The Effect of A High-Resolution Accurate Mass Spectrometer On Simultaneous Multiple Mushroom Toxin Detection

Yüksek Çözünürlüklü Kütle Spektrometrenin Eşzamanlı Çoklu Mantar Toksin Tayinleri Üzerindeki Etkisi

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Abstract

Amatoxins are deadly wild mushroom toxins that cause severe poisoning in humans, from diarrhea to organ dysfunction. Mortality can be as high as 80% if no specific treatment is applied. In this study, separation and determination methods were developed for the simultaneous determination of 7 toxins belonging to Amanita phalloides. After extraction and purification of Amanita phalloides (death cap) wild mushrooms, toxins were detected using HPLC-ESI MS and exact mass with time of flight MS (TOF-MS) in positive ionization mode. In addition, it was observed that the toxins of α- and β-amanitine could be differentiated from each other thanks to HR-MS detection in case of their close retention (Rt) in LC. The presence of two toxins at the same retention point in the chromatogram was detected by differentiating the molecular ion mass (920.3514 DA) of the α-amanitine with the HR-MS (920.3696 DA). By comparing both optimized methods with each other, faster and broad-spectrum toxin identification was achieved compared to existing methods. In this study, the rapid and accurate identification of seven toxins simultaneously with the same method is of great importance. It will make important contributions to the treatment process, especially by determining that poisoning is caused by wild mushrooms.

Keywords: Amanitin, Amatoxin, HPLC ESI/MS, LC-TOF MS, Mushroom Intoxication, Phalloidin

Öz

Amatoksinler, insanlarda ishalden organ işlev bozukluğuna kadar ciddi zehirlenmelere neden olan ölümcül yabani mantar toksinleridir. Spesifik bir tedavi uygulanmazsa mortalite %80 kadar yüksek olabilir. Bu çalışmada Amanita phalloides' e ait 7 tane toksinin eş zamanda belirlenmesi için ayırma ve tayin yöntemleri geliştirildi. Amanita phalloides (ölüm kapağı) yabani mantarının ekstraksiyonu ve saflaştırılmasının ardından toksinler, HPLC-ESI MS ve LC-doğru kütle uçuş zamanlı MS (TOF-MS) pozitif iyonizasyon modu kullanılarak tespit edildi. Ayrıca α- ve β-amanitin toksinlerinin LC'de birbirlerine çok yakın alıkonmaları (Rt) durumunda HR-MS özellikteki dedeksiyon sayesinde birbirlerinden ayırt edilmesinin mümkün olduğu görüldü. Kromatogramda aynı alıkonma noktasında iki toksin varlığı HR-MS sayesinde α-amanitinin C13 izotop kütlesi (920.3696 DA) ile β-amanitinin moleküler iyon kütlesinin (920.3514 DA) farklandırılması yapılarak tespit edildi. Optimize edilmiş her iki yöntem birbirleriyle karşılaştırılarak mevcut yöntemlere göre daha hızlı ve geniş spektrumlu toksin tanımlaması geçekleştirildi. Bu çalışmada aynı yöntem ile eş zamanda yedi adet toksinin hızlı ve doğru tanımlanması büyük önem taşımaktadır. Özellikle zehirlenmenin yabani mantardan kaynaklı olduğunun belirlenmesi ile tedavi sürecinde önemli katkılar sağlayacaktır.

Anahtar kelimeler: Amanitin, Amatoksin, HPLC ESI/MS, LC-TOF MS, Mantar Zehirlenmesi, Falloidin
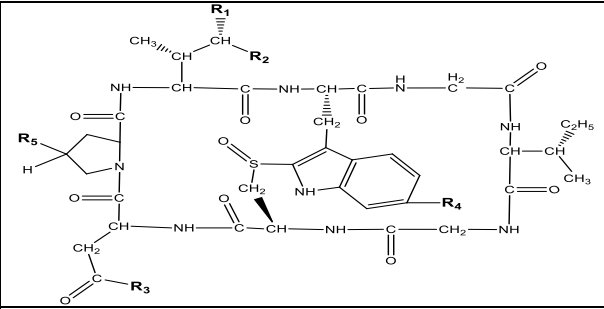
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1. Introduction

Mushroom poisoning is a seasonal phenomenon that occurs relatively frequently in areas where its consumption is common (Ventura et al., 2015). Depending on the species and amount consumed, the season involved, the geographical location, the cooking method, and the individual's response, mushroom intoxication is usually characterized by temporary gastrointestinal, allergic and central nervous system (CNS) symptoms. Ninety to ninety-five percent of all lethal mushroom poisonings are caused by Amanita species containing amatoxin causing hepatic and/or renal failure. A prominent characteristic of most Amanita species is the presence of highly toxic compounds (Vargas et al., 2011). Some Amanita species produce the most powerful peptides (toxins), known as cyclopeptides, that contain a sulfur-linked tryptophan unit and some unusual hydroxylated amino acids (Li et al., 2014). These peptides, the major toxin from amanita, are classified into three groups: amatoxins, phallotoxins, and virotoxins. Amatoxins are 10-20 times more toxic than phallotoxins and virotoxins. The virotoxins are the most recently discovered toxins in Amanita and have only been found in *Amanita virosa*. Toxicological studies have focused on the famous death cap *Amanita phalloides,* which was one of the earliest toxic mushrooms identified (Vargas et al., 2011).

Amatoxins are bicyclic octapeptides with specific properties, including heat stability, various levels of water solubility, and resistance to enzyme degradation. The molecular mechanism of toxicity involves the binding of amatoxins with eukaryotic DNA-dependent RNA polymerase II and inhibition of the elongation essential to transcription. These toxins belong to the most violent poisons from the higher fungi (Barceloux, 2008b). A single dose of 0.1 mg/kg body weight is sufficient to kill an adult. The ingestion of 20-25 g of mature *Amanita phalloides* would involve exposure to 5-8 mg of amatoxin, lethal to a 70-kg adult. Children are especially sensitive to these poisonings and have the highest mortality rates (Ventura et al., 2015). *Amanita phalloides* are white or pale-green, white-gilled mushroom with a bulbous stipe largely found in deciduous forests (Barceloux, 2008a; Vetter, 1998). These toxins in this mushroom have been identified as cyclic oligopeptides consisting of seven (phallotoxin) or eight (amatoxin) amino acids (Figures 1 and 2).

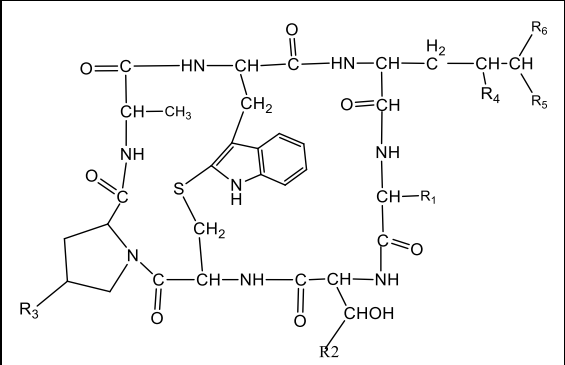|	R_1	R_2	R_3	R_4	R_5
α- AMA*	CH_2OH	OH	NH_2	OH	OH
β AMA	CH_2OH	OH	OH	OH	OH
γ AMA	CH_3	OH	NH_2	OH	OH
ε AMA	CH_3	OH	OH	OH	OH
Amanullin	CH_3	H	NH_2	OH	OH
Amanullin acid	CH_3	H	OH	OH	OH
Proamanullin	CH_3	H	NH_2	OH	H
Amanin	CHOH	OH	OH	H	OH

* Amanitin was abbreviated as AMA

Figure 1. The general structure of amanitin and a list of known amatoxins (Vetter, 1998)

Several methods have been published for the isolation and analysis of amatoxins and phallotoxins, reverse-phased high-performance liquid chromatography (RP-HPLC) being the most common (Robinson-Fuentes et al., 2008; Garcia et al., 2015; Mcknight et al., 2015; Shintani, 2014; Yilmaz et al., 2014; Kaya et al., 2015; 2013). Various analytical techniques, such as bioassays, radioimmunoassays, ultraviolet spectrometry, were formerly used. However, liquid chromatography coupled to mass spectrometry (LC-MS) can provide more sensitive and specific determinations of these toxins (Gonmori et al., 2012; 2011; Defendenti et al., 1998; Maurer et al., 1997; 2000).

The determination of *Amanita phalloides* toxins in different parts of the world is important in order to elicit the maximum information possible, ascertaining its relative morbidity and lethal potential. Gicquel et al. (2014) previously determined α- and β- AMA and phalloidin in urine samples by LC-HRMS. In this work, *Amanita phalloides* specimens collected in the Yesilyayla forest (Gumusova, Duzce, Turkey) were investigated for amatoxins and phallotoxins using reverse-phase HPLC- ESI MS and LC-TOF MS.

	R1	R2	R3	R4	R5	R6
Phalloidin**	CH$_3$	CH3	OH	H	OH	OH
Phallacidin	CH(CH$_3$)$_2$	COOH	OH	H	OH	OH

**Phalloidin was abbreviated as PHD

Figure 2. The general structure of amanitin and a list of known phallotoxins (Vetter 1998)

2. Experimental

2.1 Materials and Instruments

All reagents used were of analytical grade, except the solvents used for mobile phases, which were HPLC grade. Standard α-AMA (90% pure), acetonitrile (LC-MS grade), and methanol (HPLC grade) were obtained from Sigma–Aldrich (St. Louis, USA). Standards of β-AMA (90% pure) and PHD (90% pure) were purchased from Santa Cruz (Santa Cruz Biotechnology Inc., Heidelberg Germany). Double distilled water was purified with a Mili-Q Plus ultrapure water purification system (Millipore, Bedford, MA, USA). Nitrogen was purchased from Messner (Mechelen, Belgium). The Security Guard Column was procured from Phenomenex (Torrance, USA). Kinetex XB-C18 Column 100 mm x 2.1 mm i.d., 2.6 µm particle size (Phenomenex, Torrance. CA, USA) HPLC vials were obtained from Whatman (Maidstone, England). A 018444-L model Perkin-Elmer Sciex Instrument HPLC (2695 Waters) Applied Biosystems, and Triple Quadrupole Mass Spectrometer (MS) and, Agilent 1200 series 1290 infinity LC system (Agilent Technologies, Waldbronn, Germany) consisting of a binary pump (G1312B), degasser, autosampler and diode array detection (G1315C DADSL) and time-of-flight mass spectrometry (G6230A TOF/MS) were used for identification and confirmation of

all toxins in mushroom samples. Agilent Chemstation (version B04 03) and Mass Hunter Workstation (version A.02.02) were used for instrument control and data acquisition.

2.2 Method

2.2.1. Sample Preparation

The wild mushroom *Amanita phalloides* was collected in the Yesilyayla forest (Gumusova, Duzce, Turkey). Pieces of fresh wild mushroom pieces were dried in a 55 ºC airflow for 24 h (Kaya et al., 2015). 1 g dried wild mushroom was ground and homogenized (Waring Lab. Science blender) in 25 mL methanol 15 mL ultra pure water (UPW), pH 10 adjusted (Thermo, Orion 420 A+ pH meter) with ammonium hydroxide. The mixture was sonicated for 30 minutes (2510 Bronson Sonicator) and then centrifuged at 4000 rpm for 10 minutes. The supernatant was evaporated to dryness and reconstituted with the mobile phase, filtered with a syringe filter (0.22 µm), and then injected into HPLC.

2.2.2. Preparation of Stock Solutions, Calibration Standards, and Control Samples

Stock solutions of α- and β- AMA and PHD were prepared individually in methanol at concentrations of 1 mg mL^{-1}. These were then

serially diluted with a mobile phase solution to obtain working standard solutions for the preparation of calibration curves. All the stock solutions were stored at -20° C. Dilutions were freshly made when needed. A set of external standards ranging from 0.5 µg to 20 µg mL^{-1} were prepared in mobile phase initial conditions with appropriate amounts α-, β- AMA, and phalloidin (Figure 3).

The LOD and LOQ were determined using the calibration curve method described in the guideline on bioanalytical method validation. The minimum concentrations of α- and β- AMA and phalloidin that were reliably detected and quantified with acceptable accuracy and precision were considered the LOD and LOQ of our method, respectively. The LOD (k=3.3) and LOQ (k=10) were calculated using the following equation:

$$A = k\sigma/S$$

where A is LOD or LOQ, σ is the standard deviation of the response, and S is the slope of the calibration curve (ICH, 1996; Wharf ve Kingdom, 2012).

2.2.3. HPLC, LC and MS Measurements

The chromatographic conditions used were sample injection volume, 10 µL; flow rate, 0.6 mL min^{-1}; column temperature, 50◦C; autosampler temperature, 8 ◦C; mobile-phase solvents, (A) FA 0.1 % and TFA 0.02% in UPW, and acetonitrile; gradient elution protocol for HPLC-ESI MS, 0% B to 100% at 10 min (5.5 min hold), then 0% B and hold on 10 min to equilibrate the column. The

Triple Quadrupole Mass spectrometry method positive voltage and temperature were set at 5500 V and 475 ºC nebulizer gas, respectively: 15 psi; curtain gas: 15 psi; collision gas: 3 psi. ESI interface (Van Damme et al., 2014).

For the Agilent 1200 series 1290 infinity LC-TOF MS system, the entire flow exiting the DAD was passed directly into the mass spectrometer source operating in positive ESI mode, with a gas flow of 9 L min^{-1} at 350 ºC, and with a nebulizer pressure of 40 psi (Clarke et al., 2012). The chromatographic conditions used were sample injection volume, 10 µL; flow rate, 0.350 mL min^{-1}; column temperature, 50◦C; autosampler temperature, 8 ◦C; mobile-phase solvents, (A) FA 0.1 % and TFA 0.02% in UPW, and Methanol; gradient elution protocol, 3% (B) Methanol to 85% at 10 min to 100% at 18 min to 3% 25 min.

3. Results

The purpose of this study was improved the method employed for determining amatoxins by allowing faster broad-spectrum toxin identification via the development of HPLC-ESI– MS methods in combination with accurate mass (TOF-MS) detection. Following extraction and purification of *Amanita phalloides* (death cap) using separation methods, the toxins were detected in positive ionization mode at HPLC-ESI MS and LC-TOF MS. The toxins were determined by the two instruments are shown in Table 1. All the toxins were detected in less than 15 minutes, and no significant endogenous interferences were observed in the seven toxins' retention times. A chromatogram for seven mushroom toxins using HPLC-ESI-MS is shown in Figure 3.

Table 1. A list of ionized and detection forms in the two instruments

Compounds	Formula	Exact Mass (EM) (DA)	Rt (Minute) HPLC ESI MS	Rt (Minute) LC TOF MS	Measured Mono isotopic ion(m/z) HPLC ESI MS	Measured [M$^+$H]$^+$; EM+1 LC TOF MS	Mono isotopic ion(m/z) [M$^+$Na]$^+$ EM+23 HPLC ESI MS
1.α- AMA*	C$_{39}$H$_{54}$N$_{10}$O$_{14}$S	918.354	11,50	6.112	919.2	919.3696	941.2
2.β-AMA	C$_{39}$H$_{54}$N$_9$O$_{15}$S	919.346	11,72	6.259	920.1	920.3514	942.2
3.ɣ- AMA	C$_{39}$H$_{54}$N$_{10}$O$_{13}$S	902.359	12.16	6.520	903.3	903.4104	925.3
4. ε-AMA	C$_{39}$H$_{53}$N$_9$O$_{14}$S	903.343	12.96	5.900	904,3	904.3898	926,2
5.Amanin	C$_{39}$H$_{53}$N$_9$O$_{14}$S	903.343	12.96	7.287	904,3	905.3947	926.2
6.Amanullin	C$_{39}$H$_{54}$N$_{10}$O$_{12}$S	886.364	17.08	12.998	887,4	887.5450	909.5
7. Phalloidin	C$_{35}$H$_{48}$N$_8$O$_{11}$S	788.316	13.676	6.710	789.1	789.3704	811.2

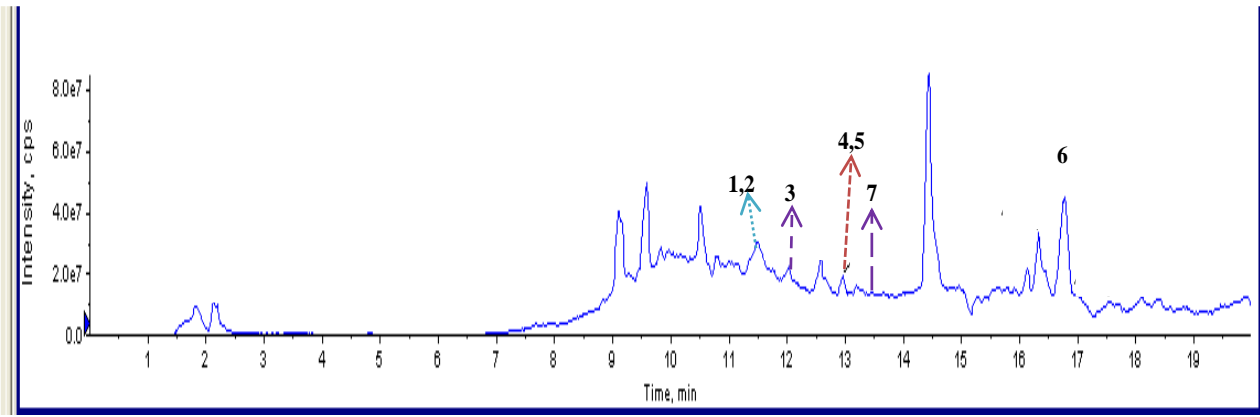**Figure 3.** Typical extracted ion chromatograms using HPLC-ESI-MS for seven toxins (Numbers refer to **1:** α- AMA; **2:** β-AMA; **3:** ɣ- AMA; **4:** ε-AMA; **5:** Amanin; **6:** Amanullin; **7:** Phalloidin)

The most toxic cyclopeptides, α and β-AMA, HPLC-ESI-MS extracted ion chromatograms and mass spectrum profiles are shown in Figures 4A and 4B. The m/z values of α and β-AMA (MH$^+$) were 919.2 and 920.2, respectively. Another MS peak with an m/z value of 920.1 in the MS profile of α-AMA was also noteworthy. However, the MS peaks with Na adducts detected in the spectrum at 941.6 and 942.0, respectively, are compatible with the masses of α and β-AMA (Figure 4). Extracted ion chromatograms and mass spectra of seven toxins obtained from the *Amanita phalloides* mushroom using LC TOF-MS are shown in Figure 5 and Figure 6.
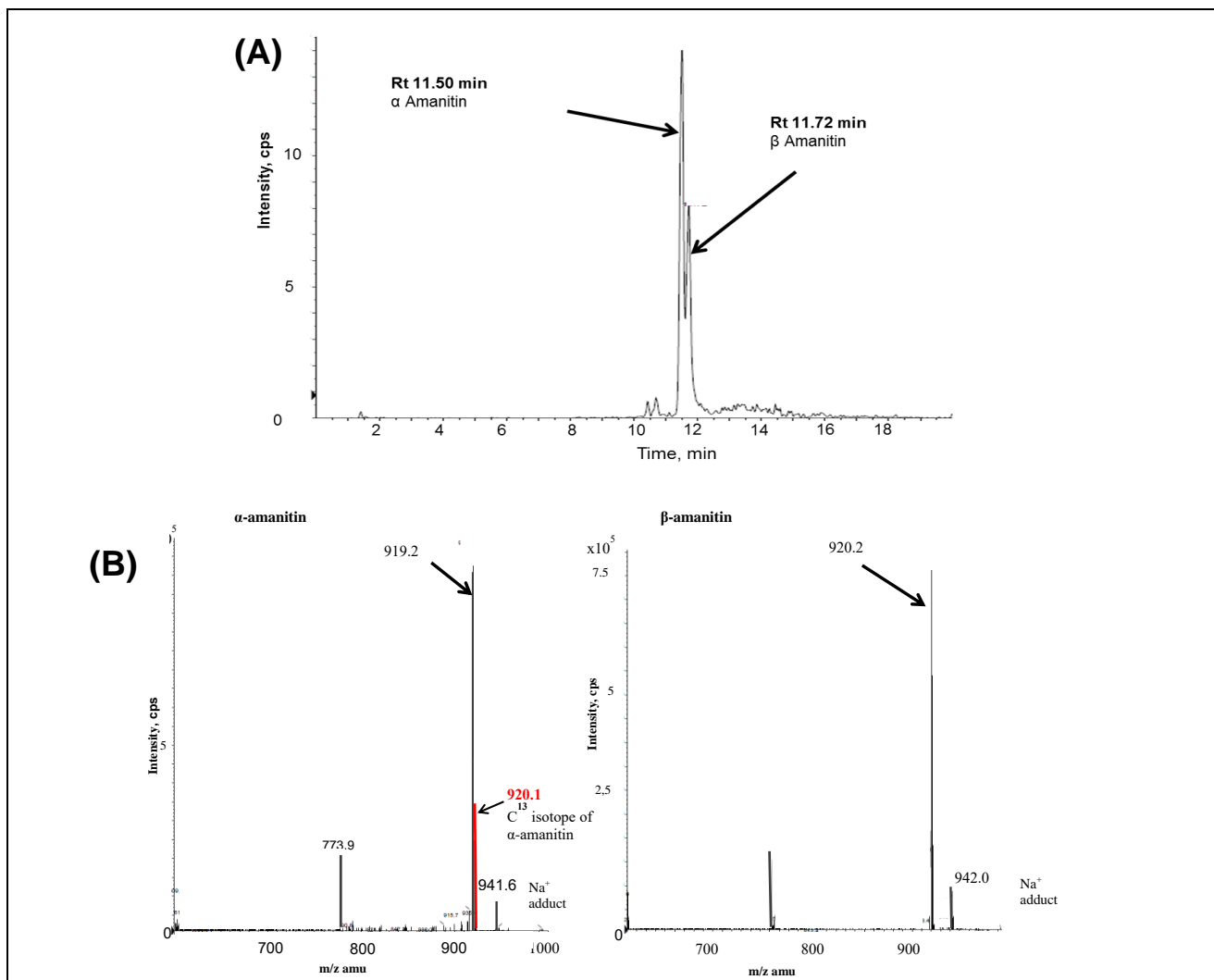

Figure 4. **(A)**Typical extracted ion chromatograms using HPLC **(B)** ESI Mass spectra of each protonated molecular ion for α and β-amanitin, obtained from *Amanita phalloides*
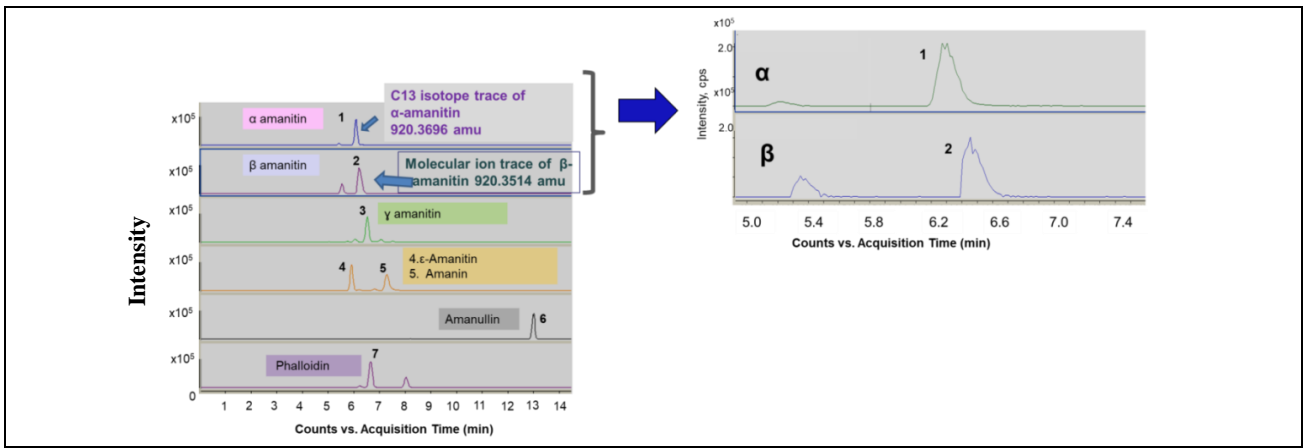
Figure 5. Extracted ion chromatograms of seven toxins with detailed α-AMA and β-AMA obtained from the *Amanita phalloides* mushroom using LC TOF-MS (Numbers refer to **1:** α- AMA; **2:** β-AMA; **3:** γ- AMA; **4:** ε-AMA; **5:** Amanin; **6:** Amanullin; **7:** Phalloidin)
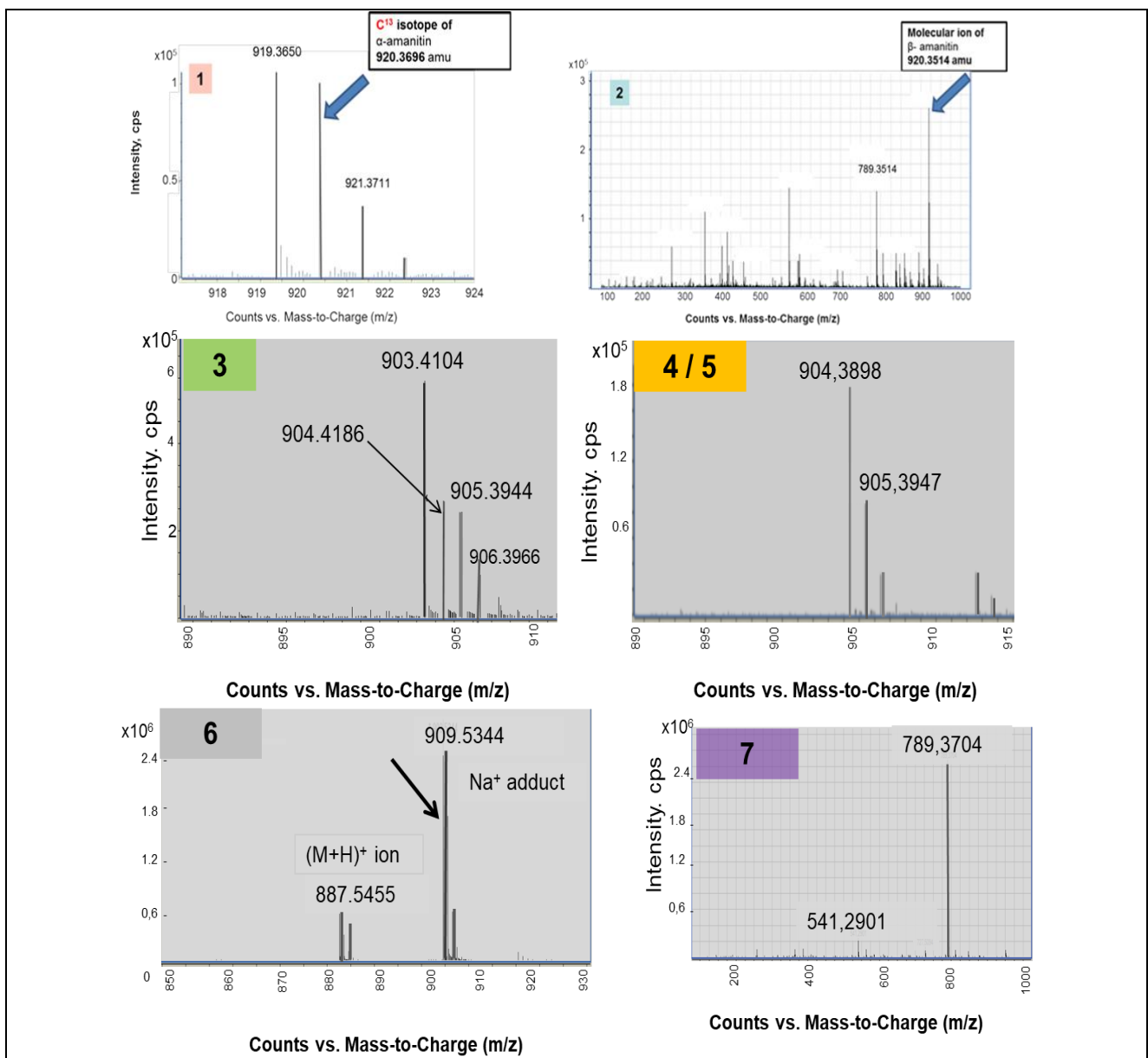

Figure 6. Mass spectra of the mono isotopic ion of the amanitins and phalloidin obtained from the *Amanita phalloides* mushroom using LC TOF MS (Numbers refer to **1:** α- AMA; **2:** β-AMA; **3:** γ- AMA; **4:** ε-AMA; **5:** Amanin; **6:** Amanullin; **7:** Phalloidin)

Calibration curves were established with LC-TOF MS for three toxins' standarts that were linear over the range of interest (Figure 7). Typical equations of calibration curves, correlation coefficient, and the LOD, LOQ values of α-AMA, β-AMA, and PHD toxins are presented in Table 2.
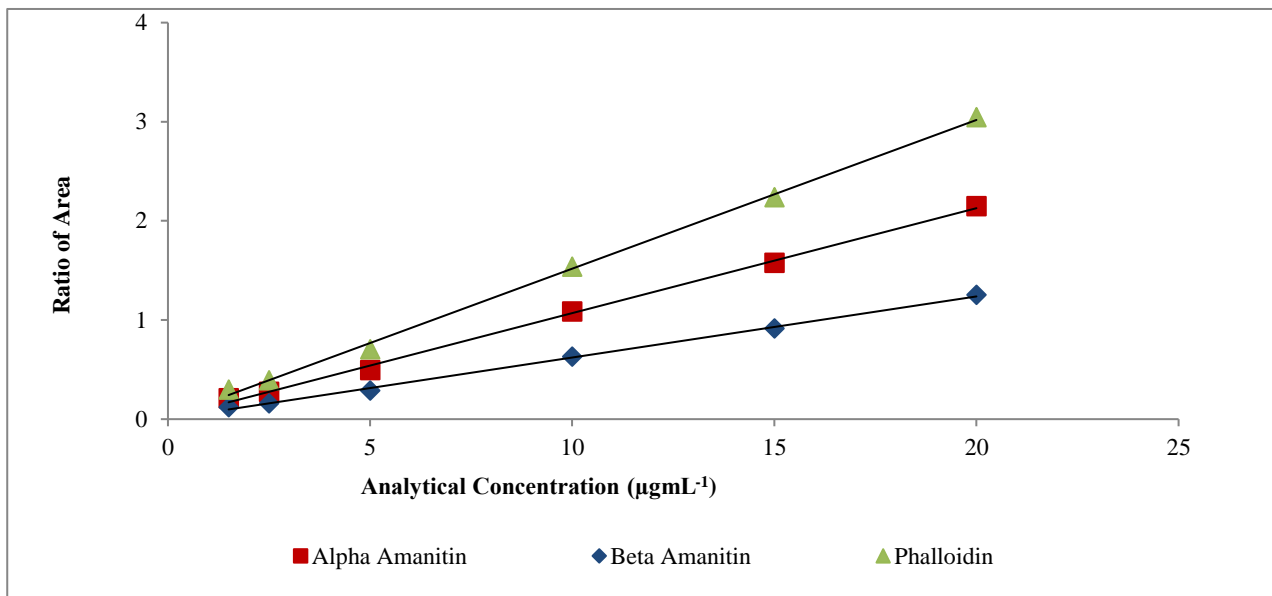

Figure 7. Calibration curves of α-AMA, β- AMA and PHD standards with LC-TOF MS based on six determinations

Table 2. The results of the calibration study for α-AMA, β- AMA and PHD standards with LC-TOF MS

COMPOUND	LINEAR RANGE (µg.mL^{-1})	REGRESSION	CORRELATION COEFFICIENT (R^2)	LOD (µgmL^{-1})	LOQ (µgmL^{-1})
α- AMA	0.5-20	y= 0.1056x+ 0.0149	0.9985	0.303	0.917
β-AMA	0.5-20	y = 0.0615x +0.0077	0.9985	0.238	0.721
PHD	0.5-20	y = 0.1497x + 0.0225	0.9986	0.297	0.901

4. Conclusion

One objective in this study is to be able to identify mushroom poisoning in patients brought to the hospital following intoxication and to make it possible for specific therapeutic procedures to be initiated without delay. We also aimed to develop appropriate methods for fast and effective mushroom toxin analyses. Rapid and accurate screening of the seven toxins is of great importance and will be time-saving in the determination of wild mushroom intoxications in particular. The simultaneous determination of amatoxin and phallotoxin compounds in *Amanita phalloides* wild mushrooms allows confirmation of both the species and the toxins involved. The accurate identification of the species involved intoxication is highly important in terms of treatment.

An isotope is an element that has the same number of electrons in the electronic layer but a different number of neutrons in the nucleus. Therefore, isotopes have the same chemical properties and only differ in mass. The ^{13}C isotopes contributions 68 times higher than that of ^{2}H deuterium and 27 times higher than that of [17]O (Nicolescu, 2017). For organic mass spectrometry the case of carbon is of highest relevance Carbon is ubiquitous in metabolic processes (Burlingame, Whitney ve Russell, 1984). Carbon contributes approximately 50% of polypeptide's mass, and its isotope distribution dominates the molecular mass distrubition observed by mass spectrometer (Beavis, 1993). Mass spectrometry has utility both in quantitative and qualitative analysis. The method is used to identify isotopic species and calculate nominal weight of compounds (Nicolescu, 2017). Mass spectrometers see the isotope peaks provided the resolution is high enough. If an MS instrument has a resolution high enough to resolve these isotopes, better mass accuracy is achieved. Obviously, mass spectrometry is ideally suited for distinguishing

between isotopic species (Burlingame, Whitney ve Russell 1984). In this study, MS differentiation between (the C^{13} isotope of) α and the molecular ion of β AMA is only possible using high resolution MS. If physical separation is achieved, then analysis can be performed using HPLC-ESI MS or LC-TOF MS in the unit resolution mode. Further work is planned to investigate the approach to toxins.

Acknowledgments

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