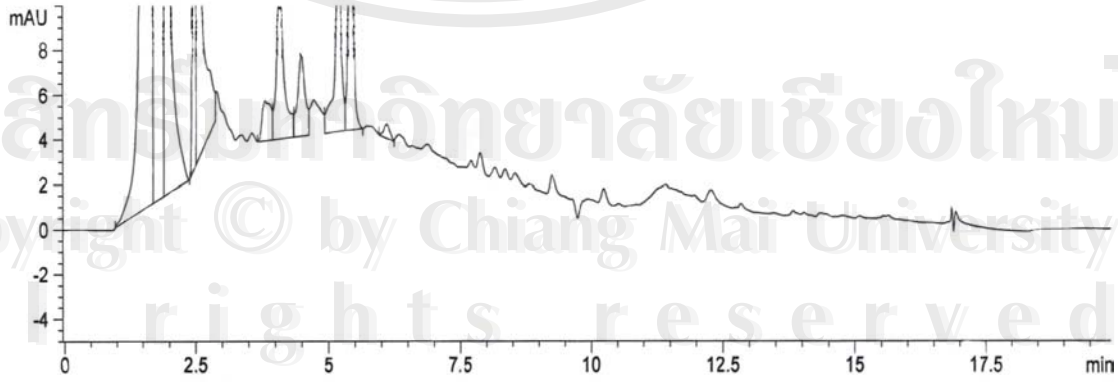


Figure 3.5 HPLC chromatogram of *Amanita* sp.1 in α - amanitin detection

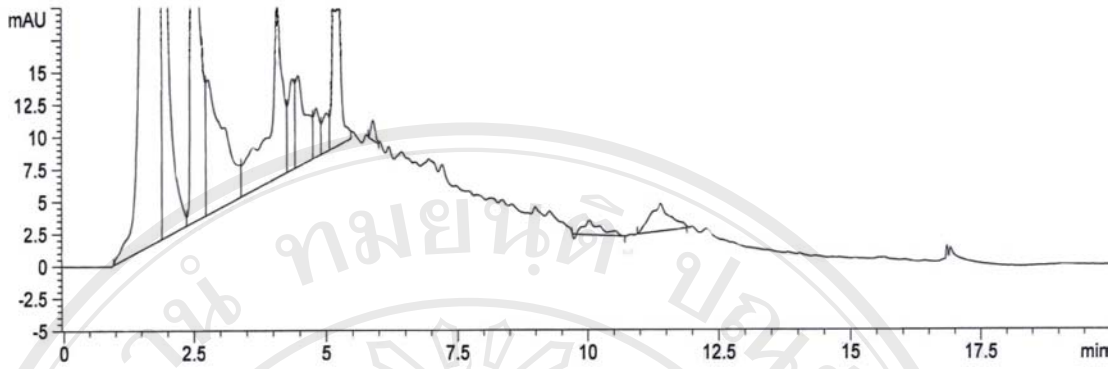


Figure 3.6 HPLC chromatogram of *Amanita* sp.2 in α - amanitin detection

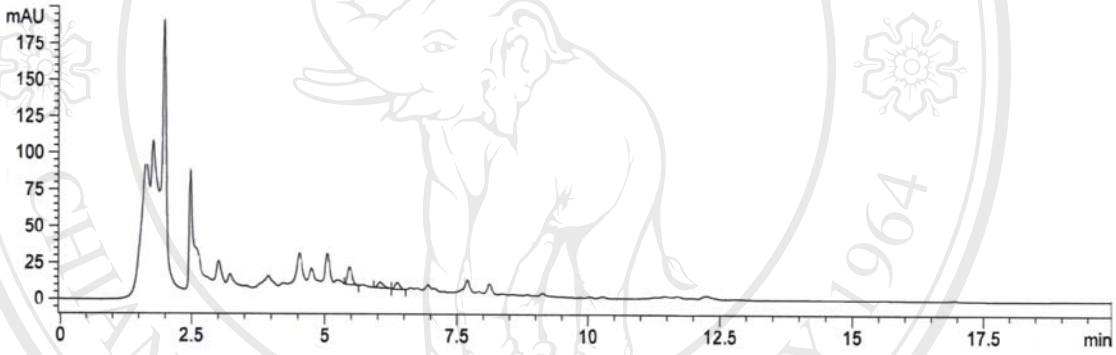


Figure 3.7 HPLC chromatogram of *Amanita* sp.3 in α - amanitin detection

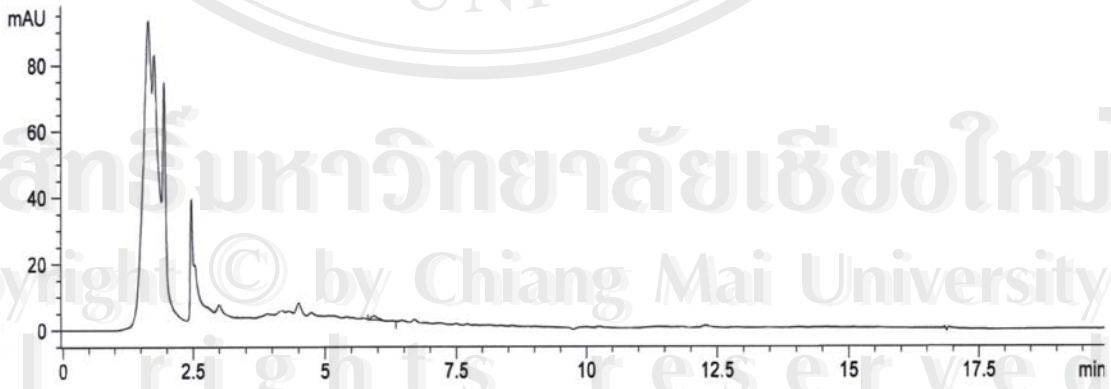


Figure 3.8 HPLC chromatogram of *Amanita* verna in α - amanitin detection

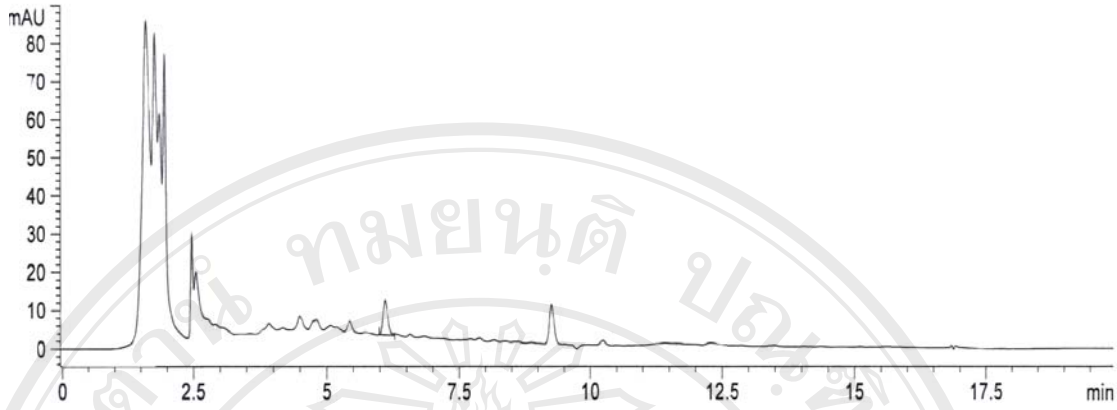


Figure 3.9 HPLC chromatogram of *Amanita virosa* in α - amanitin detection

3.3.4 Rapid screening test of α-amanitin

The rapid screening test of α-amanitin with the tested paper such as the proof paper, filter paper, glossy paper, telephone book paper, drawing paper, Sa paper, Brown paper, and filter paper + 0.01 % lignin solution (Table 3.3), revealed the absence of α-amanitin because no blue spot were observed. The inner of OTOP product box-paper, and grey/white paper revealed the presence of α-amanitin because blue spot were observed. The blue spots showed within 5-10 sec after one drop of standard was droped on the tested paper which was dropped with 8 N HCl.

Table 3.3 The results of rapid screening test of α-amanitin .

Type of paper	Weiland / Meixner test
1. Proof paper	Negative
2. Filter paper	Negative
3. High quailty, glossy paper	Negative
4. Telephone book paper	Negative
5. Drawing paper	Negative
6. Hand made paper, " Sa "	Negative
7. Brown paper from brown paper envelops	Negative
8. Filter paper + 0.01 % lignin solution	Negative

Table 3.3 (continued)

Type of paper	Weiland / Meixner test
9. Paper, inner of pineapple products (Prachub Ki Ri Kan province)	Negative
10.Lowcost, proof paper	Positive
11. Grey / white paper	Positive

A rapid screening test kit based on the Wieland or Meixer method for rapid and efficient detection of α-amanitin was modified and developed. The efficient detection of a screening test kit depends on the concentrations of α-amanitin standard solution and the lignin contents in the newsprint which reacts with the α-amanitin in the presence of concentrated acid to produce a blue color. The minimum concentration of α-amanitin standard solution was 50 ng/μl, which gave a positive Meixer test. For a suitable paper in this study were the low-cost proof paper and grey / white paper which had a good sensitivity for α-amanitin detection.

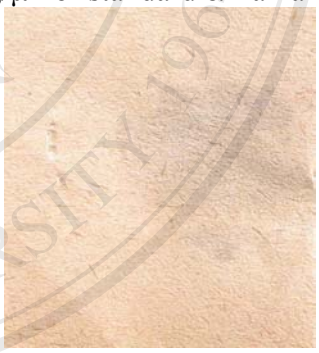
A. negative control (distilled water)

B. negative control (methanol)

C. 25 ng/µl of standard α - amanitin

D. 50 ng/µl of standard α - amanitin

E. 75 ng/µl of standard α - amanitin



F. 100 ng/µl of standard α - amanitin

Figure 3.10 Weiland / Meixner test (lowcost, proof paper)

A. negative ontrol (distilled water)

B. negative control (pure methanol)

C 25 ng/µl of standard α - amanitin

D 50 ng/µl of standard α - amanitin

E 75 ng/µl of standard α - amanitin

F 100 ng/µl of standard α - amanitin

3.5 Discussion

The qualitative tests for the presence of α-amanitin of 42 species of *Amanita* mushroom extracts revealed the presence of α-amanitin from *Amanita cokeri*, *Amanita* sp.1, *Amanita* sp.2, *Amanita* sp.3, *A. verna,* and *A. virosa.* All of them showed violet spots on the dried chromatogram which indicates the presence of α-amanitin. This results agreed with Yocum and Simons (1977) who investigated *Amanita phalloides, A. bisporegera, A. verna, A. virosa* and *A. rubescens* and detected toxins in all except *A. rubescens*.

In this study, none of the edible *Amanita* mushrooms extracts had α- amanitin. This is a negative results becauses yellow or orange spots were seen; this suggests the absence of α-amanitin in the mushrooms tested. 42 species of *Amanita* mushrooms were analysed by HPLC and compared with an α-amanitin standard solution which had results the same as the qualitative tests.

The limit of detection of α-amanitin standard was less than 1 ng/µl. Linearity was determined using extracts of negative samples spiked with α - amanitin ranging from 1 ng/µl to 100 ng/µl . The corelation coefficient (r) of 0.9962 indicated that the linearity of this method was sufficient for this research. The linearity was described by the equation $y = 0.812554 x - 1.88761$, was the concentration of α-amanitin in dried mushroom extracts (ng/µl) and y was the peak area. The recovery of α-amanitin was determined using the extracts spiked with α-amanitin at concentrations of 100 ng/µl, ranging from 93.9 – 100.0 %. The acuracy was determined by spiking extracts samples with different amounts of α - amanitin (1, 5, 10, 25, 50 and 100 ng/µl) and comparing the theoretical with measured concentrations.

The presence of α-amanitin tests are showed in paper chromatogram (Figures 3.1, Table 3.2). It was found that *Amanita cokeri, Amanita* sp.1, *Amanita* sp.2, *Amanita* sp.3, *A. verna* and *A. virosa* have α-amanitin. These species and other species were confirmed by HPLC analysis compared with the standard α-amanitin, it was found that the species with α-amanitin show peaks the same as the peak of standard α-amanitin (Figure 3.3). The content of α - amanitin in positive samples of *Amanita* mushrooms are shown in Table 3.2.

The α-amanitin content of *Amanita cokeri, Amanita* sp.1*, Amanita* sp.2*, Amanita* sp.3*, A. verna* and *A. virosa* were found to be 0.162, 0.137, 0.178 , 0.160, 1.144, and 0.237 mg/g of dry tissue respectively. Bresinsky and Besl (1990) reported that α-amanitin content of *A. phalloides*, *A. verna* and *A. virosa* varied from 1.0-2.5, 1.5-4.5 , and 1-3 mg/g dry tissue, respecitively. Enjalbert *et al*.(1992) reported that the content of toxic peptides in a single sample of *A. phalloides* was 0.07 % of the fresh tissue (123 µg /g) or 2.26 mg. Although, the α-amanitin contents of all species found in Chiang Mai community forests which had α-amanitin were less than the α-amanitin content of *Amanita verna* and *A. virosa* reported by Bresinsky and Besl (1990). These two species are very dangerous to Thai people.

Mr. Jorchitu Mingkuanjamkaet Mae Jam District Chiang Mai, was poisonned from *Amanita* mushrooms in Mae Jam District Chiang Mai Province and admitted in Maharaj Nakorn Chiang Mai Hospitial, May 2003. The author used a simple pictorial key for identification *Amanita* mushrooms and revealed his knowledge about poisonous *Amanita* mushrooms. He indicated the pictures of *Amanita virosa*. He consumed only four to fives fruiting bodies of the poisonous *Amanita* mushrooms before being admitted to the local hospital. This shows that utilisation of the simple picturial keys might be useful for the diagnosis food poisoning caused from poisonous mushroom and suppoting the primary diagnosis for proper theraphy.

The edible species, *Amanita cheapangiana, A. caesarea, A. hamibapha,* and *A. princeps* in this study had no α-amanitin. The methods in this study were analysed only α amanitin, so that other amanitin or other toxins, notably ibotenic acid or muscimol, may be present remained in the other species. Tests for other fungal toxins on need to be performed and more specimens need to be examined, before any species are considered safe to eat. The distribution of α-amanitin in *Amanita* has long been a subject of controversy. Faulstich and Cochet-Meilhac (1976) reported the presence of trace quantities of amatoxins in all species tested, including the common edible species *Agaricus bisporus* (Lange) Pilát, using radioimmunoassay (RIA).

A rapid screening test kit based on the Wieland or Meixer method for rapid and efficient detection of α-amanitin was simple and not complicate but this test was difficult in practice becauses of the lack of the suitable paper for use in Thailand.

Although the Meixer test had a good detection limit (2 μg) for toxic amounts α-amanitin, a positive Meixer reaction did not adequately distinguish between α-amanitin and other mushroom indoles (Beuhler *et al*., 2004).