# Separation of Polar Mushroom Toxins by Mixed-Mode Hydrophilic and Ionic Interaction Liquid Chromatography–Electrospray Ionization-Mass Spectrometry

**Wai-cheung Chung*, Sau-ching Tso,** and **Sai-tim Sze**

Government Laboratory, 7/F Homantin Government Offices, 88 Chung Hau Street, Homantin, Hong Kong Special Administrative Region

## Abstract

**Reversed-phase liquid chromatography (RPLC) is commonly used to analyze nonvolatile contaminants and naturally occurring toxins in foods. However, polar compounds, such as hydrophilic polypeptides and quaternary ammonium salts, are often not satisfactorily separated by RPLC and present a challenge for analytical scientists. In this study, hydrophilic interaction liquid chromatography (HILIC), on an amide-based stationary phase in combination with electrospray ionization (ESI) tandem mass spectrometry (MS–MS), is successfully employed to simultaneously separate polar mushroom toxins, including amanitins and phallotoxins, which are cyclic oligopeptides and muscarine, a quaternary ammonium compound, in mushrooms. The sensitivity of different ionization modes is studied, and the positive ionization mode is found to provide a more sensitive and effective tool for the unambiguous identification of the concerned polar toxins because of their characteristic fragmentation patterns. The properties of the mobile phase are also found to have significant impacts on the separation. At a high acetonitrile (ACN) concentration, hydrophilic interaction dominates, and all analytes under study demonstrate a much higher affinity with the stationary phase. The addition of methanol (MeOH) as a modifier could further enhance the HILIC separation for amanitins, phallotoxins, and muscarine. Valley-to-valley separation is achieved upon the optimatization of the mobile phase (comprising of ACN, MeOH, and ammonium formate buffer at pH ~ 3.5) and the solvent gradient. HILIC coupled with ESI-MS–MS is demonstrated to be a novel technique for the simultaneous separation and confirmatory analysis of the concerned polar toxins by providing an environment of solubility and retention that could not be achieved through the use of RPLC.**

## Introduction

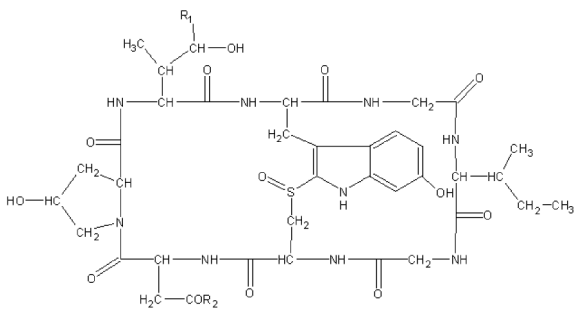
Mushroom poisoning is caused by the consumption of raw or cooked fruiting bodies of a number of species of higher fungi. The toxins involved in mushroom poisoning are produced naturally by the fungi themselves, and each individual member of a toxic species are considered equally poisonous. For individuals who are not experts in mushroom identification, there are generally no easily recognizable differences between poisonous and nonpoisonous species. Furthermore, most mushrooms that cause human poisoning cannot be made nontoxic by cooking, canning, freezing, or any other means of processing. A number of mushroom toxins, such as amanitins, phallotoxins, and muscarine (Figure 1), are extremely toxic with a lethal dose $LD_{50}$ of approximately 1.0 mg/kg of the body weight for a human (1). It is possible that the consumption of a small piece of mushroom can kill a human. The phallotoxins act relatively quickly (poisoning occurs in 1–2 h after consumption), and they bind strongly to F-actin, especially in liver cells, resulting in the high stability of the conjugate against depolymerization and degradation (2). The amatoxins are slow-acting poisons (a lethal interval is at least 15 h), but they are 10–20 times more toxic than phallotoxins. They cause cell necrosis, especially in the liver and kidney, leading to death (in humans) in 5–7 days. These toxins are treacherous because the first symptoms of intoxication arise after the irreversible conjugation of toxins with receptors (2). Muscarine is a fast-acting poison (signs and symptoms typically appear after 0.5–2 h). It causes profound activation of the peripheral parasympathetic nervous system that may end in convulsions and death.

Recently, the variety of mushrooms available for consumption in the Hong Kong Special Administrative Region has increased, and from time-to-time, food poisoning related to the consumption of a mushroom is reported. Thus, there is a need for the development of sensitive and reliable methods for food poisoning investigation and special investigation purposes.

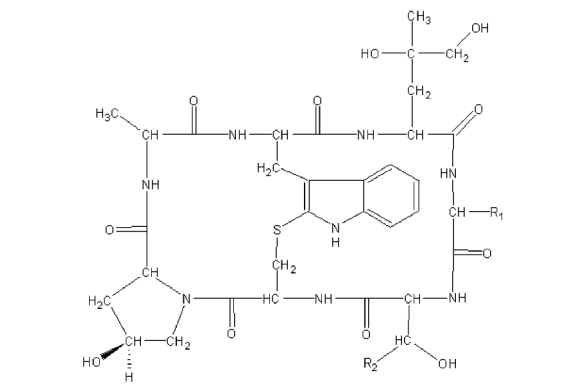
Reversed-phase (RP) liquid chromatography (LC) methods, such as octadecylsilyl, is commonly used in the analysis of contaminants and natural toxins in foods. However, polar compounds are often poorly or not even separated because of the weak retention on RP columns. Ion-exchange chromatography (3) and ion-pair RPLC (4) for polar compounds (such as small

*Author to whom correspondence should be addressed: email swcchung@govtlab.gov.hk and swcchung@walla.com.

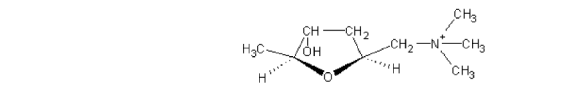
peptides) have been suggested, but interfacing with mass spectrometry (MS) is challenging because of the high salt concentrations of the mobile phase. Furthermore, the reported RPLC system for the analysis of phallotoxins and amanitins (5) was found to be sensitive to the ion strength of the sample. Hydrophilic interaction liquid chromatography (HILIC) is a method first described by Alpert in the early 1990s for the separation of proteins, peptides, amino acid, oligonucleotides, and carbonhydrates (6). The technique employs hydrophilic packings that, in the presence of mixed aqueous and organic mobile phases, will form a stagnant enriched water layer on their surface, into which analytes will partition to various degrees on the basis of their polarities. Thus, the retention times of HILIC separation increase with the hydrophilicity of the solutes. In contrast to RPLC, HILIC elution is facilitated by the aqueous components of the mobile phase, but not the organic components. The separation mechanism of HILIC is, therefore, opposite to that of RPLC, and HILIC is also different from normal-phase and polar organic mode chromatography by the fact that the retentions of the analytes are inversely proportional to the water content in the mobile phases under HILIC mode. Together with its compatibility with electrospray ionization (ESI)-MS, HILIC has been successfully applied to the analysis of polar peptides (7,8).



| | $R_1$ | $R_2$ | M.F. | Molecular weight | $LD_{50}$ in mice (mg/kg body weight) |
|---|---|---|---|---|---|
| α-Amanitin | $CH_2OH$ | $NH_2$ | $C_{39}H_{54}N_{10}O_{14}S$ | 918.971 | 0.3–0.6 |
| β-Amanitin | $CH_2OH$ | OH | $C_{39}H_{53}N_9O_{15}S$ | 919.956 | 0.5 |
| γ-Amanitin | $CH_3$ | $NH_2$ | $C_{39}H_{54}N_{10}O_{13}S$ | 902.972 | 0.2 |



| | $R_1$ | $R_2$ | M.F. | Molecular weight | $LD_{50}$ in mice (mg/kg body weight) |
|---|---|---|---|---|---|
| Phalloidin | $CH_3$ | $CH_3$ | $C_{35}H_{48}N_8O_{11}S$ | 788.869 | 1.5 |
| Phallacidin | $CH(CH_3)_2$ | COOH | $C_{37}H_{50}N_8O_{13}S$ | 846.905 | 1.5 |



| | M.F. | Molecular weight | $LD_{50}$ (mg/kg body weight) |
|---|---|---|---|
| Muscarine | $C_9H_{20}NO_2$ | 174.261 | 0.25 |

**Figure 1.** Structural and toxicity information about amanitins, phallotoxins, and muscarine.

Coupling LC to MS could be dated to 1973 by Baldwin and McLafferty (9). With new interface techniques, such as ESI, atmospheric pressure chemical ionization, and atmospheric pressure photoionization, LC–MS has become the method of choice for the analysis of nonvolatile compounds. However, ion suppression in LC–MS is not uncommon when coeluted compounds suppress the ionization of the analytes of interest in the ion source. The sodium ion, a universal component in all kinds of food, will often compromise the qualitative and quantitative analysis of food samples because its concentration varies from sample to sample. Coeluted sodium ions will often suppress the ionization of analytes and lead to an underestimation of the analytes, or even give false-negative results (10). In most cases, the ion suppression can somehow be corrected by using an isotopic dilution technique if the labeled internal standard is available or by employing more efficient sample cleanup techniques.

To the best of our knowledge, HILIC–ESI-MS has not yet been reported for the analysis of polar mushroom toxins in food samples. This study reported the development of a reliable and sensitive method for the simultaneous determination of highly potent hydrophilic mushroom toxins, ranging from the bicyclic oligopeptides and small-charged toxins (such as muscarine) using multimodal chromatography, wherein the HILIC mechanism has been demonstrated. The method involved the extraction of target mushroom toxins from the matrix by acidified ACN and cleanup by anion-exchange cartridges prior to HILIC separation. Identification and quantitation of the mushroom toxins were accomplished by HILIC–ESI-MS–MS, using an ion-trap mass analyzer as the detector.

## Experimental

### Chemicals

Amanitins, phallotoxins, and muscarine were purchased from Alexis, (Lausen, Switzerland). Ammonium formate and formic acid were from Sigma Chemical (St. Louis, MO). ACN and MeOH (both high-performance liquid chromatography grade) were purchased from Merck (Darmstadt, Germany). The water used in the experiments was deionized and purified by a Milli-Q system (Millipore, Billerica, MA). Samples were purchased from a local market and stored at –20°C prior to analysis.

### Sample extraction and cleanup

A 1.5-g portion of the finely blended sample was mixed with 10 mL of ACN and 250 µL of formic acid, homogenized for 10 s, and then sonicated for 5 min. The solution was defatted by extraction with 2 × 50 mL iso-octane, if necessary. Approximately 2 mL of the supernatant of the extract was eluted through a 3-mL Oasis MAX anion-exchange cartridge (Waters, Milford, MA).

### HILIC–ESI-MS

HILIC–ESI-MS was performed on a TSK-Gel Amide 80 column (2.0 mm i.d. × 25 cm, 5 µm, 80 Å), coupled with a corresponding guard column from Toshi Bio Sep (Tokyo, Japan) (2.0 mm i.d. × 1.0 cm), using a Finnigan LCQ$^{DUO}$ ion-trap MS (Thermo Finnigan, San Jose, CA) under the ESI mode. The LC system consisted of a quaternary SpectraSYSTEM P4000 pump with an on-line degasser and a SpectraSYSTEM AS3000 autosampler. Helium was used as the collision gas, and nitrogen was used as both the sheath and auxiliary gases, which were set at 60 and 10 arbitrary units, respectively. The spray voltage for ESI was 5.5 kV, and the heated capillary of the ESI source was maintained at 250°C. Mass detection was done in segments with a one scan event designated for each analyte under study. Details of the experimental conditions are given in Table I. The LC effluent was diverted to waste before and after the selected retention times window in which analytes of interest were eluted. Data acquisition and processing were carried out using the Xcalibur software version 1.2 (Thermo Finnigan). The mass scale of the instrument was calibrated using a solution containing caffeine, the tetrapeptide L-methionyl-arginyl-phenylalanyl-alanine (MRFA), and the Ultramark 1621 (Thermo Finnigan), according to the standard procedure described in the instrument manual. MS–MS experiments were performed with relative collision energy. Samples were introduced into the LC via a 20-µL injection loop. Mobile phases comprised of different mixtures of a buffer containing ammonium formate (2mM) and formic acid (5mM) at pH 3.5 (A), ACN (B), and MeOH (C) were tested at flow rates of 0.2 mL/min under ambient temperature for HILIC separation. The solvent program was set as follows: 4% A and 96% B, from 0–8 min; ramped up to 10% A, 78% B, and 12% C at 30 min; then the solvent composition was held until 50 min; and finally the solvent was ramped up to 10% A, 70% B, and 20% C at 60 min.

**Table I. Mass Spectrometric Conditions for Analysis of the Concerned Mushroom Toxins**

| Analyte | Event time (min) | Collision energy | Scan range* |
|---|---|---|---|
| α-Amanitin | 46–52 | 25 | 600–930 |
| β-Amanitin | 38.5–40 | 25 | 600–930 |
| γ-Amanitin | 40–42 | 28 | 600–930 |
| Phalloidin | 36–38.5 | 25 | 500–800 |
| Phallacidine | 42–46 | 26 | 500–850 |
| Muscarine | 52–60 | 26 | 90–180 |

* Scan rate was 1 scan per second.

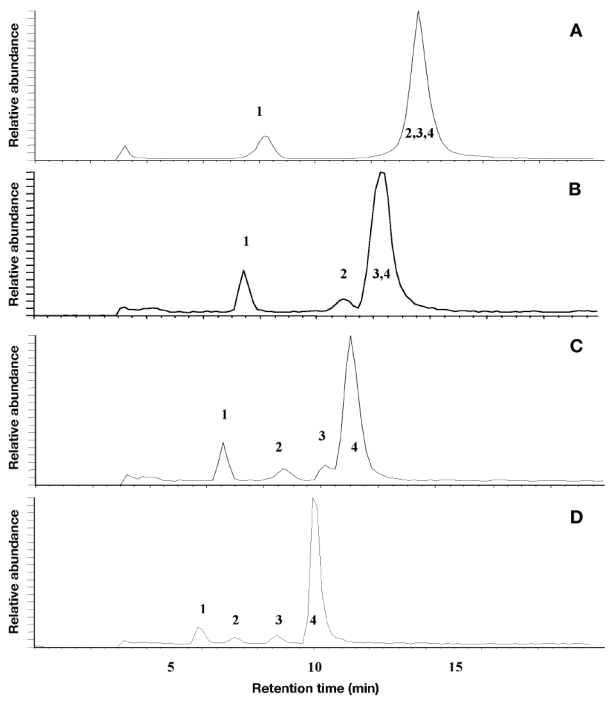



**Figure 2.** Effect of changing the relative amount of organic concentration in the mobile phase on the separation of peaks: phalloidin, 1; β-amanitin, 2; α-amanitin, 3; and muscarine, 4 on TSK gel Amide-80 column (2.0 mm i.d., 25 cm, 5 µm, 80Å). The flow rate was set at 0.2 mL/min, and the mobile phase of ACN–MeOH–water: 85:5:10, (A); 80:10:10, (B); 75:15:10, (C); and 70:20:10, (D).

## Results and Discussion

### Effect of ACN

Four of the representative mushroom toxins (α-amanitin, β-amanitin, phalloidin, and muscarine) were chosen as test analytes for this study because of their toxicities. To test whether the toxins under study could be separated by mixed-mode hydrophilic and ionic-interaction LC, a column with polyhydroxyethyl aspartamide (TSK-Gel Amide 80) packing was chosen as the stationary phase. Isocratic elution at a flow rate of 0.2 mL/min was performed using the mobile phase composed of a mixture of an aqueous buffer [2mM ammonium formate and 5mM formic acid at (pH ~ 3.5)] and ACN. Owing to the strong ion-exchange interaction with the stationary phase, sodium ions were found to have distinctly longer retention times than the four analytes throughout the changes in the mobile phase composition from 71–86% (v/v) of ACN. All the tested oligopeptides became positively charged under an acidic medium, and the singly-charged muscarine would probably behave similar to the cyclic oligopeptides. Comparatively speaking, β-amanitin was found to be the most sensitive analyte to the change of organic concentration than the other toxins under study. This may be attributed to the presence of the ethylenediol moiety in β-amanitin, which enhanced the stereo charge-to-volume ratio of the compound. Minimal separation of the four compounds was observed when 71% (v/v) of ACN was used. The retention of the analytes increased significantly when the organic concentration increased, and in 86% ACN, all of the concerned compounds doubled their retention times. Though the HILIC mechanism has clearly been demonstrated with a decrease of the retention times of analytes with an associated increase of hydrophilicy of the solvent, good separation of the toxins were not obtained under such conditions.

### Effect of the modifier

To study the solvent properties as proposed by Karger et al. (11), MeOH was selected in the study as the modifier of the mobile phase for fine tuning the magnitude of the system in terms of proton-donor and acceptor strength. To maintain an environment that favored the formation of positively-charged ions, an ammonium formate buffer (at pH ~ 3.5) was used in the mobile phase as the aqueous portion. While keeping the same flow rate (0.2 mL/min) and overall composition of the buffer at 10% (v/v), the amount of ACN and MeOH was varied, and the results of the isocratic separation of phalloidin, α- and β-amanitin, and muscarine on the same stationary phase (TSK-Gel Amide 80) are shown in Figure 2. Under the mobile phase of the ACN–MeOH–ammonium formate buffer (85:5:10, v/v), the latter three toxins eluted as an unresolved peak. The gradual decreasing of the relative composition of ACN from 85% to 70% while increasing the MeOH portion from 5% to 20% enabled a valley-to-valley of all four toxins under study.

Further experiments were conducted to study the effects of the pH and solvent gradient on the separation. A gradient elution using a mixture of ACN–MeOH–ammonium formate buffer (pH ~ 3.5) was performed. The results are shown in Figure 3. It was discernible that increases in the percentage of MeOH could reduce the retention times of amanitins, phallotoxins, and muscarine. The retention time of muscarine was found to have an approximate linear relationship with the percentage of MeOH in the mobile phase. Both being neutral cyclic oligopeptides, amanitins and phallotoxins were observed to have a similar sensitivity towards the modifier. If the major mechanism for separating the oligopeptides was merely hydrophilic interaction, the elution order should not be significantly altered by replacing ACN with MeOH. Hence, ion-exchange was also believed to exist as a second separation mechanism. To this end, increasing the amount of MeOH would increase the magnitude of the mobile phase by favoring the hydrogen bonding between MeOH and the analytes, and in turn, decrease both the ion-exchange interaction and hydrophilic interactions between the stationary phase and the amanitins or phallotoxins. Apart from a very minor exception (the relative elution order of β-amanitin against γ-amanitin was reversed when MeOH was increased from 5% to 10%, v/v), the elution order of the toxins under study remained unchanged upon changing the relative volume ratio of MeOH to ACN. As the relative changes in the retention times for the oligopeptides were similar to that of muscarine, albeit because of variation of the MeOH–ACN content, it was likely that the oligopeptides existed as charged species under the acidic conditions.

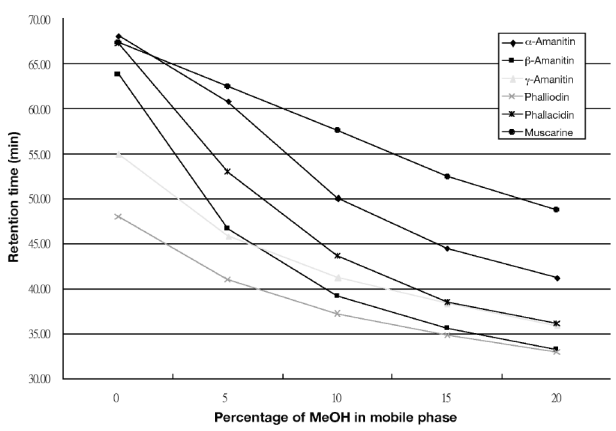



**Figure 3.** A plot of the retention times of α-amanitin, β-amanitin, γ-amanitin, phallacidin, phalloidin, and muscarine against the percentage composition of MeOH in the mobile phase under the gradient elution of a mixture of ACN, MeOH, and ammonium formate buffer (pH ~3.5) at a flow rate of 0.2 mL/min on a TSK gel Amide-80 column. The ammonium formate buffer in the mobile phase was increased linearly from 0% to 10% in the initial 8 min and set at 10% until the end of the gradient program as the proportion of ACN and MeOH was varied.

### Effect of the buffer salt concentration

Buffers have often 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. Although volatile buffering agents were compatible with ESI-MS, it was known that ESI was adversely affected by buffer salts because of the ionization suppression that could occur if the buffer concentration was too high (12). In some cases, the actual buffer concentrations were selected to maximize the response of the target analyte(s). According to Strege (13), a number of different types of compounds, including polypeptides, appeared to be stable on Amide-80 packing with ammonium acetate buffers at concentrations of 3.3mM or greater. During this study, the retention of the tested oligopeptides was found to have insignificant changes upon increasing the concentrations of ammonium formate and formic acid in the buffer from a few mM to approximately 50mM. Henceforth, in order to minimize the suppression of ESI signals, the buffer system with 2mM ammonium formate and 5mM formic acid was employed.

### Positive and negative ESI-MS

The positive ionization mass spectrum of muscarine was dominated by the $[M]^+$ ion, and no significant fragmentation was evident. The positive ionization ESI mass spectra of the oligopeptides were very similar with the base peaks arising from the $[M+H]^+$ ions and the sodium ion adducts, with $[M+Na]^+$ being the second most abundant ions. Doubly-charged molecular ions were not observed for the amanitins and phallotoxins. The relatively low abundance of the mass fragments corresponding to the $[M+H–H_2O]^+$ ions suggested that protonated molecules of amanitins were quite stable in the gas phase. Amanitins were relatively more stable than the phallotoxins that formed both the $[M+H–H_2O]^+$ and the $[M+H–2H_2O]^+$ ions. Probably owing to the presence of an additional carboxyl group, phallacidin could also form di-sodium adducts that were not observed in the spectrum of phalloidin.

It was evident from the mass spectra of the oligopeptides under study that mainly the $[M–H]^-$, $[M+Na–2H]^-$, and $[M–H–H_2O]^-$ (for the amanitins), or $[M–H–CO_2]^-$ (for phallacidin) ions were formed under a negative ESI mode. Apparently, the deprotonated molecules were relatively not stable, and smaller mass-to-charge fragments involving the loss of water and carbon dioxide molecules for amanitins and phallotoxins were formed. When compared with positive ionization, the negative ionization of the analytes under study was less effec-

tive, resulting in a less sensitive detection. To this end, positive ionization was chosen for the MS–MS experiments.

The most abundant and protonated molecular ions were selected for MS–MS experiments, and their relatively low energy collision induced dissociation (CID) spectra are shown in Figure 4. The amanitins, which are bicyclic octapeptides having relatively stable structures, produced $[M+H–H_2O]^+$ as the dominant daughter ions. The $[M–44]^+$ ions were also observed in all amanitins. Because only the β-amanitin contained an intact –COOH group and the dissociation of an ethenyl alcohol from the pyrrolidionol ring was not uncommon, it was suggested that the $[M–44]^+$ should be $[M+H–CH_2=CHOH]^+$ instead of $[M+H–CO_2]^+$. The mass fragments $[M–195]^+$ at *m/z* 724 and 725 for α- and β-amanitin probably arose from the loss of the hydroxyl indole side chains from the parent compounds, forming cyclic peptides (Figure 5A). Phallotoxins, which are bicyclic heptapeptides, were less stable and produced relatively more complex mass spectra. The $[M+H–H_2O]^+$ ions were the major daughter ions of phallotoxins, and $[M+H–CH_2=CHOH]^+$ ions were also observed. The ring opening mechanism leading to the formation of $[M–145]^+$ ions from the phallotoxins is shown in Figure 5B. Other mass fragments likely to arise from the ring opening of the cyclic phallotoxins or loss of the peptides chains were also observed.

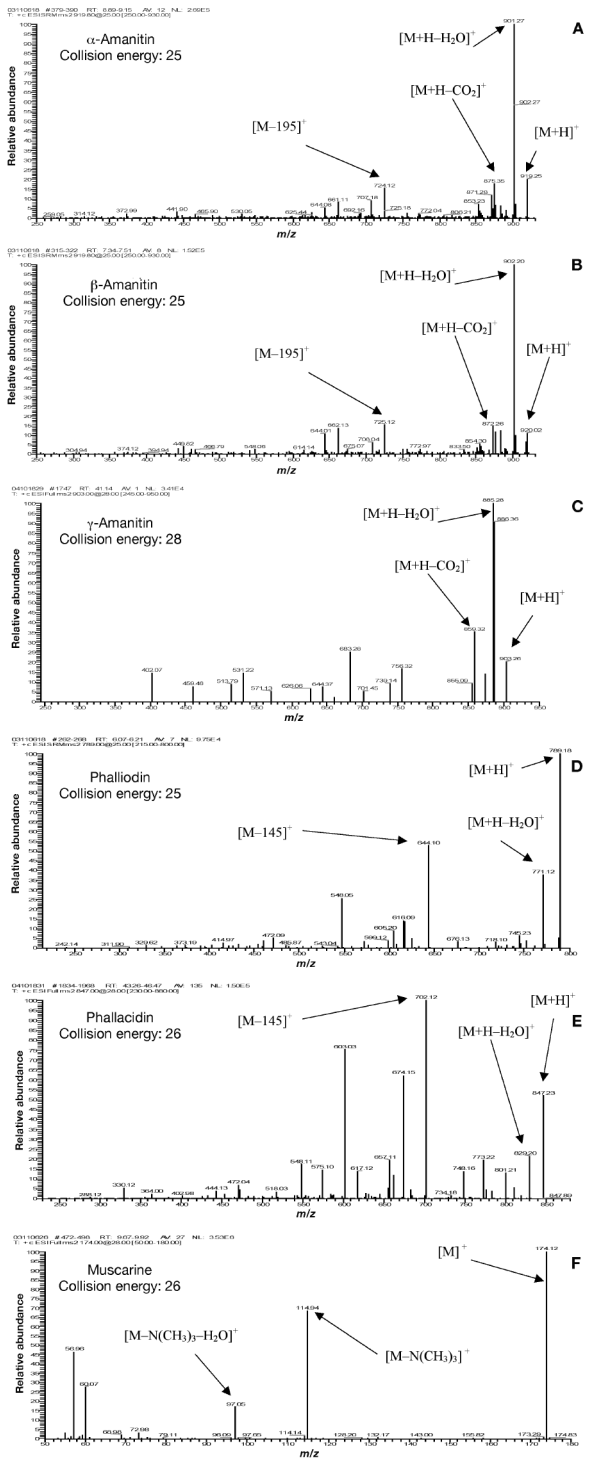



**Figure 4.** HILIC–ESI-MS–MS spectra of the protonated molecular ion of α-amanitin, (A); β-amanitin, (B); γ-amanitin, (C); phalloidin, (D); phallacidin, (E); and muscarine, (F) obtained under the positive ionization mode.

Figure 6 shows the proposed dissociation mechanism that muscarine, upon CID, lost the quaternary ammonium group and formed the respective $[M–N(CH_3)_3]^+$ and $[M–N(CH_3)_3–H2O]^+$ ions. The mechanism involved first the formation of hydroxyfuran ions, which then lost one water molecule, giving the disubstituted furans with *m/z* at 97 and other smaller mass fragments. The fragmentation mechanism of muscarine was found to be quite independent of the collision energy. Irrespective of the increase in collision energy, similar mass fragments were formed; however, there were minor changes in the ion abundance ratios.

The sodium adducts of amanitins and phallotoxins were also selected for MS–MS analysis, and the respective CID spectra are shown in Figure 7. It was evident from the spectra that $[M+Na–H_2O]^+$ were the major daughter ions of amanitins and phallotoxins upon the CID collision of their sodium adducts. The absence of the [M]+, arising from the dissociation of the sodium adducts, suggested that the ionization potential of the compounds was higher than that of the adducts.

## Quantitation of toxins

As discussed previously, the oligopeptides ionized under ESI mode predominantly formed the protonated molecular ions and the respective sodium adducts. Owing to the presence of ion suppression, the quantitation of analytes was made difficult if the content of sodium ions in samples could not be controlled. With

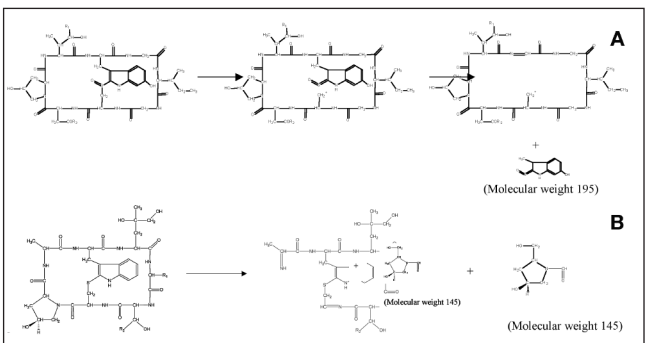



**Figure 5.** Proposed dissociation reaction mechanism for the CID of the protonated molecular ions of amanitins (A), and proposed dissociation reaction mechanism for the CID of the protonated molecular ions of phallotoxins (B).

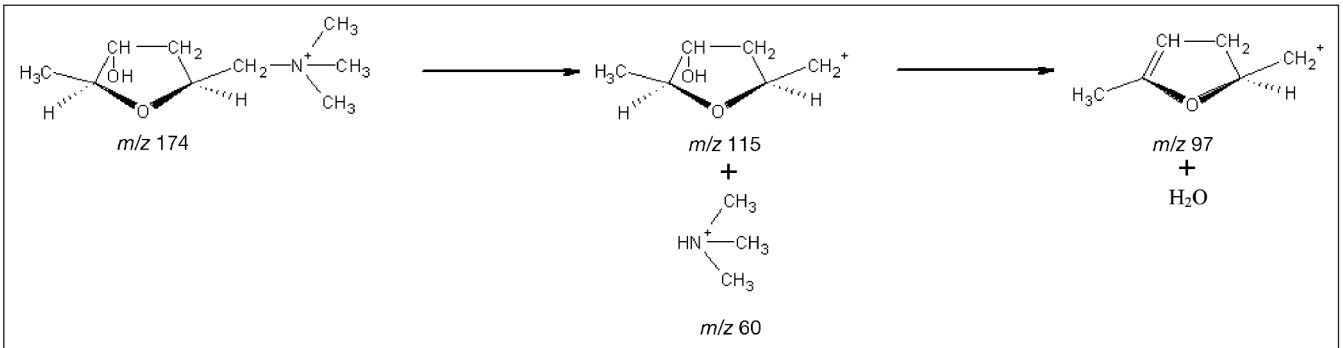



**Figure 6.** Proposed dissociation reaction mechanism for the CID analysis of muscarine.

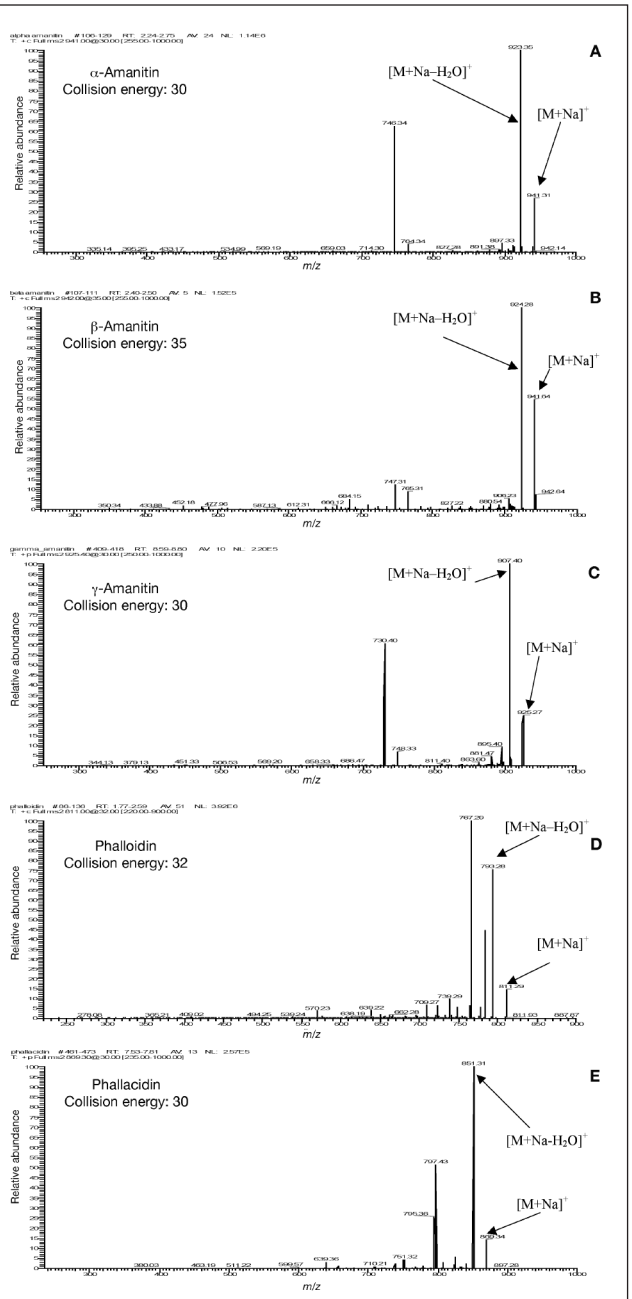



**Figure 7.** Product ion spectra of the sodium adducts of α-amanitin, (A); β-amanitin, (B); γ-amanitin, (C); phalloidin, (D); and phallacidin, (E) obtained under the positive ionization mode.

the HILIC, the sodium ions could be separated from the analytes and expected to produce a constant mass fragment ratio between the protonated molecular ions and the respective sodium adducts. Table II shows the ratios of the protonated molecular ions and their respective sodium adducts for some of the tested analytes at different spiked levels of sodium ions. It is obvious from Table II that both the retention times and the ratios of the oligopeptides and those of the respective sodium adducts were not affected by the increase of sodium ion concentrations from 10 to 500 mg/kg. At a high concentration (1000 mg/kg) of sodium ions the retention times of the oligopeptides were not affected, but the ratios of the oligopeptides and their sodium adducts were slightly altered. High concentrations of sodium ions at 1000 mg/kg, however, affected the retention time of muscarine significantly.

A labeled standard of the target analytes was not available on the market; therefore, the external calibration method was employed for the quantitative analysis. The responses of the five calibration standards for oligopeptides and muscarine were found to be linear for the tested concentration range of 0.02–0.50 and 0.005–0.050 µg/mL, respectively, with correlation coefficients ($r$) greater than or equal to 0.995. The retention times of the analytes in all valid injections were found to be very constant with relative standard deviation (RSD) less than or equal to 1%, demonstrating the stability of the HILIC–ESI-MS system during the course of analysis.

The limits of detection (LODs) were estimated to be at 20 and 5 µg/kg for oligopeptides and muscarine, respectively, and were the lowest concentration calibration standards that gave signals with signal-to-noise ratios of 3:1. The limit of quantitation (LOQ), as illustrated in Table III, was determined by replicate analyses of fortified samples (two species of common edible mushrooms available in the Far East) with spiked concentrations at 40 to 200 µg/kg. The LOQ values for oligopeptides and muscarine were found to be less than 30 µg/kg in the mushrooms under study. Henceforth, the present method should be sufficiently sensitive to measure these toxins in mushroom samples below their respective $LD_{50}$ levels. The concentrations of all the analytes in reagent blanks, matrix blanks, and sample blanks were found to be below the LODs. The precision (repeatability) of the method was assessed by the RSD of replicate analyses of spiked samples. At concentrations around the LOQ, the precision of measurement was less than 20%. In the absence of internal standards, the overall recovery (63–94%) and precision (RSD ranging from 2.1% to 18%) were considered to be acceptable.

## Conclusion

A novel HILIC–ESI-MS method was developed for the separation and characterization of three groups of mushroom toxins, including the polar cyclic polypeptides of amanitins and phallotoxins, and the singly-charged muscarine. The method was relatively simple without the need of derivatization. The selectivity and separation power of the adopted HILIC system was studied. Unambiguous identification was possible because the analytes showed different fragmentation patterns during CIDs in MS–MS experiments. The technique was successfully applied to both reference compound mixtures and complex food samples.

Currently, there are only two published RPLC methods on the determination of α-amanitin, β-amanitin, and phalloidine (14,15). This study clearly illustrated the suitability of HILIC–ESI-MS as an analytical tool for the determination of different types of mushroom toxins. The described HILIC–ESI-MS–MS method offers a number of significant improvements over the previously published RPLC methods (14,15) by providing a baseline separation of all compounds. Though the LODs were found to be of the same levels as the single quadrupole MS detector (15), this study enabled sensitive analyses under multiple reaction monitoring mode of a tandem MS. Moreover, the use of structure-diagnostic product ions, generated by MS–MS, instead of the non-confirmative signals from the normal LC detectors (14) or single quadrupole MS detector (15), offered enhanced specificity for the analysis. Background interference was found to be negligible when compared with UV detection. Finally, the MS–MS methodology utilized in the study did not necessitate special and tedious sample cleanup steps during the sample preparation step. This enables a much faster sample turnaround with good sensitivity for surveillance purposes.

**Table II. Average Mass Fragment Ratios (*n* = 5) of the Protonated Molecular Ions and Respective Sodium Adducts for some of the Test Analytes at Different Spiked Levels of Sodium Ions**

| Spiked concentrations of sodium ions in mg/kg | Analytes | | | |
|---|---|---|---|---|
| | α-Amanitin | β-Amanitin | Phalloidin | Muscarine* |
| 10 | 3.59 (7.22)† | 3.66 (8.94) | 2.43 (6.06) | – (9.88) |
| 100 | 3.50 (7.26) | 3.75 (8.98) | 2.31 (6.07) | – (9.50) |
| 500 | 3.59 (7.12) | 3.59 (8.93) | 2.37 (6.10) | – (9.60) |
| 1000 | 2.92 (7.12) | 3.78 (8.93) | 1.76 (6.10) | – (8.95) |

* Muscarine is a quaternary ammonium salt and does not form sodium adducts.
† Average retention times (min) were reported in parenthesis.

**Table III. Recovery of Toxins in Fortified Samples (Two Different Species of Mushrooms)**

| Analyte | Spiked level (µg/kg) | Mean recovery (%) | Precision* (%) | LOQ† (µg/kg) |
|---|---|---|---|---|
| **(A) Fresh *Lentinula edodes* (Shiitake, winter mushroom, black oat mushroom) (*n* = 5)** | | | | |
| α-Amanitin | 150 | 63 | 5.7 | 26.8 |
| β-Amanitin | 200 | 65 | 5.3 | 33.3 |
| γ-Amanitin | 100 | 91 | 6.8 | 21.3 |
| Phalloidin | 40 | 97 | 11 | 13.8 |
| Phallacidine | 40 | 77 | 18 | 22.6 |
| Muscarine | 40 | 84 | 4.1 | 5.1 |
| **(B) Fresh *Pleurotus pulmonarius* (phoenix mushroom, hsiu tseng mushroom) (*n* = 5)** | | | | |
| α-Amanitin | 150 | 75 | 3.2 | 15.1 |
| β-Amanitin | 200 | 65 | 4.8 | 30.1 |
| γ-Amanitin | 100 | 72 | 4.1 | 12.9 |
| Phalloidin | 40 | 92 | 6.2 | 7.8 |
| Phallacidine | 40 | 88 | 5.7 | 7.2 |
| Muscarine | 40 | 94 | 2.1 | 2.6 |

* The precision was defined as the RSD of the five replicates measurements.
† The LOQs were determined from the analyses of five replicates of spiked sample processed through the entire analytical method. LOQ= $t(n-1, 0.99) \times SD$, where t(n–1, 0.99) is the student *t* value appropriate for a 99% confidence interval and a standard deviation with $n-1$ degrees of freedom [$n = 5$; $t(n-1, 0.99) = 4.60$].

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