Cryptogamie, Mycologie, 2016, 37 (2): 217-239

Decision-making for the detection of amatoxin poisoning: a comparative study of standard analytical methods

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Abstract – Amatoxin-containing mushroom poisonings are recorded worldwide and the frequency increases due to confusion with other macrofungi. Affected regions are characterized by important disparities in relation to available technological equipment for analytical identification of amatoxins. In this context the present study was designed to define advantages and disadvantages of the most accessible standard analytical methods for amatoxin detection. Several methods were compared: (1) a commercialized immunoassay kit, (2) standard high-performance liquid chromatography (HPLC) and (3) high-performance thin layer chromatography (HP-TLC). For each method, linearity, limit of detection (LOD), limit of quantification (LOQ) and recovery were determined. Six macrofungi were analysed using these compared methods, three known to contain amatoxins: *Amanita phalloides*, *Amanita virosa*, *Lepiota josserandii*, and three free-amatoxin containing macrofungi: *Amanita muscaria*, *Macrolepiota procera* and *Omphalotus olearius*. Our results will allow for a choice of method with full knowledge of advantages and disadvantages of each technique as a function of local technological possibilities when facing suspected poisoning due to amatoxin-containing mushrooms. The final aim is to be able to reach faster and effective diagnosis in order to save a patient's life.

Amanita* / Amatoxins / Analytical methods / Decision-making / *Lepiota* / Method validation / Mushroom Poisoning / *Omphalotus

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doi/10.7872/crym/v37.iss2.2016.217

Résumé – Les intoxications dues aux champignons contenant des amatoxines surviennent dans le monde entier. Les régions touchées sont caractérisées par des disparités importantes en matière d'équipement technologique disponible pour l'identification des amatoxines. Cette étude définit les avantages et les inconvénients des méthodes d'analyse les plus accessibles pour la détection des amatoxines. Le choix de la méthode doit être fait en pleine connaissance des avantages et inconvénients de chaque technique et en fonction des possibilités technologiques locales. Plusieurs méthodes ont été comparées : (1) un kit commercial de dosage immunologique, (2) la chromatographie liquide à haute performance et (3) la chromatographie haute performance sur couche mince. Chaque méthode a été validée (linéarité, limites de détection et de quantification) et appliquée à des matrices de champignons : trois champignons connus pour contenir des amatoxines : *Amanita phalloides*, *Amanita virosa*, *Lepiota josserandii*, et trois champignons ne présentant pas d'amatoxines : *Amanita muscaria*, *Macrolepiota procera* et *Omphalotus olearius.* Les résultats fournissent aux cliniciens des données comparatives pour appuyer leur prise de décision concernant le choix des méthodes analytiques. Cette étude est utile dans l'interprétation des résultats face à un empoisonnement présumé aux amatoxines. L'objectif final est d'être en mesure d'atteindre un diagnostic plus rapide et efficace afin de sauver la vie des patients.

***Amanita* / Amatoxines / Intoxication par les champignons / *Lepiota* / Méthodes analytiques / *Omphalotus* / Prise de décision / Validation de Méthodes**

INTRODUCTION

The frequency of mushroom fatal poisonings recorded in emergency medicine units increases worldwide (Enjalbert *et al.*, 2002; Berger & Guss, 2005; Diaz, 2005; Giannini *et al*., 2007; Barceloux, 2008; Assisi *et al*., 2009; Benítez-Macías *et al.,* 2009; Isiloglu *et al*., 2009; Epis *et al.*, 2010; Patowary, 2010; Trabulus & Altiparmak, 2011; Oeckinghaus *et al*., 2012; Schenk-Jaeger *et al.*, 2012; Lawton & Ni Bhraonain, 2013; Roberts *et al*., 2013; Chen *et al.*, 2014; Gawlikowski *et al*., 2015; Gok *et al.,* 2015). Over 90% of human casualties are caused by the ingestion of amatoxin-containing species of the genus *Amanita*, i.e., mainly *Amanita phalloides* (Vaill. ex Fr.) Link in Europe and *A. bisporigera* G.F. Atk., *A. exitialis* Zhu L. Yang & T.H. Li, *A. ocreata* Peck, *A. phalloides*, *A. verna* (Bull.) Lam. and *A. virosa* (Fr.) Bertill. in the United States, Canada, South America, Australia and China (Benjamin, 1995; Enjalbert *et al*., 2002; Karlson-Stiber & Persson, 2003; Lamoureux, 2006; McNeil, 2006; Escudié *et al*., 2007; Giannini *et al.*, 2007; Madhok, 2007; Ferenc *et al*., 2009; Deng *et al.*, 2011; Mendez-Navarro *et al.*, 2011; Vargas *et al*., 2011; Xue *et al.*, 2011; Hu *et al.*, 2012; Santi *et al*., 2012; Roberts *et al.*, 2013; Ward *et al.*, 2013; Yilmaz *et al.*, 2014; Varvenne *et al.*, 2015). Fatalities also occur by the consumption of several amatoxin-containing species of the genera *Galerina* and *Lepiota* (Klisnick *et al.*, 2000; Enjalbert *et al.*, 2004; Roux *et al.*, 2008; Delacour *et al*., 2009; Ben Khelil *et al*., 2010; Kervegant *et al*., 2013; Sgambelluri *et al.*, 2014; Kose *et al*., 2015; Varvenne *et al.*, 2015). These mushrooms contain biotoxins, i.e., toxic secondary metabolites, which can be classified in three types: amatoxins, phallotoxins and virotoxins (Wieland, 1986; Bresinsky & Besl, 1990; Li & Oberlies, 2005; Barceloux, 2008). Amatoxins are a group of nine bicyclic octapeptides (with an indole-(*R*)-sulphoxide bridge) resistant to heat, freezing, drying and digestion. They are absorbed in the gastro-intestinal tract and are considered as the agent responsible for poisoning (Vetter, 1998; Rittgen *et al.,* 2008; Allen *et al*., 2012; Clarke *et al.,* 2012). Phallotoxins are a group of seven bicyclic heptapeptides (with

an indole-thio-ether bridge) unstable to heat. They are not absorbed in the gastro-intestinal tract and are therefore not considered to be responsible for poisoning. Virotoxins are a group of seven cyclic heptapeptides (without intra-molecular bridge) whose properties have been less studied than the phallotoxins; the virotoxins do not generate poisoning (Clarke *et al.,* 2012).

Therefore *Amanita* fatal poisonings are associated with amatoxins (α-, β- and γ-amanitins accounting for 40% of the amatoxin content) which are considered as one of the most violent natural poisons (Barceloux, 2008; Clarke *et al.,* 2012; Yilmaz *et al.*, 2015). Human adult lethal dose (LD_{50}) *per os* is 0.1-0.3 mg/kg body weight (Scheurlen *et al*., 1994; Leist *et al.*, 1997); these doses correspond to 50-300 g of amatoxin-containing mushrooms (Barceloux, 2008). Therefore, a medium-sized *Amanita* specimen containing 10-15 mg of amatoxins may be fatal (Faulstich, 1980; Wieland, 1986). The mortality rate depends also on the applied treatment (Faulstich, 1980; Enjalbert *et al.*, 2002; Saviuc *et al*., 2003; Zilker, 2009; Evrenoglou *et al*., 2010; Poucheret *et al.*, 2010; Jansson *et al.,* 2012; Mengs *et al*., 2012). Amatoxins are absorbed in the intestinal tract and follow the enterohepatic cycle thereby increasing toxins half-life and intoxication severity (Santi *et al*., 2012; Erden *et al*., 2013). Toxins accumulate in the liver. Excretion is mainly urinary (Jaeger *et al.,* 1993). Acute tubular necrosis may occur in the kidney after ingestion of amatoxin-containing mushrooms (Ganzert *et al*., 2008; Ferenc *et al.,* 2009; Garrouste *et al*., 2009). Pharmacological actions of amatoxins include inhibition of RNA polymerase II (Michelot & Labia, 1988; Karlson-Stiber & Persson, 2003; Poucheret *et al.*, 2010; Santi *et al.,* 2012) and induction of apoptosis through synergic action with cytokines such as tumor necrosis factor (TNF) (Leist *et al.,*1998). These molecular actions lead to three successive clinical stages: (1) an asymptomatic latency period lasting for 6-10 hours (Vetter, 1998), (2) a gastro-intestinal phase lasting for 24-72 hours with vomiting and cholera-like diarrhea, and (3) a hepatic-kidney final stage lasting for 6-16 days leading to liver failure (massive hepatocyte necrosis) and kidney damages (Enjalbert *et al*., 2002; Saviuc *et al*., 2003; Berger & Guss, 2005; Karakayali *et al.*, 2007; Unverir *et al.*, 2007; Santi *et al.*, 2012). Without appropriate medical care, patients may develop encephalopathy followed by coma and death (Ronzoni *et al.*, 1991; De Carlo *et al.,* 2003; Allen *et al.*, 2012).

Amatoxin clinical symptomatology being non-specific (i.e., vomiting, diarrhea, damage to liver and kidneys), toxin analysis is mandatory to rule out other gastro-intestinal and hepatic pathologies or poisonings. In addition it is now widely accepted that amatoxin quantitative dosage, in classical biological fluids and biopsy, is of no interest for clinical prognosis. Indeed no correlation could be established between amatoxin tissue (blood, urines) concentration and either intoxication stage or poisoning severity (Allen *et al.*, 2012; Erden *et al*., 2013; Santi *et al.*, 2012). Therefore, for clinical purposes, the main interest of qualitative and quantitative amatoxin analyses lays in differential diagnosis (Butera *et al.*, 2004; Tomkova *et al.*, 2015). The main objective is to certify, without false positive or negative results, the presence or absence of amatoxins, mostly α-amanitin (Beutler & Vergeer, 1980; Staack & Maurer, 2000; Karlson-Stiber & Persson, 2003).

To this end, researchers develop various analytical methods on different types of samples for amatoxin detection. Amatoxin analyses may be performed using liquid chromatography (Enjalbert *et al.*, 1992; Maurer *et al.*, 2000; Nomura *et al*., 2012; Kaya *et al*., 2013; Leite *et al.,* 2013; Gicquel *et al*., 2014; Helfer *et al.*, 2014; Yilmaz *et al.*, 2014; Garcia *et al*., 2015a, 2015b; Parnmen *et al.*, 2016), thin layer chromatography (Wieland, 1964, 1986; Andary *et al.*, 1977; Stijve & Seeger, 1979; Plancke *et al*., 1980), enzyme-linked immunosorbent assay (ELISA) (Butera

et al., 2004; Parant *et al.*, 2006; Gomolka *et al.*, 2011), electrophoresis (Rittgen *et al.*, 2008; Robinson-Fuentes *et al*., 2008), fluorescent complex, artificial receptors and molecular biology (Gausterer *et al.*, 2014; Parnmen *et al.*, 2016).

In addition, amatoxin poisonings being recorded in diverse parts of the world with high disparities of scientific equipment, there is a real and clear need for: (1) a comparative assessment of the most accessible analytical methods for amatoxin detection and (2) the validation of these analytical methods in the aqueous matrix since the first sources of mushroom material for amatoxin detection are mushroom harvest remains, cooking residues and vomiting.

Therefore, the aim of the present study was to fill this void, thereby helping decision-making regarding the choice for the most appropriate analytical method and proper interpretation of results for early clinical diagnosis of amatoxin-containing mushroom poisonings to save lives.

MATERIAL AND METHODS

Chemical standards/Material

Alpha- and β-amanitins were purchased from Sigma-Aldrich (Deisenhofen, Germany). Acetonitrile was HPLC grade from Carlo Erba Reagents (Val de Reuil, France). Other chemicals were from Sigma-Aldrich (Deisenhofen, Germany). Ultrapure water was obtained using Simplicity® Water Purification system from Millipore (Darmstadt, Germany).

The stability of α- and β-amanitins over a time period of six months at – 7°C was already proven by Maurer *et al.* (2000) and therefore no additional tests were performed here.

Mushroom material

The mushrooms were collected in the Montpellier area, France. Three mushrooms known to contain amatoxins were selected: *A. phalloides* (Fr.) Link, *A. virosa* (Fr.) Bertillon and *Lepiota josserandii* Bon & Boiffard (synonym: *L. subincarnata* J.E. Lange). Three mushrooms that do not contain amatoxins were used in the present study as negative controls: *Amanita muscaria* (L.:Fr.) Hook., *Macrolepiota procera* (Scop.) Singer and *Omphalotus olearius* (DC.) Sing. The two former mushrooms are selected because they are classified in mushroom genera similar to those containing amatoxins (Enjalbert *et al.*, 2002); the latter belongs to a distant mushroom genus (Courtecuisse & Duhem, 2013). After morphological identification of the mushrooms, they were dried and preserved at room temperature in the dark to improve preservation of the fungal materials. Voucher specimens are conserved in the laboratory.

Extraction procedure: 50 mg of dried mushroom were grounded then extracted with 2 mL of ultrapure water. Ultrasonic extraction was performed at room temperature for 5 min. After filtration, extracts were preserved at 4°C until analysis.

Sample preparation: Preliminary assays were conducted in hydroalcoholic solution (50:50, v/v), nevertheless, ethanol interacts with immunoassay method by giving false positive results, and so we choose to compare the three methods using

water as solvent extraction. Samples were diluted in pure water and then directly injected without any sample pretreatment. Extemporaneous dilutions were done in pure water: 1/2 for immunoassay method, 1/10 for HPLC method, no dilution for HP-TLC.

Immunoassay method

BUHLMANN Amanitin ELISA kit (BUHLMANN Laboratories AG, Switzerland) was used in accordance with manufacturer's specifications. The aqueous mushroom samples were dilute 1:25 with Incubation Buffer. The wells, coated with a polyclonal specific antibody for α- and β-amanitins, were washed twice using wash buffer. 50 µL of α-amanitin calibrators, low control and high control were deposited in duplicate into wells. 50 µL of each sample were deposited in triplicate into wells. 50 µL Biotin Conjugate were added to all wells. The plate was covered with a plate sealer, incubated for 30 ± 5 min at 18-28°C on a plate rotator (500 rpm). The wells were then washed three times with Wash Buffer. 100 µL of the TMB Substrate Solution were distributed into all wells. The plate was cover again with a plate sealer, incubated for 15 ± 5 min at 18-28°C on a plate rotator (500 rpm) and protected from direct light. 100 µL of Stop Solution were added into all wells. Within 30 min absorbance was read at 450 nm in a microtiter plate reader UVMAX Molecular Devices (MDS Inc., Toronto, Canada). All experiments were repeated three times.

Linearity/work range: Standard curves were generated by increasing amounts of α- and β-amanitins corresponding to a concentration range of 1-100 ng/mL. Peak areas of α- and β-amanitins were integrated and a calibration curve constructed. Regression lines of best fit were constructed and deemed acceptable if the regression coefficient, r, was > 0.99.

Limit of detection/Limit of quantification (LOD/LOQ) were specified by the manufacturer.

Recoveries of pure amanitins were assessed by analysing prepared samples at each of the five concentrations available in the kit (1-3-10-30-100 ng/mL). The accuracy was expressed as percent error [(mean of measured/mean of expected] $\times 100$, while the precision was given by the coefficient of variation (C.V. in %).

Applicability: Extracts of mushrooms without amanitins (*A. muscaria, M. procera, O. olearius*) and extracts of mushrooms containing amanitins (*A. phalloides, A. virosa, L. josserandii*) were prepared and analysed.

HPLC method

Chromatographic separation and detection for quantitative analysis were performed on a SpectroSYSTEM® which included a P4000 pump, a SCM1000 degasser, an AS3000 automatic sampler and an UV6000LP DAD detector (Thermo Fisher Scientific Inc., San José, USA). The system was operated using ChromQuest Software version 5.0. Chromatographic separation was achieved on an ODS Hypersyl C18 column (250 mm × 4.6 mm, 5 µm, Thermo Fisher Scientific Inc., San José, USA). Column temperature was maintained at 30°C.

Chromatography analyses were performed using a modified separation based on Enjalbert *et al.* (1992). Briefly, elution was achieved at a flow rate of 1 mL/min (initial back pressure of approximately 105 bar), with a gradient from 10:90 (acetonitrile/aqueous ammonium acetate 2 mM, pH 5), with an initial hold of 7 min

followed by a linear increase in organic mobile phase to 24:76 at 16 min, which was sustained for a further minute (until 20 min) before re-equilibration, with a total run-time of 21 min. Retention times for β- and α-amanitins internal standards were approximately 8.3 and 9.3 min, respectively. Absorbances were monitored between 200 and 400 nm. For each peak, UV spectrum was extracted and compared with literature data for amanitins (Enjalbert *et al*., 1992, 2002, 2004; Wieland, 1964, 1986) to confirm identification.

***Linearity/work range*:** Standard curves were generated by increasing amounts of α- and β-amanitins corresponding to a concentration range of 2-100 µg/mL (n = 6). Peak areas of α- and β-amanitins were integrated and a calibration curve constructed. Regression lines of best fit were constructed and deemed acceptable if the regression coefficient, *r*, was > 0.99.

Limit of detection/Limit of quantification (LOD/LOQ): The LOD was defined as the sample concentration resulting in a response of three times the noise level. The LOQ was defined as the sample concentration resulting in a response of ten times the noise level.

Recoveries of pure amanitins were assessed by samples analysis at each of three concentrations (6-18-50 µg/mL). The accuracy was expressed as percent error [(mean of measured)/mean of expected] × 100, while the precision was given by the determined coefficient of variation (C.V. in %).

Applicability: Extracts of mushrooms without amanitins (*A. muscaria, M. procera, O. olearius*) and extracts of mushrooms containing amanitins (*A. phalloides, A. virosa, L. josserandii*) were analysed. UV spectrum of each peak was visualized to confirm the presence/absence of amanitins or interfering compounds.

Recovery by the addition of standard known amounts: *A. muscaria* and *A. phalloides* water extracts were analysed by HPLC to quantify α- and β-amanitins concentrations and compare with the same extracts spiked with known concentrations of pure amanitins. Recoveries were determined as [(mean of measured in the mushroom extract spiked-measured in the mushroom extract without spiked)/(expected concentration) × 100].

HP-TLC method

Pre-coated HP-TLC plates of silica gel 60 on glass (20 × 10 cm) with fluorescent indicator F254 were purchased from Merck (Darmstadt, Germany). Spots of samples and standards were applied on the TLC plate by Automatic TLC Sampler 4 (CAMAG, Muttenz, Switzerland). The system was operated using CAMAG WinCATS Software version 1.4.9. Plates for the analyses were developed in a tank containing 10 mL of solvent system [2-butanol/ethyl acetate/water (28:24:10, v/v/v)]. Chromatographic plates were observed under CAMAG TLC Scanner 3 (CAMAG, Muttenz, Switzerland) at 254 nm and 294 nm. The plates were sprayed using cinnamaldehyde reagent extemporary prepared as follows: cinnamaldehyde/methanol/HCl (0.3:15:5, v/v/v), then heating at 50°C during 2 min and directly analyses under scanner at 500 nm.

Linearity/work range: Standard curves were generated by increasing amounts of α- and β-amanitins corresponding to a concentration range of 100-1000 ng/spot. Peak areas of α- and β-amanitins were integrated and a calibration curve constructed. Regression lines of best fit were constructed and deemed acceptable if the regression coefficient, *r*, was > 0.99.

Limit of detection/Limit of quantification (LOD/LOQ): The LOD was defined as the sample concentration resulting in a response of three times the noise level. The LOQ was defined as the sample concentration resulting in a response of ten times the noise level.

Recoveries of pure amanitins were assessed by analysing samples at each of three concentrations (400-600-800 ng/spot). The accuracy was expressed as percent error [(mean of measured)/mean of expected] × 100, while the precision was given by determined coefficient of variation (C.V. in %).

Applicability: Extracts of mushrooms without amanitins (*A. muscaria, M. procera, O. olearius*) and extracts of mushrooms containing amanitins (*A. phalloides, A. virosa, L. josserandii*) were analysed. UV spectrum of each peak was visualized to confirm the presence/absence of amanitins or interfering compounds.

Recovery by the assay of known added amounts: *A. muscaria* and *A. phalloides* water extracts were analysed by HP-TLC to quantify α- and β-amanitins concentrations and compare with the same extract spiked with known concentrations of pure amanitins. Recoveries were determined as (mean of measured in the mushroom extract spiked-measured in the mushroom extract without spiked)/(expected concentration) × 100.

RESULTS

Immunoassay method

Enzyme-linked immunosorbent assay (ELISA) is the most used in clinical setting for detecting α-amanitin in biological samples (Butera *et al*., 2004; Parant *et al.*, 2006; Gomolka *et al.*, 2011; Gausterer *et al.*, 2014).

In the present study, Bühlmann kit is used to determine presence of amanitins directly in mushroom samples. Reconstitution of reactive products contained in the kit, preparation of the samples, kit procedure and analyses are performed in 4 hours.

Hydroalcoholic extraction of the mushrooms revealed false positive results for all the samples tested following manufacturer's procedure (data not shown). Hence aqueous extractions were conducted to avoid false positive. Consequently, the three methods were conducted using aqueous extraction to be compared.

Linearity

Calibrators give a curve fit y = (A-D)/(1+(x/C)^B)+D, with :
A = 1.83; B = 0.453, C = 1.27, D = – 0.236
Correlation Coefficient (R^2) is calculated as 1.00 for the three experiments.

LOD/LOQ

Limit of detection was established by the manufacturer as 0.22 ng/mL (Functional Sensitivity) whereas LOQ is 1.5 ng/mL (Analytical Sensitivity). It should be noted that β-amanitin is not detected by the kit.

Table 1. Recovery of pure amanitins standards

Amatoxins	*Expected concentration (ng/mL)*	*Measured concentration in ng/mL (C.V.* in %) n = 3*	*Recovery (observed/expected)*
α-amanitin	0	0.893 (5.5%)	–
	1	1.360 (2.7%)	136.0%
	3	2.516 (1.3%)	83.87%
	10	8.886 (3.1%)	88.86%
	30	27.57 (6.7%)	91.90%
	100	145.9 (9.3%)	145.9%
β-amanitin	0	0.517 (5.4%)	–
	1	0.789 (13.1%)	78.90%
	3	0.738 (9.8%)	24.60%
	10	0.780 (6.5%)	7.800%
	30	0.839 (4.9%)	2.796%
	100	0.995 (3.2%)	0.995%

* C.V. = Coefficient of Variation.

Recovery of pure standards

Quantitative analysis of known concentrations of α- and β-amanitins was done to confirm the exactitude of the kit. We observed a good correlation between absorbance measured for α-amanitin samples prepared in the laboratory and the kit's calibrator with recovery range between 83.4% and 145.9% (Table 1). Similar range is presented in the manufacturer's specifications (80.0%-127.5%).

β-amanitin reacts with only 1% of the highest concentration (recovery of 0.995% with 100 ng/mL). This value is consistent with the low specificity for β-amanitin manufacturer's procedure (specificity of 0.1% for β-amanitin). For lower concentrations the recoveries are aberrant; this is in accordance with functional sensitivity of 1.5 ng/mL for the kit. In consequence no correlation can be done for lower concentrations.

Applicability on mushrooms (Immunoassay method)

As presented in Table 2, the mushrooms containing α-amanitin react with the Bühlmann kit (α-amanitin concentrations up to 100 ng/mL). Conversely, mushrooms without amanitin appeared negative for the presence of α-amanitin: in other words, no false positive results were recorded. The mushrooms tested were chosen for being known to belong to various genera. Nevertheless, further investigations should be carried out with a larger diversity of mushroom species to confirm the absence of false positive when using the immunoassay method on mushroom matrix.

At the tested concentration (dried mushrooms: 25 mg/mL, then dilution ½) *A. phalloides* solution contains up to 100 ng/mL of α-amanitin (the assay has a range of 1-100 ng/mL). We selected this concentration for several reasons. First, to avoid false negative results the mushrooms concentration must be elevated. Second, no correlation can be made between ingested mushroom amount and prognosis. Therefore, a secured qualitative test (limiting probability of false negative) is more useful than a quantitative test without any recommendation for therapeutic guidance.

Table 2. Analysis of amanitins in mushrooms by immunoassay method

Mushrooms	*Expected concentration*	*Measured concentrations (means in ng/mL)*
A. muscaria	0	< LOD
M. procera	0	< LOD
O. olearius	0	< LOD
A. phalloides	+	> 100
A. virosa	+	> 100
L. josserandii	+	> 100

Table 3. LOD and LOQ for amanitins at 294 nm

	α-amanitin	*β-amanitin*
LOD	20.5 ng/mL	29.1 ng/mL
LOQ	68.5 ng/mL	97.1 ng/mL

Table 4. Recoveries measurements for pure amanitins at 294 nm

Amatoxins	*Level*	*Observed concentration (C.V. in %)*	*Recovery*
	6 µg/mL	5.30 (1.17)	88.3%
α-amanitin	18 µg/mL	18.38 (0.63)	102.1%
	50 µg/mL	51.50 (0.20)	103.0%
	6 µg/mL	5.1 (1.38)	84.4%
β-amanitin	18 µg/mL	18.3 (1.28)	101.9%
	50 µg/mL	52.3 (2.53)	104.6%

HPLC method

Linearity

Calibration curves for α-amanitin (Fig. 1) and for β-amanitin (Fig. 2) are presented at 294 nm. For both amanitins, correlation factors were calculated as $R^2 > 0.999$ in the range 2-100 µg/mL.

Limits of detection/quantification for α- and β-amanitins

LOD and LOQ were calculated at 294 nm for both amanitins diluted in H_2O. Results are presented in Table 3.

Recovery of pure amanitin standards

Measurements have been performed for three concentrations, i.e., 6 µg/mL, 18 µg/mL and 50 µg/mL. Recoveries of both amanitins were in the acceptable range 84.4-104.6% (Table 4).

Applicability on mushrooms (HPLC method)

Three amanitin-containing mushrooms and three amanitins-free mushrooms were analysed using HPLC method at 294 nm. The results are presented in Table 5, and are in accordance with literature (Ahmed *et al*., 2010; Sgambelluri *et al*., 2014).

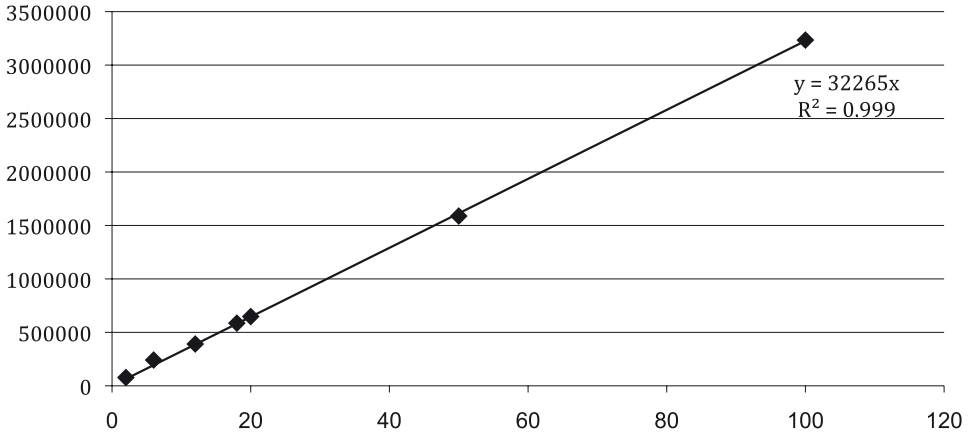

Fig.1. Calibration curve of α-amanitin in water at 294 nm (2-100 µg/mL).

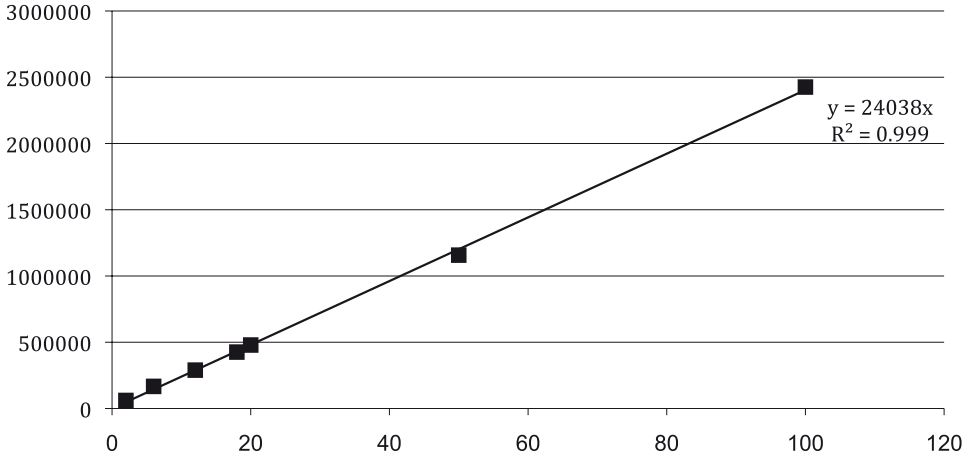

Fig. 2. Calibration curve of β-amanitin in water at 294 nm (2-100 µg/mL).

Table 5. Analysis of amanitins in mushrooms by HPLC-UV

Mushrooms	*Expected concentrations*	*Amount in dried mushroom (µg/g)*	
		α-amanitin	*β-amanitin*
A. muscaria	0	< LOD	< LOD
M. procera	0	< LOD	< LOD
O. olearius	0	< LOD	< LOD
A. phalloides	+	2026	1585
A. virosa	+	1031	< LOD
L. josserandii	+	3031	< LOD

Table 6. Amanitins recovery by the assay of known added amounts

	Amanita muscaria	
	α-amanitin	*β-amanitin*
Concentration difference	5.0 µg/mL	7.4 µg/mL
Recovery (%)	83.0%	124.1%
	Amanita phalloides	
	α-amanitin	*β-amanitin*
Concentration difference	17.6 µg/mL	25.0 µg/mL
Recovery (%)	87.9%	124.8%

Recovery by the assay of known added amounts

A. phalloides (with amanitins) and a mushroom without amanitins (*A. muscaria*), were spiked with known concentration of α- and β-amanitins. Recoveries for both amanitins are in the range 83.0-124.8% (Table 6).

HP-TLC method

Linearity

Calibration curves for α-amanitin (Fig. 3) and for β-amanitin (Fig. 4) were performed at 254 nm: a nonspecific wavelength, but present in all laboratories worldwide. Linearity is also presented at 294 nm corresponding to the wavelength absorbance for amanitins. For both wavelengths, correlation factors were calculated as $R^2 > 0.994$ in the range 100 ng-1000 ng/spot.

The calibration curve was also presented at 500 nm, after spraying a cinnamaldehyde reagent (Fig. 5), a classical reagent used for detection of amanitins (Stijve & Seeger, 1979). In this case, the linearity is presented in the range 1-20 µg. This is different from linearity at 254 nm and 294 nm, because of a decreased

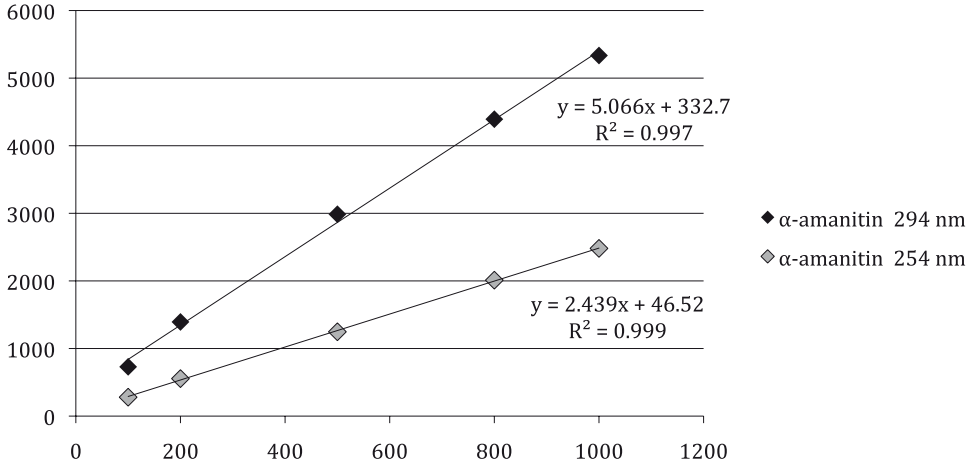

Fig. 3. Calibration curve for α-amanitin at 254 nm and 294 nm (100-1000 ng/spot).

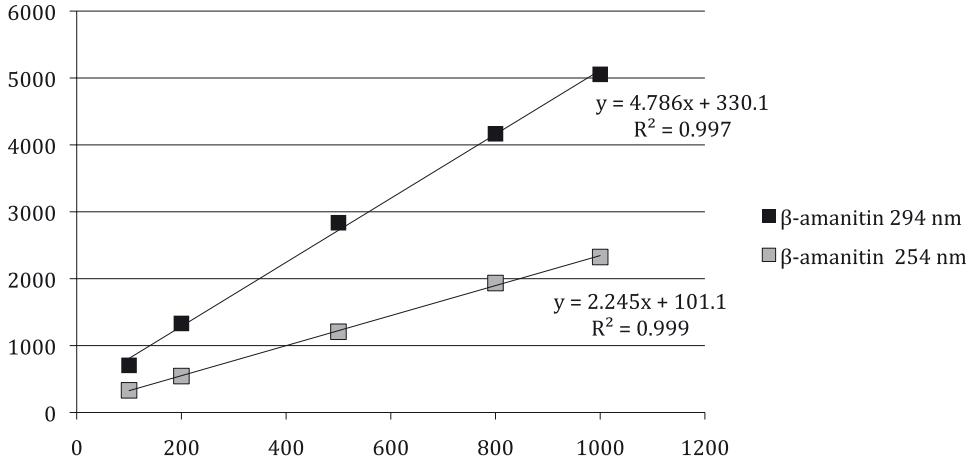

Fig. 4. Calibration curve for β-amanitin at 254 nm and 294 nm (100-1000 ng/spot).

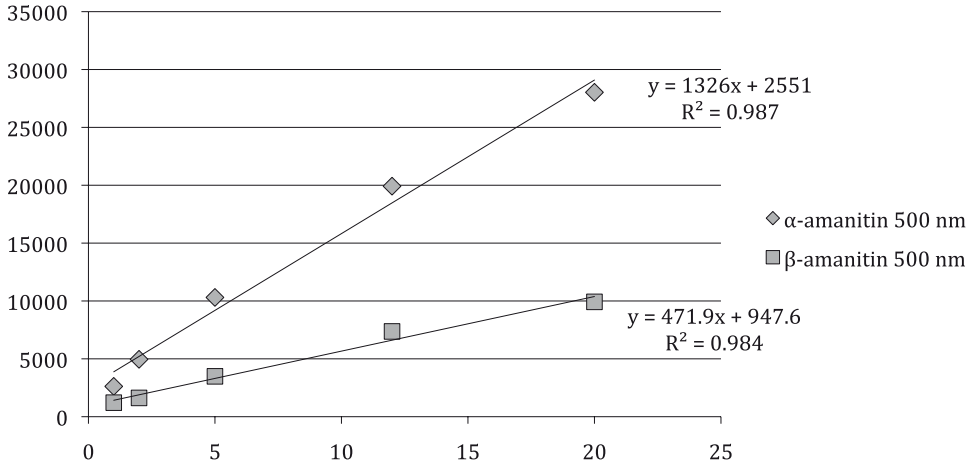

Fig. 5. Calibration curve for α- and β-amanitins at 500 nm after spraying cinnamaldehyde reagent (1-20 µg/spot).

sensibility after reagent spraying. Correlation factors (R^2) are > 0.98 in the range 1-20 µg/mL.

LOD and LOQ

LOD was defined as 3 times signal-to-noise ratio, whereas LOQ was 10 time signal-to-noise. Determination of signal-to-noise was performed on 20 blank samples. LOD/LOQ is expressed in ng/spot (Table 7).

As presented in Table 7, 294 nm should be the wavelength of choice for the lowest detection and quantification of amanitins.

Recovery of pure amanitins

Recovery with pure amanitins was evaluated at 294 nm and 254 nm (Table 8). For α-amanitin, recovery was calculated in the range 99.4-118.8% at

Table 7. LOD and LOQ for amanitins at 254 nm, 294 nm, and at 500 nm after spraying cinnamaldehyde reagen

	UV 254 nm		*UV 294 nm*		*UV 500 nm after cinnamaldehyde spraying*	
	α-amanitin	*β-amanitin*	*α-amanitin*	*β-amanitin*	*α-amanitin*	*β-amanitin*
LOD (ng/spot)	47	59	23	31	272	388
LOQ (ng/spot)	157	197	82	104	905	1293

Table 8. Recoveries measurements for pure amanitins at 254 nm and 294 nm

Amatoxins	*Expected amount (ng/spot)*	*Wavelength*	*Found amount in ng/spot (C.V. in %)*	*Recovery (%)*
α-amanitin	400 ng	294 nm	467.53 (1.20)	118.8
		254 nm	440.20 (1.00)	110.0
	600 ng	294 nm	651.20 (1.24)	108.5
		254 nm	635.24 (2.08)	105.9
	800 ng	294 nm	795.34 (1.85)	99.4
		254 nm	805.98 (3.34)	100.8
β-amanitin	400 ng	294 nm	433.97 (1.33)	108.5
		254 nm	413.61 (2.46)	103.4
	600 ng	294 nm	609.56 (1.10)	101.6
		254 nm	609.86 (0.97)	101.6
	800 ng	294 nm	754.42 (2.99)	94.3
		254 nm	775.52 (4.53)	96.9

Table 9. Recoveries for pure amanitins at 500 nm after spaying with cinnamaldehyde

Amatoxins	*Nominal value (ng/plot)*	*Found amount in ng/spot (C.V. in %)*	*Recovery (Found amount/ Nominal value) (%)*
α-amanitin	400 ng	1.38 (22)	27.7
	600 ng	3.54 (25)	35.4
	800 ng	5.23 (24)	34.9
β-amanitin	400 ng	18.13 (15)	362.5
	600 ng	29.80 (12)	298.0
	800 ng	37.12 (10)	247.5

294 nm and 100.8-110.0% at 254 nm (three amounts, three experiments). For β-amanitin, recovery was in the range 94.3-108.5% at 294 nm and 96.3-103.4% at 254 nm.

As presented in Table 9, using cinnamaldehyde reagent then absorbance measurement at 500 nm led to low recovery for α-amanitin, whereas recovery for β-amanitin was above 300%. So, this method should not be used for quantitative determination of amanitins. Nevertheless, cinnamaldehyde spraying can be useful for enhanced specificity during basic TLC analysis.

Table 10. Analysis of amanitins in mushrooms by HP-TLC

Mushrooms	*Expected concentrations*	*Amount in dried mushroom (µg/g)*	
		α-amanitin	*β-amanitin*
A. muscaria	0	< LOD	< LOD
M. procera	0	< LOD	< LOD
O. olearius	0	< LOD	< LOD
A. phalloides	+	3464	700
A. virosa	+	1400	< LOD
L. josserandii	+	5100	< LOD

Table 11. Recovery by the assay of known added amounts

Amanita muscaria				
	α-amanitin		*β-amanitin*	
Wavelength	254 nm	294 nm	254 nm	294 nm
Extract/spot	2 µL	2 µL	2 µL	2 µL
Amount difference/spot *	220.7	204.2	236.5	208.6
(C.V. in %)	(11.3)	(10.0)	(9.0)	(15.5)
Recovery (%)	110.4	102.1	118.2	104.3
Amanita phalloides				
	α-amanitin		*β-amanitin*	
Wavelength	254 nm	294 nm	254 nm	294 nm
Extract/spot	2 µL	2 µL	2 µL	2 µL
Amount difference/spot *	238.4	226.1	259.9	207.9
(C.V. in %)	(13.1)	(10.9)	(3.3)	(9.8)
Recovery (%)	119.2	113.1	129.9	104.0

* Amount difference /spot is calculated as the difference of amount in amanitins detected in mushroom spiked with 200 ng and amount in amanitins detected in mushroom alone.

Applicability on mushrooms (HP-TLC method)

Three mushrooms containing amanitins and three free of amanitin (not containing amanitin) mushrooms were analysed using HP-TLC method at 294 nm. The results are presented in Table 10, and are in accordance with the literature (Ahmed *et al*., 2010; Sgambelluri *et al*., 2014).

Recovery by the assay of known added amounts

Recoveries were calculated in *A. muscaria* and *A. phalloides*, spiked with a known amount of amanitins (200 ng). As presented in Table 11, recoveries for both amanitins (α-and β-amanitins) are in the range 110.4-129.9% at 254 nm and in the range 102.1-113.1% at 294 nm. It should be noticed that recovery measured for β-amanitin at 254 nm for *A. phalloides* is particularly high (129.9%), because of the non-specificity of this wavelength. For both mushrooms, the wavelength 294 nm should be preferably used for a quantitative analysis, whereas 254 nm can be used for a semi-quantitative analysis when a scanner (with 294 nm as wavelength) is not available in laboratory.

DISCUSSION

Amatoxin poisoning is potentially fatal or may lead to severe health injury (e.g. liver failure). Initial semiology is non-specific and there is no antidote. Therefore medical emergency focuses on: (1) preliminary medical care and supportive measures to maintain the vital functions, (2) specific treatments protocols based on one drug or various combinations, and (3) liver transplantation (Allen *et al*., 2012; Bergis *et al*., 2012; Escudié *et al.,* 2007; Enjalbert *et al*., 2002; Karakayali *et al.*, 2007; Evrenoglou *et al*., 2010; Jander & Bischoff, 2010; Mengs *et al*., 2012; Poucheret *et al*., 2010; Ward *et al*., 2013; Zhang *et al*., 2014; Bakirci *et al*., 2015; Garcia *et al.*, 2015c). Therefore early differential diagnosis is crucial. It allows medical care to act earlier and focus on limiting as much as possible the detrimental effects of toxin enterohepatic recirculation on liver (Santi *et al.*, 2012; Erden *et al*., 2013; Thiel *et al*., 2011; Zhang *et al*., 2014) and later on kidney (Das *et al*., 2007; Unverir *et al.*, 2007; Garrouste *et al*., 2009). Also a general clinical consensus was reached regarding the absence of prognostic correlation between amatoxins concentration in biological fluids (blood and urines) or biopsy (Rieck & Platt, 1988; Filigenzi *et al.*, 2007) and severity of pathophysiological damages. Taken together, these combined parameters suggest that in terms of clinical intervention, the qualitative detection of amatoxins is more critical than their actual quantification. The main objective of our work is to provide reliable proof for either presence or absence of amatoxins in mushroom materials after ingestion.

Indeed amatoxin-containing mushroom poisonings are worldwide distributed and the frequency increases due to confusion with other macrofungi. Affected regions are characterized by important disparities in term of available technological equipment for analytical investigations. In this context the present study was designed to define advantages and disadvantages of standard most accessible analytical methods for amatoxin detection. Results presented are intended to provide clinicians with comparative data. It will support their decision-making in relation to the chosen methods and potential of results interpretation when facing suspected poisoning due to amatoxin-containing mushrooms. The final aim is to be able to reach faster and effective diagnosis in order to optimize patient care for saving life. In consequence, choice of the method will be made with full knowledge of advantages and disadvantages of each technique as a function of local technological possibilities. Several methods were compared: (1) a commercialized immunoassay kit, (2) standard high-performance liquid chromatography (HPLC) and (3) high-performance thin layer chromatography (HP-TLC). For each method, linearity, limit of detection (LOD), limit of quantification (LOQ) and recovery were determined. In addition a challenge test in "real conditions", of all methods, on mushrooms containing or not amatoxins was performed before general conclusion.

To the best of our knowledge, this is the first time that basic amatoxin detection methods are compared to provide rational support for decision-making. During the past few years, much work was performed and reported in the literature on detection and quantitation of amatoxins in biological samples (Chen & Hu, 2014). Most methods used HPLC with sophisticated and elegant protocols in combination with associated devices (Garcia *et al*., 2015a; Gicquel *et al.*, 2014; Helfer *et al*., 2014; Leite *et al*. 2013) thereby leading to major improvements in decreasing the detection/quantification limit. Even though these methods are of high analytical quality, not all clinicians dealing with amatoxin poisoning in various parts of the world have access to such sophisticated protocols and expensive equipment.

Indeed, in situations of emergency, the choice of the appropriate, accessible, affordable and easy to use techniques are necessary to support early diagnosis, Taking into account the great disparity of logistic constraints and equipment between health care facilities around the world, an integrative comparison of cost effective basic methods of amatoxin qualitative analysis might help decision-making in choosing appropriate techniques based on knowledge of pros & cons for optimal clinical result exploitation.

Our investigations were performed on aqueous extracts of mushrooms for two main reasons. Firstly homogeneity and compatibility between techniques had to be respected for unbiased comparison. Indeed immunoassay sample preparation requested aqueous extraction to avoid false positive or false negative results. This protocol requirement was compatible with all three methods studied. Secondly, water is more available and easier to handle than any other expensive organic solvent.

Immunoassay method. It showed that amatoxin detection kit is useful for detecting α-amanitin directly in aqueous mushroom samples. It is efficient in urine, serum and plasma as specified by the manufacturer. Following the provided procedure, this method is straightforward and relatively easy to perform. Beside a microplate reader (450 nm), required material to perform the assay is very common and widespread. Sample pre-treatment (aqueous extraction) is quick and simple. Linearity, limit of detection (LOD), limit of quantification (LOQ) and recovery are validated and provided by the kit supplier. Results obtained by this technique are reproducible. α-Amanitin is efficiently detected with a detection range as low as 0.22 ng/mL. Nevertheless, immunoassay method is costly and potentially difficult to obtain in remote and/or limited budgets area. A significant supplier restriction "not intended for use in diagnostic procedure" limits its use to research purpose only, thereby as part of post-clinical care investigations. It requires freezing for kit storage. α-Amanitin being the only toxin detected, other toxins, contributing to the phalloid syndrome poisoning such as β-amanitin, are not detected. Moreover, the range of detection compatible with the validated calibration curve is quite restrictive (1-100 ng/mL) leading to potential repetition of sample preparation and/or full assay repetition.

In conclusion, immunoassay main pros include: "all included & ready to use" feature, ease of use, sensitivity in the low nanogram range, efficiency for α-amanitin detection. Kit main cons include: restriction to research use, high price, assays duration (over 4 h), storage conditions (– 20°C), potential false positive/false negative results if supplier protocol conditions are not precisely respected. Immunoassay kit can be used in α-amanitin detection with mushrooms samples with a major precaution: extraction process has to be done only with water. Approximately concentration of 25 mg/mL of dried mushrooms is enough for the kit.

HPLC method. It confirmed the High-Performance Liquid Chromatography method usefulness for detection of α- and β-amanitins in aqueous mushroom samples (from residue of harvest, cooking or vomiting). The procedure is straightforward and easy to perform under the condition of a preliminary set up for suitable conditions. Beside HPLC machine, required material is common and widespread. Sample pre-treatment, after aqueous extraction, needs to be properly performed but remains simple upon reagent availability (solvents and amatoxins standards). Linearity, LOD, LOQ and recovery should be set up before facing an actual poisoning case. Results are reproducible; both α- and β-amanitins are efficiently detected. Nonetheless, HPLC method needs to have an operating HPLC apparatus which can sometimes be

not as evident as it seems, mainly in remote area, even if this material is now very common. Local procedure preliminary validation is mandatory before use for clinical purpose. The range of detection indicates middle range sensitivity, around the 20 ng/mL minimum. Both α- and β-amanitins can be detected. The range of concentration for calibration curve is large, allowing easier matching with samples concentration, thereby limiting the necessity to multiply HPLC runs to obtain results.

In conclusion, HPLC method main pros include: compatibility with clinical purpose in diagnosis, range of detection compatible with expected sample concentration, efficient detection of both α- and β-amanitins, middle range assay duration (2-3 h) and absence of false positive and/or false negative results. Main cons include: operating HPLC material availability, amatoxins references permanent storage, necessity to pre-set up the method, middle range sensitivity.

HP-TLC method. It suggested that the High-Performance Thin Layer Chromatography technique or TLC can be used to detect both α- and β-amanitins in aqueous mushroom samples. Procedure is simple and easy to perform. This method requires material that is usually common in most laboratories. Sample pretreatment is fast and easy upon access to adequate reagents (solvent, amatoxins standards and spraying reagent). Linearity, LOD, LOQ and accuracy should be set before use in an actual poisoning case. Results are reproducible, and both α- and β-amanitins are detected. Nonetheless, sensitivity remains pretty low (over 100 ng/spot) and local procedure validation is mandatory before considering its use for diagnosis purpose. Cinnamaldehyde was used in the present study to improve sensibility and specificity. Other reagents such as sulfanilic acid can also be used as spraying reagent (Andary *et al.*, 1977). As for HPLC, the range of amatoxin detection makes it more compatible with significant amounts of test sample thereby limiting the necessity of re-assay.

In conclusion, TLC method main pros include: a short assay duration (1-2 h) to obtain an analytical response, compatibility with clinical diagnosis purpose, efficient detection of α- and β-amanitins in the high concentration range (over 100 ng/spot) and absence of false positive and/or false negative results. Main cons include: requested pre-setup of this method, a low sensitivity and elution solvents and spraying reagents availability.

In order to finalize the assessment of the three detection methods of α- and β-amanitins, they were applied in "real conditions" on harvested residue from six macrofungi species: three amatoxin-containing species and three amatoxin-free species. All three methods properly identified presence or absence of amatoxins in the tested mushrooms. The results suggest that upon availability, any method can be used to detect α-amanitin, but not β-amanitin since it was only detected by HPLC and TLC methods. This validates the possibility to use immunoassay, HPLC and TLC on aqueous extract of mushroom residue from harvest, cooking or vomiting origin. Nonetheless it should be noted that immunoassay is not intended to be used on mushroom aqueous extract therefore caution must be considered for its use. In addition further investigations should be carried out to confirm the presence of false negative results (Gicquel *et al.*, 2014).

CONCLUSION

Major information and data to remember are summarized in Table 12. It was produced for clinicians to have comprehensive data for decision-making in their choice of amatoxin detection method for differential diagnosis. This choice should

Table 12. Comparison of immunoassay method, HPLC and TLC for α- and β-amanitins detection for physician decision-making

	Amatoxins	*Immunoassay method*	*HPLC method (at 294 nm)*	*HP-TLC method (at 294 nm)*
Linearity	α-amanitin	Range : 0-100 ng/mL R^2 = 1.00	2-100 µg/mL R^2 = 0,9996	100 ng-1 µg/spot R^2 = 0.9978
	β-amanitin	N.D.*	2-100 µg/mL R^2 = 0,9992	100 ng-1 µg/spot R^2 = 0.9978
LOD/LOQ	α-amanitin	LOD = 0.22 ng/mL LOQ = 1.5 ng/mL	LOD = 20,5 ng/mL LOQ = 68,5 ng/mL	LOD = 23 ng/spot LOQ = 82 ng/spot
	β-amanitin	N.D.	LOD = 29,1 ng/mL LOQ = 97,1 ng/mL	LOD = 31 ng/spot LOQ = 104 ng/spot
Recovery of pure amanitins	α-amanitin	84-146%	88.3-103.0%	99.4-118.8 %
	β-amanitin	N.D.	84.4-104.6 %	94.3-108.5%
Recovery in mushrooms	α-amanitin	N.D.	83.0-87.9 %	102.1-113.1 %
	β-amanitin	N.D.	124.1-124.8 %	104.0-104.3 %
Qualitative analysis in mushroom	α-amanitin	+++	+++	+++
	β-amanitin	–	+++	+++
Quantitative analysis in mushroom	α-amanitin	+/– but in the range 0-100 ng/mL	+++	+
	β-amanitin	–	+++	+
Specificity	α-amanitin	+++	++	++
	β-amanitin	–	++	++
Time of analysis		3-4 h	2-3 h	1-2 h
Cost of the analysis		+++	++	+
Storage		Amanitins at – 20°C (available into the kit) Others reagents at -20°C	Amanitins at – 20°C Others reagents at R.T.**	Amanitins at – 20°C Others reagents at R.T.

*N.D.: Not Determined
**R.T.: Room Temperature

be made depending on local logistic constraints, reagents availability, expected result outcome and type of use (clinical applied analyses or fundamental research). These comparative data on analytical methods for amatoxin detection and amatoxin poisoning diagnosis along with comparative data on treatment decision-making (Poucheret *et al*., 2010; Trabulus & Altiparmak, 2011; Allen *et al.,* 2012; Roberts *et al.*, 2013; Gores *et al*., 2014; Zhang *et al.*, 2014; Laita *et al.*, 2015) should provide clinician comprehensive tools that may contribute to save human lives.

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