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Author: Marta Leite Andreia Freitas Anabela Marisa Azul Jorge Barbosa Saul Costa Fernando Ramos

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DEVELOPMENT, OPTIMIZATION AND APPLICATION OF AN ANALYTICAL METHODOLOGY BY ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY FOR DETERMINATION OF AMANITINS IN URINE AND LIVER SAMPLES

Marta Leite[a]; Andreia Freitas[b]; Anabela Marisa Azul[c]; Jorge Barbosa[b]; Saul Costa[a]; Fernando Ramos*[a]

[a]CEF – Center for Pharmaceutical Studies, Health Sciences Campus, Pharmacy Faculty, University of Coimbra, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal

[b]INIAV, Instituto Nacional de Investigação Agrária e Veterinária, Unidade Estratégica de Investigação e Serviços de Tecnologia e Segurança Alimentar
Estrada de Benfica, 701, 1549-011 Lisboa – Portugal

[c]Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, POBox 3046, 3001-455 Coimbra, Portugal

***Corresponding author:** Fernando Ramos

Postal address: CEF – Center for Pharmaceutical Studies, Health Sciences Campus, Pharmacy Faculty, University of Coimbra, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal

Telephone: + (351) 239 488492 **Fax:** + (351) 239 488503

E-mail adress: fjramos@ci.uc.pt

Abstract

Amanitins, highly toxic cyclopeptides isolated from various *Amanita* species, are the most potent poisons accounting for the hazardous effects on intestinal epithelium cells and hepatocytes, and probably the sole cause of fatal human poisoning.

The present study was focused on the development, optimization and application of an analytical methodology by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), following urine and liver sample preparation by protein precipitation with organic solvents, and solid phase extraction (SPE) procedure, for the determination of the amatoxins, α- and β-amanitin. Linearity, detection and quantification limits, selectivity, sensitivity, intra and inter-assay precision and recovery were studied, in order to guarantee reliability in the analytical results. The developed method proved to be specific and selective, with LOD (Limit of Detection) values for α- and β-amanitin of 0.22 and 0.20 ng mL^{-1} in urine and 10.9 and 9.7 ng g^{-1} in liver, respectively. LOQ (Limit of Quantification) values ranged from 0.46-0.57 ng mL^{-1}in urine and 12.3-14.7 ng g^{-1} in tissue, for both amanitins. Linearity, in the range of 10.0 to 200.0 ng mL^{-1}or ng g^{-1}, shows that coefficients of correlation were greater than 0.997 for α-amanitin and 0.993 for β-amanitin. Precision was checked at three levels during three consecutive days with intra-day and inter-day coefficients of variation not greater than 15.2%. The extraction recovery presents good results for the concentrations analyzed, with values ranging from 90.2-112.9% for both matrices.

Thus, the proposed analytical method is innovative, presents a high potential in the identification, detection and determination of α- and β-amanitins in urine and tissue samples, as well as in other biological samples, such as kidney and mushrooms.

Keywords: Amanitin; Tilmicosin; Urine; Liver; Method validation.

1. Introduction

The *Amanita phalloides* mushroom poisoning is a rare but severe cause of human fatal intoxication, since they are responsible for over than 90% of the lethal cases [1,2]. Amatoxins, a group of bicyclic octapeptides produced also by other *Amanita* species, such as *A. virosa* and *A. verna*, are the cause for the high toxicity in these fungi, with α- and β-amanitin being the main toxins (Figure 1) [2-8]. These amatoxins interfere with DNA transcription by binding and inhibiting eukaryotic RNA polymerase II in hepatocytes. The progressive decrease in mRNA results in a marked arrest of protein synthesis with subsequent induced cellular necrosis, especially in the liver and kidneys. Death can occur due to fulminant hepatic failure (FHF) and renal damage [9-14].

Amatoxins are rapidly absorbed by a direct intestinal process, in high proportion [6,15,16]. They disappear rapidly from plasma and do not bind to plasma proteins, being free in circulatory system [6,17]. The volume of distribution is close to the extracellular space [6,17,18] and the total body clearance corresponds to the creatinine clearance [17]. The most important route of elimination is renal, since about 80-85% of the amatoxins dose absorbed is excreted in the urine within 6 hours and less than 10% in the bile [6,15,17]. The early elimination of the amatoxins through the kidney can be explained by the low molecular weight of the toxins, allowing an easy glomerular filtration [15]. In experimental findings, it was observed that the urine was completely out of toxins in 24-36 hours, in patients treated with forced diuresis, but they were still being eliminated from the gastric and the duodenal aspirates for longer periods of time, decreasing gradually after 24-48 hours [15,17]. In *post-mortem* investigations, amanitins have been detected in kidney and liver up to 22 days after ingestion of toxic mushrooms [6,17]. Therapeutic implications may occur due to enterohepatic recycling of amanitins [10,19].

Amatoxin intoxication presents a long incubation period of 6 to 24 hours and, in extreme cases, up to 36 hours, before the sudden appearing of the violent gastrointestinal symptoms [6,15]. In the intestinal phase or cholera like period, symptoms are typically intense, such as aqueous diarrhea, abdominal pain and vomiting [4,10,16,19,20]. There are no signs of liver toxicity and biochemical tests are normal. On the second day post-ingestion (24 to 48 hours), the symptoms appear to be diminished; though it is a false improvement state [15,19]. This is followed by the visceral involvement phase, in which hepatic and renal dysfunctions appear [10,16,20]. In severe cases, the clinical deterioration may continue, with symptoms of hepatic encephalopathy, coma and death (2 to 7 days) [4,20]. This short time development of symptomatology requires a rapid intervention in order to avoid severe organ damages and to achieve a successful outcome [17].

Unambiguous detection of amanitins in biological fluids turns to be essential for the early diagnosis of mushroom intoxication due to the invasive and extensive therapy needed for treatment. Therefore, it is important to establish a rapid and specific methodology for analysis of these toxins in appropriate samples. Gas chromatography coupled with mass spectrometry (CG-MS) is the gold method in toxicological analyses, though nonvolatile compounds cannot be identified by this method, such as the toxins in this study [21]. Several methods have been described for these purposes, each one with its own drawbacks, such as radioimmunoassay (RIA) [22-25], enzyme-linked immunosorbent assay (ELISA) [26-27] and capillary zone electrophoresis (CZE) [1,28]. RIA and ELISA, despite being the main analytical approaches for objective diagnosis, require generation and purification of antibodies, which is not possible in most of the laboratories, as well as the commercial availability of the kits, underneath the mushroom season only, with a tracer stability of two months or less for RIA [29]. CZE methods, on the other hand, present reproducibility issues, which can be problematic in routine quantitative analysis [29]. According to literature, Liquid

Chromatography (LC) is the method of excellence for amanitin separation [30,31]. It fills in all the requirements in terms of sensitivity, precision and specificity bound with operating simplicity and rapid detection of α- and β-amanitin in biological samples. A considerable number of reports make use of LC coupled to various detection methods like ultraviolet (UV) [17, 32-35], electrochemical (ECD) [36-38] and mass spectrometry (MS) [21,31,39-44]. From the following, LC-MS(-MS) techniques are the most sensitive, powerful and reliable for these analyses [31,38,41,44]. However, only a few methods using LC coupled to tandem mass spectrometry have been reported [30,31,42-44].

In the present paper, a fast and sensitive method was developed for simultaneous determination of α- and β-amanitin in urine and liver by combining ultra performance liquid chromatography (UPLC) to a triple quadrupole (TQ) mass spectrometry (MS) instrument with an electrospray ionization interface in positive mode (ESI^+), after a two-phase extraction procedure.

2. Experimental

2.1. Reagents and Materials All reagents used were of analytical grade with the exception of solvents used for mobile phase that were high-performance liquid chromatography grade. Standards of α-amanitin (≥ 90% purity), β-amanitin (≈ 90% purity), and internal standard (IS) tilmicosin (Figure 1) were purchased from Sigma-Aldrich (Steinheim, Germany). Ultrapure water was obtained from Millipore System (France). Methylene chloride, chloroform, ammonium acetate and glacial acetic acid were obtained from Merck (Darmstadt, Germany); acetonitrile and methanol were obtained, respectively, from JT Baker (Deventer, Holland) and Fischer Chemical (Leicestershire, United Kingdom). Monobasic and dibasic potassium phosphates (minimum 99% pure) were obtained from Sigma-Aldrich (Steinheim, Germany).

Oasis® HLB 6cc (500 mg) and Oasis® HLB 3cc (60 mg) polymeric sorbent cartridges were purchased from Waters (Milford, MA, USA); Clean Screen® DAU (1000mg, 6 mL) from Agilent (Santa Clara, CA, USA). ACQUITY UPLC® HSS T3 1.8 μm (2.1×100 mm i.d.) and ACQUITY UPLC® BEH C18 1.7 μm (2.1×100 mm i.d.) separation columns were purchased from Waters (Milford, MA, USA). HPLC vials and *Syringeless Device Mini-UniPrep* filters (PVDF, polypropylene) were obtained from Whatman (Maidstone, England).

2.2. Instrumentation

For identification and confirmation of amanitins in biological samples, a liquid chromatographic system coupled to a tandem mass detector was used. In the chromatographic system, an ACQUITY UPLC® HSS T3 1.8 μm (2.1×100 mm) separation column was used for analyte separation. The UPLC ACQUILITY system (Waters, Milford, MA, USA) consisted of binary pumps, a variable-volume autosampler with refrigeration system for the samples and a thermostated column compartment.

Amanit toxins were identified and quantified in biosamples using a XEVO TQ MS detector (Waters, Milford, MA, USA) coupled to the prior chromatographic system. The TQ MS was operated using an electrospray interface in positive ion mode (ESI^+) – TurboIonspray – and a triple quadrupole analyzer. Data acquisition was controlled by the MassLynx® software, version 4.1 SCN 714 (Waters, Milford, MA, USA).

The TQ MS conditions were: capillary voltage, 1.5 kV; extractor voltage, 3 V; ion source temperature, 150ºC; desolvation gas (1000 L h^{-1}, 500ºC), nitrogen; collision gas (0.22 mL min^{-1}), argon. Multiple Reaction Monitoring (MRM) was used to measure the target compounds. MRM parameters for each compound and ion transitions were optimized, as summarized in Table 1.

To obtain MS/MS spectra of the compounds and to optimize ESI conditions, as well as to define the ideal voltage for fragmentation of the toxins, a methanol solution containing α- and β-amanitin (4 μg mL^{-1} each) was directly infused into the mass spectrometer at a flow rate of 0.02 mL min^{-1}.

The chromatographic conditions used were: sample injection volume, 20 μL; flow rate, 0.5 mL min^{-1}; column temperature, 40ºC; autosampler temperature, 10ºC; mobile-phase solvents, (A) 0.02M ammonium acetate pH 5 and (B) acetonitrile; gradient elution protocol, 100% A to 87.5% A/ 12.5% B (5 min, 1-min hold), 87.5% A/ 12.5% B to 100% B (7 min), 100% B to 100% A (8 min, 2-min hold); total run time, 10 min.

2.3. Samples

Human urine samples were obtained from healthy volunteers for blank control. Fresh porcine liver was purchased from a local market for use as a blank matrix. It should be noted that a single source of liver matrix was used for the preparation of fortified control samples. Thus, this investigation does not take into account potential differences in control matrix composition for liver that could affect measurements of amanitin levels between individuals.

2.4. Preparation of Calibration Standards and Fortified Samples

Separate stock solutions of 40 μg mL^{-1} of each standard were prepared in methanol. Working solutions used for fortifying control samples were prepared by successive dilution process of the stock solutions with methanol to 0.4, 2 and 4 μg mL^{-1} of α- and β-amanitin. All of the standard solutions were stored at -20±2ºC, protected from light. All fortified samples were prepared by adding an appropriate level of standard solution of α-amanitin, β-amanitin and IS in methanol to aliquots of 1 mL of blank urine samples and aliquots of 1 g of blank chopped and homogenized liver tissue.

174

175 **2.5. Extraction of Amanitin from Urine**

176 An aliquot of 1 mL of urine sample was combined with 2 mL of acetonitrile and
177 vortexed for approximately 30 seconds. The sample was centrifuged for 10 min. at 719 *g*, at
178 4ºC using a Megafuge 1.0R Labcare centrifuge, and the supernatant was decanted into a 15-
179 mL centrifuge tube. Five milliliters of methylene chloride was added, and the tube was
180 inverted several times and then centrifuged for 5 min. at 719 *g*, 4ºC. One milliliter of the
181 aqueous (top) layer was transferred to a 15-mL centrifuge tube.

182 An Oasis® HLB 6cc (500 mg) cartridge was preconditioned with 2 mL of methanol
183 and 2 mL of ultrapure water. The aqueous extract (1 mL) was then applied to the
184 preconditioned cartridge. It was washed with 1 mL of 5% methanol in chloroform. The toxins
185 were eluted with 6 mL of methanol. The eluate was evaporated to dryness under nitrogen, at
186 60ºC, using a QTB2 dry block heating system, and the dry residue was reconstituted in 300-
187 µL of 0.02M ammonium acetate pH 5. The 300-µL extract solution was filtered to HPLC
188 vials and 20 µL of the reconstituted extract was injected in the UPLC-MS/MS system.

189

190 **2.6. Extraction of Amanitin from Liver**

191 Liver tissue was finely chopped. One gram of the chopped tissue was weighed into a
192 15-mL centrifuge tube, followed by combination with 100 ng mL^{-1} of IS, and homogenized
193 with 5 mL of 30% 0.1 M phosphate buffer pH 6 (500-mL 0.1M monobasic potassium
194 phosphate/ 150-mL 0.1M dibasic potassium phosphate) in acetonitrile using an Ultra-Turrax
195 homogenizer. The homogenate was centrifuged at 719 *g* for 10 min, at 4ºC. The supernatant
196 was transferred to a 15-mL centrifuge tube and the pellet was rinsed twice with 5-mL aliquots
197 of 0.1 M phosphate buffer. Each rinse pellet was centrifuged at 719 *g* for 10 min, at 4ºC. The
198 two rinses were then combined with the initial supernatant and 10 mL of methylene chloride

was added to the centrifuge tube. The tube was inverted several times and centrifuged at 719 *g* for 5 min, at 4ºC. The top layer was transferred to a 15-mL centrifuge tube. The aqueous layer was then centrifuged at 2204 *g* for 5 min at 4ºC and the supernatant was transferred to a test tube.

An Oasis® HLB 6cc (500 mg) cartridge was preconditioned with 2 mL of methanol, 2 mL of ultrapure water and 2 mL of 0.1 M, pH 6, phosphate buffer. All the aqueous extract (15 mL) was then applied to the preconditioned cartridge. It was washed with 1 mL of 5% methanol in chloroform. The toxins were eluted with 6 mL of methanol. The eluate was prepared for injection on UPLC-MS/MS system as described for urine samples.

2.7. Assay validation for urine and liver analysis.

The present method was validated for the determination of α- and β-amanitin in multiple matrices according to the guidelines established by FDA, ICH Q2 (RI) and Relacre [45-47]. For method validation and quantification of the compounds, peak areas of both the analyte and the IS were measured, and the analyte/internal standard ratios were determined. The analyte concentrations were calculated by using a linear regression procedure.

2.7.1. Specificity/ selectivity

Twenty blank urine samples from healthy volunteers were analyzed for peaks that could interfere with the detection of the analytes. All the samples were analyzed with the previous analytical method described, including the extraction procedure. Equally, twenty samples of blank liver samples were analyzed for the same purpose [45-47].

2.7.2. Linearity/ Work range

Quality calibration samples were studied at six concentration levels, between 0 and 200 ng mL^{-1} of α- and β-amanitin. Spiked control samples at concentrations of 10, 25, 50, 100 and 200 ng mL^{-1} of α- and β-amanitin were assayed. One calibration curve was analyzed each day for three days. Linearity was evaluated by calculation of the regression equations through the method of least squares for each curve. Correlation coefficients were obtained and residual analysis showed the straight line model is correct [45-47].

2.7.3. Detection and Quantification Limits

Quality control blank samples (n = 20) were assayed for the determination of the limit of detection (signal-to-noise ratio 3:1) and of the limit of quantification (signal-to-noise ratio 10:1). To confirm the values of LOD and LOQ, 10 blank samples fortified at LOD level and 10 blank samples fortified at LOQ level were assayed [45-47].

2.7.4. Extraction recovery

The extraction recoveries were assayed by analyzing spiked control samples of α- and β-amanitin at the levels of concentration of 10, 50 and 200 ng mL^{-1} (n = 6). This parameter was determined as follows [45-47]: Recovery (%) = A_{ex}/A_{th} x 100 (Eq. 1), in which A_{ex} is the average concentration of the six replicates measured for each quality control point and A_{th} is the theoretical concentration assayed.

2.7.5. Precision

Spiked control samples of α- and β-amanitin at three levels of concentration (10, 50 and 200 ng mL^{-1}) were assayed against a calibration curve to determine the intra-day (n = 6)

and inter-day (n = 3) precision. The coefficient of variation (*CV*) was calculated to estimate precision, according to the following equation [45-47]: $CV\ (\%) = \sigma/\mu \times 100$ (Eq. 2), in which σ is the standard deviation at each calibration level in the six spiked blank samples and μ is the mean concentration at each calibration level in the six spiked blank samples.

3. Results and Discussion

3.1. Optimization of the extraction and UPLC-MS/MS conditions

Several preliminary studies were assayed for chromatographic-spectrometric conditions and extraction procedures using different mobile phases and extraction columns/ reagents to develop a proper and advantageous analytical methodology for the separation and detection of amanitins in biological samples.

For UPLC separation, studies were developed in isocratic conditions and elution gradients with ammonium acetate 0.01M / methanol, acetonitrile, methanol:water (40:60), 0.1% formic acid in acetonitrile, ammonium acetate 0.02M / acetonitrile and 0.1% formic acid in water / 0.1% formic acid in acetonitrile. At a high acetonitrile concentration, hydrophilic interaction dominates, and the analytes under study demonstrate a much higher affinity to the stationary phase. To study the effect of the mobile phase modifier, several other solvents were selected for fine tuning the magnitude of the system in terms of proton-donor and acceptor strength. Buffers have been applied in the chromatographic separation of charged species because electrostatic interactions affecting the retention between the analytes and stationary phases were often influenced and controlled by the buffers. To avoid adverse affects in ESI by buffer salts, which could lead to ionization suppression, low buffer concentrations were used. To maximize the response of the target analyte, an acetate buffer was employed. The retention of the tested toxins was found to have significant changes upon increasing the concentrations of the buffer from 0.01M to 0.02M. Therefore, the combination of acetonitrile and acetate

buffer at 0.02M pH 5 by elution gradient, as described above, gave the best chromatographic resolution and the sharpest peaks. It was discernible that increasing the percentage of buffer could reduce the retention times of amanitins. It also allowed a good separation of both toxins and IS. In what concerns LC columns, ACQUITY UPLC® BEH C18 1.7 µm (2.1×100 mm) and ACQUITY UPLC® HSS T3 1.8 µm (2.1×100 mm) were tested, giving both good separation and high intensities. However, ACQUITY UPLC® HSS T3 gave the best results towards identification and quantification of amanitins.

In what concerns extraction procedures, several SPE cartridges were tested, namely *Clean Screen*® DAU (1000mg, 6 mL), *Oasis*® HLB (60 mg, 3 cc) and *Oasis*® HLB (500 mg, 6 cc). The lowest background noise and highest recoveries were achieved with the hydrophilic-lipophilic columns, constituted of N-vinylpyrrolidone-divinylbenzene copolymer. *Oasis*® HLB columns reveal a great capacity to retain efficiently polar and hydrophilic analytes as the ones analyzed in the present report, through a reverse-phase mechanism of retention. In consequence, we were able to obtain analytical results with good recoveries and sensibility. It was also observed a better fluidity in what concerns reagents flux through the column. Sorbent weight of Oasis HLB columns was also tested, concluding that the 500 mg sorbent per cartridge column gave better results in regard to recovery rates comparing to the 60 mg column.

Structurally, amatoxins are characterized as cyclopeptides with a tryptophan residue substituted in position 2 of the indole ring by a sulphur atom, and some unusual hydroxylated amino acids [4]. The only difference between α- and β-amanitin is the presence/absence of an amine group on an aspartic residue, in which α-amanitin presents the $–NH_2$ radical, and β-amanitin contains a –OH group as seen in Figure 1 [29,41]. Being an acidic compound, β-amanitin elutes first than α-amanitin, a neutral compound due to the $–NH_2$ group. Thus, solvents for extraction must be carefully tested in order to obtain both toxins and IS in the

same fraction. To accomplish this step, various solvents were also tested (data not shown), in which the elution with methanol reveal to be the best one concerning recovery rates and sensitivity.

Due to the complexity of liver samples in what refers to its composition, a prior step to SPE was also added to this procedure, which allowed a cleaner extract from interfering compounds, enabling a better signal-to-noise ratio, with gains in sensitivity and specificity. To proceed to the sample preparation prior to SPE, a protein precipitation procedure with organic solvents was applied. Acetonitrile was used to remove protein content from the matrices, with further adding of methylene chloride to eliminate the first solvent. Due to the different dielectric constants of water and acetonitrile, the last one allows the prevalence of protein-protein interactions present in aqueous solution over water solvation (protein-water interaction), with subsequent protein precipitation. Further centrifugation, allowed a better separation of the supernatant and the precipitate, thus facilitating the removal of proteins, susceptible of blocking the column pores. Methylene chloride, as a medium polarity solvent, removes acetonitrile from the initial solution. When the second organic solvent is added, two phases are formed, one aqueous and other organic. Since methylene chloride does not form hydrogen bonds with water, dipole-dipole forces are favorable to the interaction between this solvent and acetonitrile with similar polarity. Finally, an aqueous extract without proteins is obtained. This preparation step is essential since it prevents extraction columns to block and provides a cleaner sample for further extraction by SPE.

3.2. Internal Standard Studies

Due to the importance of internal standards in the analysis of biological samples by LC-MS methods, its selection has to be rigorous. Several different tetracycline and macrolides were used: tetracycline, doxycycline, chlortetracycline, oxytetracycline and

demethyltetracycline as tetracyclines, erythromycin, spiramycin, tilmicosin and tylosin as macrolides. Tilmicosin gave the most reproducible results, with similar chromatographic behavior as the toxins studied, being thus used as the internal standard (IS). Maurer *et al* [39] recommended γ-amanitin methyl ether as IS in determination of α- and β-amanitin in urine after immunoaffinity extraction by LC-ESI-MS. In this report, the absolute recovery for IS at 25 ng mL^{-1} was 60%. The absolute recoveries of α- and β-amanitin were 63% and 58% at 5 ng ml^{-1} and 61% and 57% at 75 ng mL^{-1}. Ahmed *et al* [41] mentioned microcystin RR as IS for quantitating amanitins by LC-TOF-MS in toxic mushrooms. In these report, the recovery rates at 100, 500 and 1000 ng g^{-1} were in the range of 53.1-69.6%. Gonmori *et al* [44] also used microcystin RR as IS for determination of amanitins in urine samples, with recovery values ranging from 60-80% for the levels of concentration of 10, 50 and 500 ng mL^{-1}. The last reported method for determination of amanitins in urine, plasma and serum used virginiamycin B as IS, with recovery rates in the range of 91.3-110.0% [42]. In the present study, we obtained better recovery results with tilmicosin than those observed in the previous reports, as shown in section 3.4.

3.3. Compounds Identification

In the present study, α-amanitin, β-amanitin and tilmicosin were identified and confirmed by their relative retention time (RRT), ion transitions and mass spectrum. In Table 1, conditions used for the confirmation and quantification of the analytes are described. RRT and ionic transitions (precursor ion > product ion) presented are those who produced the most intense signal in MRM mode.

The protonated molecular ions were chosen as precursors of the product ions. To confirm unequivocally the presence of the compounds by UPLC-MS/MS, two ionic transitions corresponding to the main ion fragmentation were used (919.48 > 901.53 > 259.13

and 920.48 > 902.44 > 259.13 for α- and β-amanitin, respectively). For quantification purposes, the ionic transitions were 919.48 > 901.53 for α-amanitin and 920.48 > 902.44 for β-amanitin. The ionic chromatograms and fragmentation mass spectra of tandem MS for α-amanitin, β-amanitin and tilmicosin are shown in Figures 2 and 3, respectively.

The positive ionization mode chosen for the present method was found to provide a more sensitive and effective tool for the identification of the concerned polar toxins because of their characteristic fragmentation patterns. Despite having very closed structures, since the only structural difference between α- and β-amanitin is an R substituent (R = NH_2 for α-amanitin; R = OH for β-amanitin), they do not respond in the same manner under ESI [28,30]. Amanitins as bicyclic octapeptides are relatively stable, producing $[M+H - H_2O]^+$ as dominant daughter ions due to the MS/MS dissociation of the $[M+H]^+$ ion. The mass spectra obtained for both α- and β-amanitin gave base peaks at m/z 901 and 902, respectively, produced by dehydration of the protonated ions. Fragmentation of $[M+H]^+$ ions by MS/MS mode also gave the base peaks at m/z 259 for both toxins (Figure 3), a characteristic fragment already reported by Ali Ahmed *et al* [41]. The chemical structural fragmentation for both toxins is proposed in Figure 4. Cleavage of peptide bonds on dihydroxy-Ile - Gly (a), Asn - Cys (b) and hydroxy-Pro - Asn (c) produces the ion peaks m/z 661 [fragment a to c + H]$^+$, 547 [fragment a to b + H]$^+$, 373 [fragment b to a + H]$^+$ and 259 [fragment c to a + H]$^+$ observed on the fragmentation mass spectra characteristic of amanitins (Figure 5). The latter product ion is therefore likely to be the protonated fragment resulted from the cleavage at c) and b) (Figure 4) with peak ion m/z 259 as shown in Figure 5. Fragment nomenclature is according to Ngoka and Gross [48].

This is the first reported method in which it is possible to obtain product ions of amanitins with high intensity and reproducible patterns that allows the possibility of

monitoring two ionic transitions for guaranteeing unambiguous identification of the substances.

3.4. Validation

The selectivity/specificity was verified by analyzing different blank urine and liver samples. At the expected retention times for the analytes, no interfering peaks could be detected in any of the analyzed samples, which could lead to false identification of the compound. This fact can be confirmed through the comparative analyses between fortified samples chromatograms with IS only and with both toxins, as seen in Fig. 2. A good chromatographic separation was obtained, which allowed for the quantification of the two compounds separately. Thus, the extraction and cleanup procedures used for biological fluids and tissues combined with UPLC-MS/MS provided chromatograms with minimal background interference.

Calibration curves were obtained in the range of 10-200 ng mL^{-1} or ng g^{-1}, at five different concentrations for each toxin in both matrices (10, 25, 50, 100 and 200 ng mL^{-1} or g^{-1}). For each level of fortification, the sample was extract as described above. All calibration curves gave good linearities for both toxins in urine and liver, with correlation coefficients greater than 0.997 and 0.993, respectively.

Values of LOD and LOQ were obtained from the intensity of background noise signal of twenty blank samples at RT of 5.73±0.5 min for α-amanitin and 5.27±0.5 min. for β-amanitin. LOD values were 0.22 and 0.20 ng mL^{-1} for α- and β-amanitin in urine, as for in the liver, these values were 10.9 and 9.7 ng g^{-1}. For LOQ, the values in urine were 0.57 ng mL^{-1} for α-amanitin and 0.5 ng mL^{-1} for β-amanitin; in liver, these values were 14.7 ng g^{-1} and 12.3 ng g^{-1} for α- and β-amanitin, respectively.

As shown in Table 2, LOD values in previous reports, were 0.05 ng mL^{-1} for serum [22], 1 ng mL^{-1} for urine and 0.1 ng mL^{-1} for plasma [24] by RIA, 10 ng mL^{-1} for urine, stomach washings and mushrooms, 10, 3 and 6 ng mL^{-1} for serum by HPLC-UV [29,33,35], 2 ng mL^{-1} for plasma and urine [37,38] by LC-ECD, 1000 ng mL^{-1} [1] or 2.5 ng mL^{-1} [28] for urine samples by CZE-PDA, 30 ng g^{-1} [41] for mushrooms, 2.5 ng mL^{-1} and 10 ng mL^{-1} for urine [21,39] by LC-MS, 5 ng mL^{-1} for urine by MALDI-TOF-MS [44], 20 ng g^{-1} for mushrooms [40], 0.26 ng g^{-1} for serum, 0.50 ng g^{-1} for liver [30], 0.5 ng mL^{-1} for plasma [31], 0.5-1.5 ng mL^{-1} for urine, serum and plasma [42], 29 ng g^{-1} for food [43] by LC-MS/MS. However, most of these methods were not able to detect both amanitins, were time-consuming relative to amanitins separation or presented ambiguous parameters for detection and confirmation of the compounds.

As described above, the detection and quantification limits obtained in the present report for urine are much lower compared to previous reports. For liver, in comparison with the only reported method for this matrix [30], the values obtained with the present method are greater than that. However, the present method offers a number of significant improvements over the previously one by providing a baseline separation of all compounds and enabling sensitive analyses under MRM mode of a tandem MS. Thus, the structure-diagnostic product ions, generated by MS/MS, instead of the non-confirmative signals from the normal LC detectors or single quadrupole MS detector, offered enhanced specificity for the analysis. We succeeded in detecting and quantifying both amanitins unequivocally in concentrations lower than required for clinical purposes, using an essential component for a robust high throughput bioanalytical method, which is the internal standard. According to the statements reported by Jaeger *et al*. [17], amanitins concentrations were 48-4820 ng mL^{-1} for α-amanitin and 75-7103 ng mL^{-1} for β-amanitin in urine, during the 6-72h following ingestion. In the liver, these values were between 0-19 ng g^{-1} for α-amanitin and between 0-3298 ng g^{-1} for β-amanitin.

Therefore, the present method is sensitive enough to determine amanitins in urine and liver in poisoning cases, and can unequivocally contribute for the diagnosis of amanitin exposure and intoxication in humans.

The extraction recoveries were evaluated from fortified samples at three levels of concentration and determined in each level by the mean concentration measured with further application of Eq. 1. As shown in Table 3, the recovery values for both amanitins ranged between 90.4-105.2 ng mL^{-1} and 90.2-112.9 ng g^{-1} for urine and liver, respectively. The method developed presents good extraction efficiency with reproducible values of recovery, since the losses of both toxins were minimal during the extraction procedure either in urine or in liver, even for the lowest concentration evaluated.

Table 3 also shows the values of coefficient of variation (*CV*) obtained in intraday and interday analyzes. For this parameter, spiked samples of urine and liver were evaluated in the same day (n=6) during 3 consecutive days (n=3). To represent the variability of the results, expressed in *CV*, Eq. 2 was applied. The *CV* values were not greater than 15.2%, ranging from 5.4 to 11.6% for urine samples and 4.1 to 7.8% for liver, in intraday analyzes; and from 6.9 to 15.2% for urine samples and 3.2 to 12.1% for liver, in interday analyzes. The present method reveals low variation between individual assays, corresponding, therefore, to a repeatable and reproducible methodology for the determination of α- and β-amanitin in both matrices, since the values obtained are found to be inside the control limits considered acceptable (±20%) [47].

4. Conclusion

Currently, there is only one published UPLC-MS/MS method for the determination of α- and β-amanitin in urine, serum and plasma samples [42]. Nevertheless, the previous report was not able to detect amanitins through MS-MS mode, since they did not obtain product ions

of amanitins with high intensity, neither obtained reproducible patterns of these ions. The present methodology successfully allowed combining the determination of both α- and β-amanitins in human specimen, making full use of tandem spectrometry, since it succeeded in obtaining product ions using UPLC-MS/MS method. Thus, the developed methodology permits the identification of m/z 259 product ion with high intensity, allowing a sensitive detection of amanitins. Structural characterization of the fragmentation pattern for both amatoxins leading to the product ion mentioned was also proposed in this study.

Effectively, the mass detector on MRM mode for data acquisition provides an excellent specificity, allowing an unequivocal detection and confirmation of amanitins at low levels in biological matrices with commercially available reagents, which was not achieved in the previous report using MS/MS spectrometry [30,31,42,43]. LOD and LOQ values obtained demonstrated the method capacity to determine very low concentrations of the toxins, frequently found in urine and liver matrices. Ultra performance liquid chromatography, in comparison with conventional liquid chromatography techniques, exhibits an increasing efficiency, strength and pH range, which offers the development of methodologies with higher velocity, sensibility and resolution parameters. Combination of both techniques allowed more rapid and efficient separation, with symmetrical chromatographic peaks, which guarantee great evaluation of the analytes, as well as the possibility to obtain accurate mass measurement. The use of tilmicosin as IS in this method also presents a high credibility to quantification measures, since it is an essential component of a robust high throughput bioanalytical method, being one of the few methods in which this type of compound is used [39,41,42,44].

The present methodology offers a significant improvement in diagnosis and *postmortem* confirmation of amatoxin intoxications, since it provides great advantages for the determination of amanitins in urine and liver. Urine turns out to be the gold matrix for this

diagnosis since it is a suitable specimen to examine the intoxication at an early stage and to follow the toxins for a longer time. Thus, it can be also successfully applied to kidney and mushroom samples. The lack of an antidote for these intoxications and a standardized accepted treatment for this poisoning leads to the need of an early and unambiguous prognosis which can be achieved with the method developed, allowing, therefore, to take immediate measures in cases with late hospital incoming, preventing aggressive treatment like liver transplantation. Concerning *postmortem* analyses, a reliable method becomes important for clarifying or interpreting an eventual cause of death.

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551

551 **Figure Captions**

552

553 **Figure 1 –** Chemical structures of α-amanitin, β-amanitin and tilmicosin.

554

555 **Figure 2 –** MS/MS chromatograms of α-amanitin, β-amanitin and tilmicosin extracted from
556 (a) urine and (b) liver.

557

558 **Figure 3 –** Mass spectra and fragmentation pattern of (a) α-amanitin, (b) β-amanitin and (c)
559 tilmicosin.

560

561 **Figure 4 –** Fragmentation of $[M+H]^+$ ions by MS/MS mode to m/z 259 ion.

562

563 **Figure 5** – Ion peaks resulted from cleavage of the peptide bonds proposed on Figure 4.

564

565

565 **Table 1 –** Multiple Reaction Monitoring (MRM) parameters and Relative Retention Times
566 (RRT) for α-amanitin, β-amanitin and tilmicosin.

Compound	Precursor ion (m/z)	Product ion (m/z)	Cone Voltage (V)	Collision energy (eV)	RTT (min.)
α-amanitin	919.48	901.53[a,b]	44	28	5.73±0.5
		259.13[a]		44	
β-amanitin	920.48	902.44[a,b]	42	26	5.27±0.5
		259.13[a]		42	
Tilmicosin	869.60	696.50	50	40	7.09±0.5

567 [a] Confirmation assays; [b] Quantification assays.

568

569

569 **Table 2 –** Limits of detection and quantification for determination of mushroom toxins in
570 biological samples.

571

Toxins	Analytical Method	Matrices	LOD ($ng\ mL^{-1}/ng\ g^{-1}$)	LOQ ($ng\ mL^{-1}/ng\ g^{-1}$)	Reference
α-amanitin β-amanitin γ-amanitin	RIA	Serum	0.05	-	[22]
α-amanitin	RIA	Urine	1	-	[24]
α-amanitin	RIA	Plasma	0.1	-	[24]
α-amanitin	HPLC-UV	Serum	3	10	[29]
α-amanitin β-amanitin	HPLC-UV	Serum Urine Stomach washings	10	-	[33]
α-amanitin β-amanitin γ-amanitin	HPLC-UV	Mushrooms	10	-	[35]
α-amanitin	LC-ECD	Plasma	2	-	[37]
α-amanitin	LC-ECD	Urine	2	10	[38]
α-amanitin β-amanitin	CZE-PDA	Urine	1000	-	[1]
α-amanitin β-amanitin	CZE-PDA	Urine	2.5	5	[28]
α-amanitin β-amanitin	LC-MS	Mushrooms	30	-	[41]
α-amanitin β-amanitin	LC-MS	Urine	10	-	[21]
α-amanitin β-amanitin	LC-MS	Urine	2.5	5	[39]

α-amanitin β-amanitin	MALDI-TOF-MS	Urine	5	-	[44]
α-amanitin β-amanitin γ-amanitin	LC-MS/MS	Mushrooms	20	15.1 30.1 12.9	[40]
α-amanitin	LC-MS/MS	Serum Liver	0.26 0.50	-	[30]
α-amanitin β-amanitin	LC-MS/MS	Plasma	0.5	-	[31]
α-amanitin β-amanitin	UPLC-MS/MS	Urine Serum Plasma	0.5-1.5	-	[42]
α-amanitin β-amanitin	UPLC-MS/MS	Food	29	-	[43]
α-amanitin	UPLC-MS/MS	Urine Liver	0.22 10.9	0.57 14.7	Current Method
β-amanitin		Urine Liver	0.20 9.7	0.50 12.3	Current Method

572
573

573 **Table 3** Recovery, intra-day and inter-day precision values for α- and β-amanitin in urine and
574 liver.

Compound	Matrices	Concentration (ng mL^{-1})	Recovery (%) (n=6)	Precision (% CV)	
				Intraday (n=6)	Interday (n=6)
α-amanitin	Urine	10	90.4	7.12	15.23
		50	92.2	7.00	13.69
		200	105.2	5.36	8.48
	Liver	10	110.0	6.54	11.02
		50	90.2	5.40	12.06
		200	99.0	4.14	4.70
β-amanitin	Urine	10	97.8	9.20	12.61
		50	93.5	11.57	12.74
		200	93.3	5.68	6.85
	Liver	10	112.9	7.84	12.14
		50	92.8	2.66	9.65
		200	93.4	4.23	3.21

575
576

576 Highlights

577 Unequivocal confirmation and determination of α- and β-amanitins in urine and liver samples

578 Structural characterization of the fragmentation pattern for both amatoxins

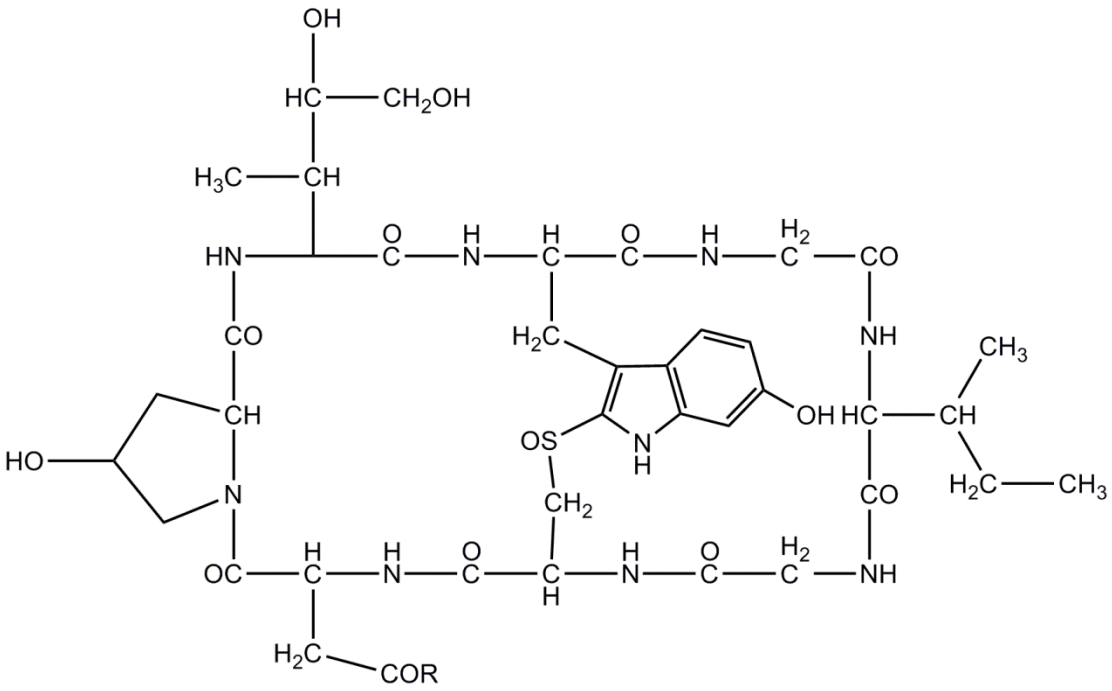
579 LOD and LOQ in urine are much lower compared to previous reports

580 Diagnostic tool for mushroom intoxication

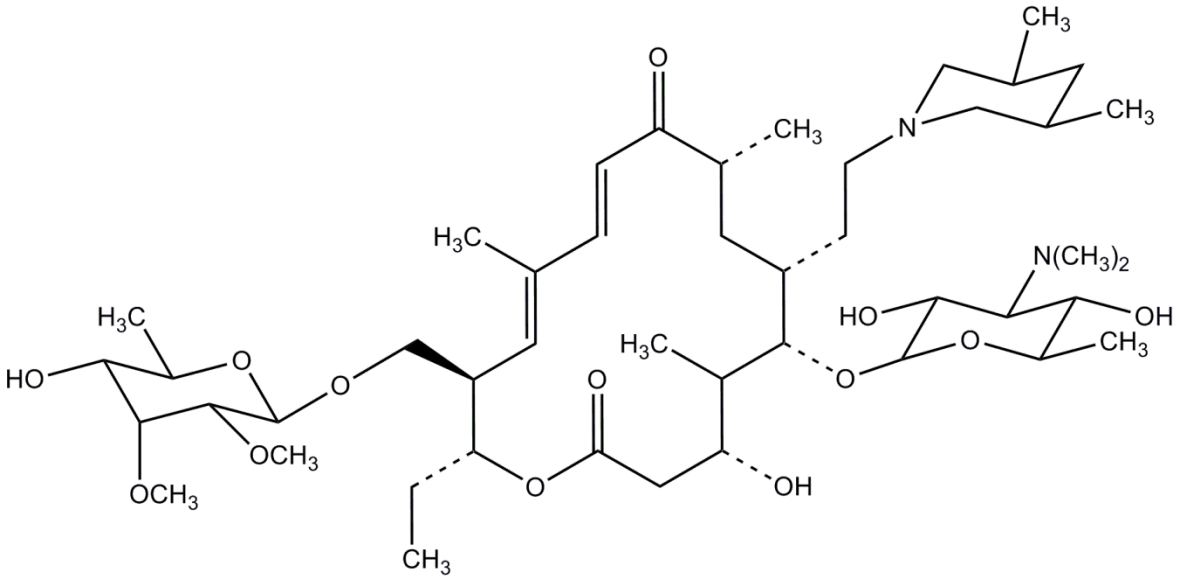
581

582

583

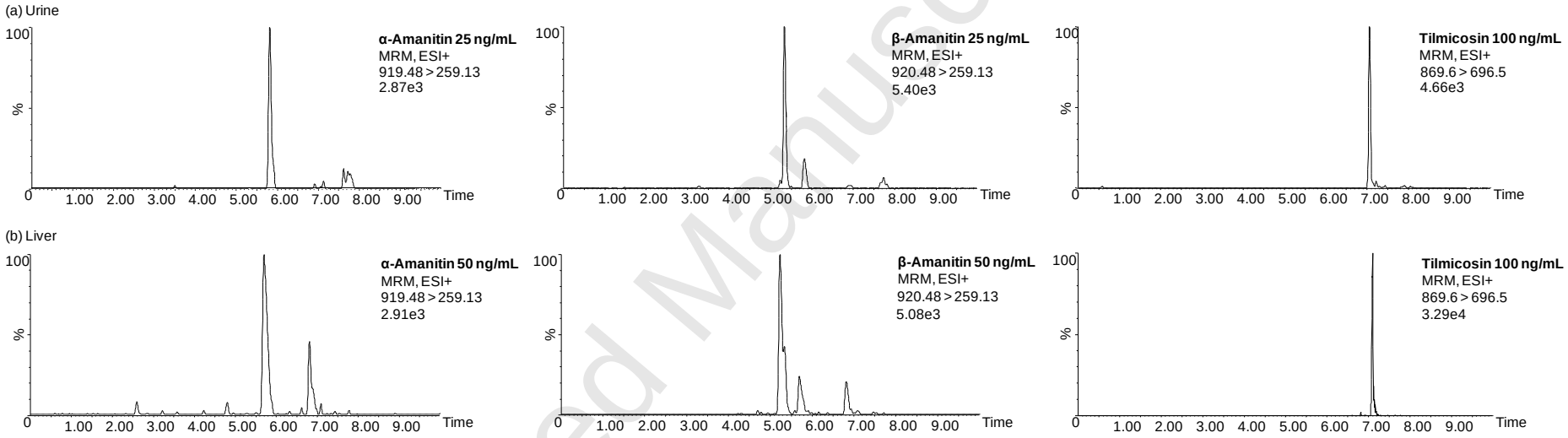


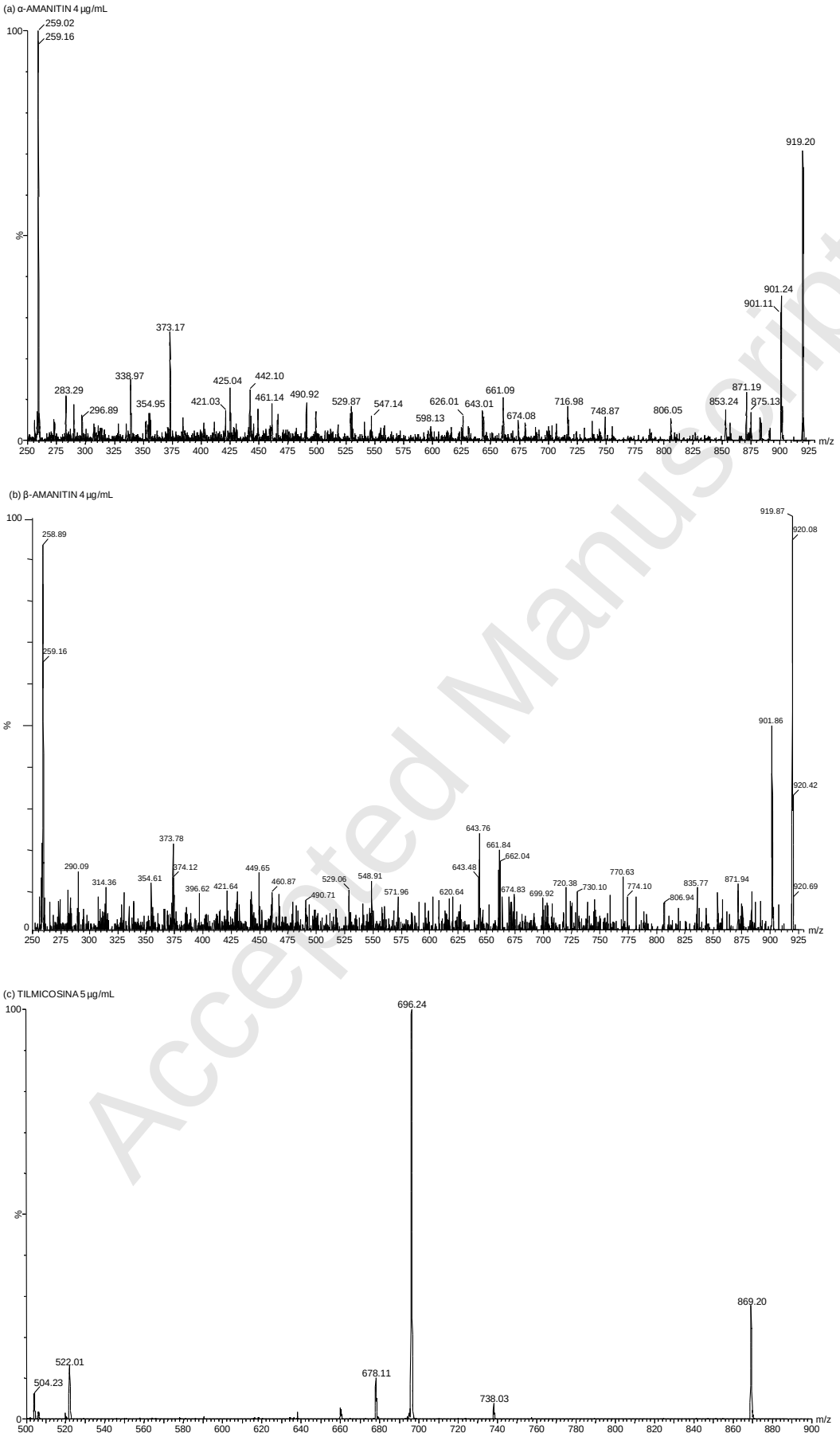
α-Amanitin R= NH_2
β-Amanitin R= OH

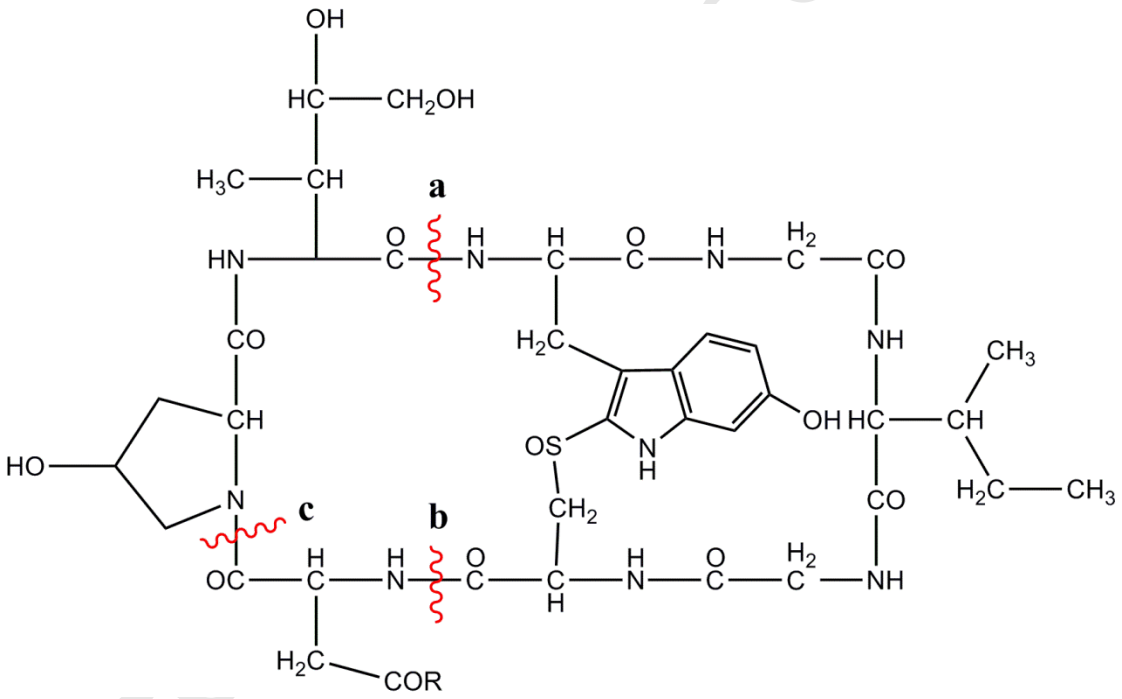


Tilmicosin

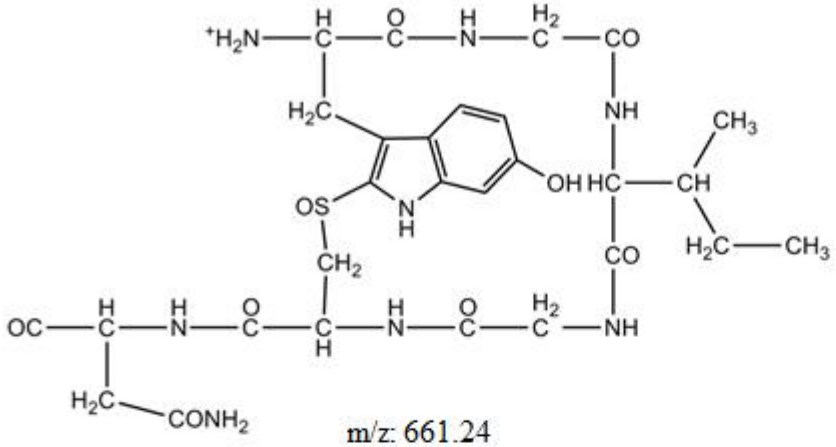
Figure 2







α-Amanitin R= NH_2



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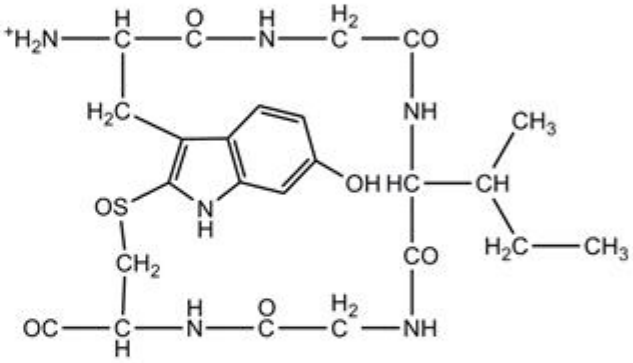

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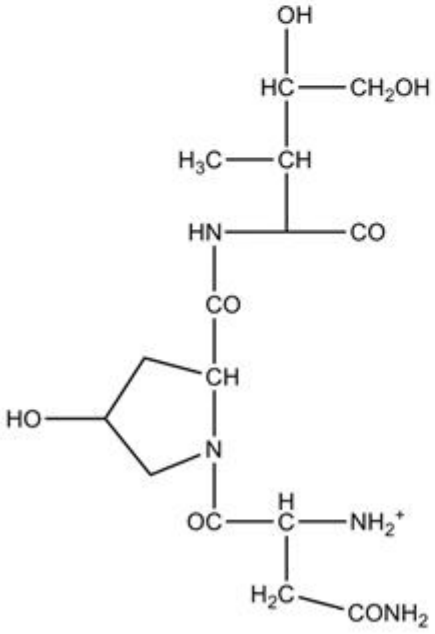

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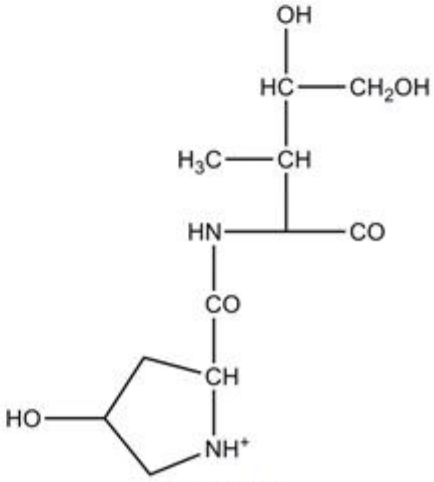

m/z: 259.13

*Graphical Abstract

Structural characterization of the fragmentation pattern for amatoxins