Identification of forensically relevant oligopeptides of poisonous mushrooms with capillary electrophoresis-ESI-mass spectrometry

J. Rittgen, M. Pütz, U. Pyell

Abstract

Over 90% of the lethal cases of mushroom toxin poisoning in man are caused by a species of Amanita. They contain the amatoxins α-, β-, γ- and ε-amanitin, amanin and amanullin together with phallotoxins and virotoxins. The identification of the named toxicants in intentionally poisoned samples of foodstuff and beverages is of significant forensic interest.

In this work a CE-ESI-MS procedure was developed for the separation of five forensically relevant oligopeptides including α-, β- and γ-amanitin, phalloidin and phallacidin. The running buffer consisted of 20 mmol/l ammonium formate at pH 10.8 and 10% (v/v) isopropanol. Dry nitrogen gas was delivered at 4 l/min at 250°C. The pressure of nebulizing nitrogen gas was set at 4 psi. The sheath liquid was isopropanol/water (50:50, v/v) at a flow rate of 3 µl/min. A mass range between 600 and 1000 *m/z* and negative as well as positive polarity detection mode was selected. The separation of the five negatively charged analytes was achieved at 23°C within 8 min using a high voltage of + 28 kV. The method validation included the determination of the detection limits (10 – 40 ng/ml) and the repeatability of migration time (0.2 – 0.3% RSD). The CE-MS procedure was successfully applied for the identification of amatoxins and phallotoxins in extracts of fresh and dried mushroom samples.

1. Introduction

Over 90% of the lethal cases of mushroom toxin poisoning in man are caused by a species of Amanita [1]. They contain the amatoxins α-, β-, γ- and ε-amanitin, amanin and amanullin together with phallotoxins and virotoxins [2].
The amatoxins, which rank among the most harmful poisonous substances in nature, are bicyclic octapeptides containing a central tryptophane ring-system, a sulphide-bridge and an isoleucine side chain [3]. The molecule framework of the amatoxins is shown in Figure 1 and the substitution patterns as well as toxicity data for the known amatoxin compounds are listed in Table 1.

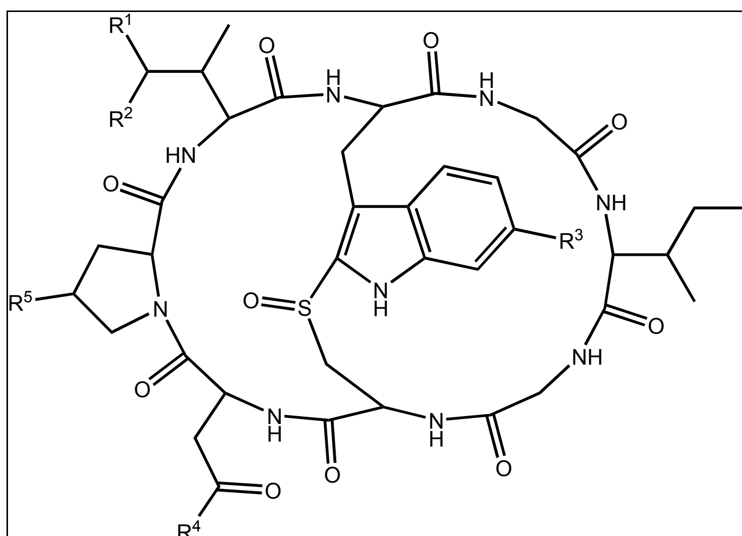

Figure 1: Molecule framework of the amatoxins [3]

Table 1: List of the known amatoxins; LD_{50}-values in mg/kg white mouse [3]

Name	**R^1**	**R^2**	**R^3**	**R^4**	**R^5**	**LD_{50}**
α-amanitin	CH_2OH	OH	OH	NH_2	OH	0.3
β-amanitin	CH_2OH	OH	OH	OH	OH	0.5
γ-amanitin	CH_3	OH	OH	NH_2	OH	0.2
ε-amanitin	CH_3	OH	OH	OH	OH	0.3
amanullin	CH_3	H	OH	NH_2	OH	> 20
amanullin acid	CH_3	H	OH	OH	OH	> 20
proamanullin	CH_3	H	OH	NH_2	H	> 20
amanin	CH_2OH	OH	H	OH	OH	0.5

The amino acid (4*R*)-4,5-dihydroxy-L-isoleucin, which was first discovered in the amatoxins, is a structural part of α- and β-amanitin and amanin [3]. Generally, the lethality of Amanita is mainly attributable to the amatoxins, potent inhibitors of RNA polymerase II, especially in hepatocytes [2].

The phallotoxins are bicyclic heptapeptides with an amatoxin-like structure and they are the cause for the chronic component of an Amanita poisoning [3, 4]. Figure 2 shows the molecule framework of the phallotoxins and the substitution patterns as well as toxicity data for the known phallotoxin compounds are listed in Table 2.

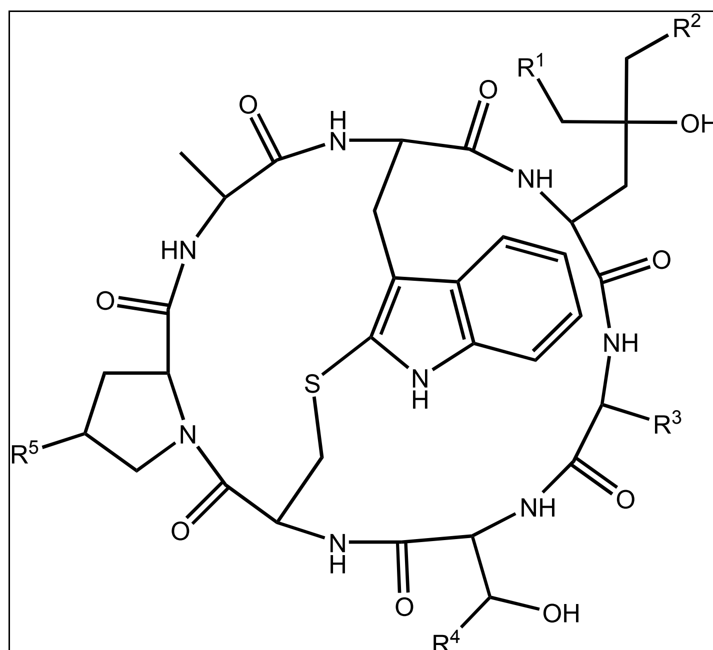

Figure 2: Molecule framework of the phallotoxins [3]

Table 2: List of the known phallotoxins; LD_{50}-values in mg/kg white mouse [3]

Name	**R^1**	**R^2**	**R^3**	**R^4**	**R^5**	**LD_{50}**
phalloidin	OH	H	CH_3	CH_3	OH	2.0
phalloin	H	H	CH_3	CH_3	OH	1.5
prophalloin	H	H	CH_3	CH_3	H	>100
phallisin	OH	OH	CH_3	CH_3	OH	2.5
phallacin	H	H	$CH(CH_3)_2$	COOH	OH	1.5
phallacidin	OH	H	$CH(CH_3)_2$	COOH	OH	1.5
phallisacin	OH	OH	$CH(CH_3)_2$	COOH	OH	4.5

Amatoxins and phallotoxins are known for their unusually high chemical and thermal stability. Amanitin is e.g. not destroyed by boiling in aqueous solutions and preserves its toxic activity after prolonged storage below 0°C [1]. The peptide ring-structure is even not decomposed by the alimentary tract.

Capillary electrophoresis (CE) coupled to mass spectrometry (MS) is a powerful choice for the separation and identification of peptides like the named Amanita toxins in complex matrices. It has proven to meet a high throughput, outstanding certainty in peptide/protein identification, exceptional resolution, and quantitative information in proteome research [5]. The high suitability of CE-MS for the direct analysis of main and minor active compounds in complex mixtures

of biogenic drugs has been demonstrated [6]. Soft ionisation techniques, such as electrospray ionisation (ESI), due to successful multidimensional CE-MS experiments facilitate the analysis and structure elucidation of biomolecules.

2. Experimental

2.1 Chemicals

Ammoniumformate (Biochemika ultra, ≥ 99%), was purchased from Fluka (Taufkirchen, D). Wasser/2-propanol 50:50 (v/v) (LC/MS-grade) and 2-propanol (LC/MS-grade) were purchased from Riedel-de Haën (Seelze, D). α-Amanitin, β-amanitin and phalloidin were purchased from Sigma-Aldrich Chemie (Steinheim, D). γ-Amanitin and phallacidin were purchased from Axxora GmbH (Lörrach, D). Ammonia 25% (p.a.) and methanol (hypergrade for LC/MS) were purchased from Merck (Darmstadt, D). The analysed mushroom samples were provided by the Bundeskriminalamt. Deionised water from a Milli-Q system was used to prepare the running buffer.

2.2 Instrumentation

The samples were introduced into a Beckman-Coulter P/ACE 5000 system by hydrodynamic injection with 34.5 mbar (0.5 psi) for 5 s. Separation was performed in a bare 75 µm I.D. (363 µm O.D.) fused silica capillary from Polymicro Technologies LLC (Phoenix, AZ, USA) with a length of 84 cm. For separation, the capillary inlet was put on a voltage of +28 kV, keeping the sprayer on ground potential. For CE system control the software Beckman P/ACE Station 1.2 was used. The CE unit was connected to an HCT plus ion-trap mass spectrometer (Bruker Daltonik, Bremen, D) via an Agilent coaxial sheath-liquid sprayer interface (Agilent Technologies, Palo Alto, CA, USA). Electrospray ionisation (ESI) was performed at 4500 V. The sheath liquid isopropanol/water (50:50) was supplied a flow rate of 3 µl/min by a syringe pump (Cole-Parmer, Vernon Hill, IL, USA). Nebulizer gas pressure was set to 4 psi. Flow and temperature of dry gas (nitrogen) were 4.0 l/min and 250°C. ESI-MS spectra were obtained in the negative-ion mode with a scan speed of 26000 *m/z* per second in the mass range 600-1000 *m/z* with a target mass of 920 *m/z*. Auto-MS^n experiments were performed by isolation and subsequent fragmentation. Postprocessing software DataAnalysis (version 3.2, Bruker Daltonik) was used for data processing.

2.3 Separation conditions

To achieve the separation of five forensically relevant oligopeptides including α-, β- and γ-amanitin, phalloidin and phallacidin, the running buffer consisted of 20 mmol/l ammonium formate at pH 10.8 (adjusted with ammonia) and 10% (v/v) isopropanol. The separation of the five negatively charged analytes was achieved at 23°C using a high voltage of +28 kV.

2.4 Sample preparation

The solutions of the reference substances (α-, β- and γ-amanitin, phalloidin and phallacidin) consisted of a 0.2 mmol/l solution in methanol. For the CE-MS measurements these solutions were diluted 1:16.67 in the described buffer.

3. Results and discussion

3.1 Developed procedure

The separation of the five oligopeptides was achieved within 8 min. An electropherogram is shown in Figure 3.

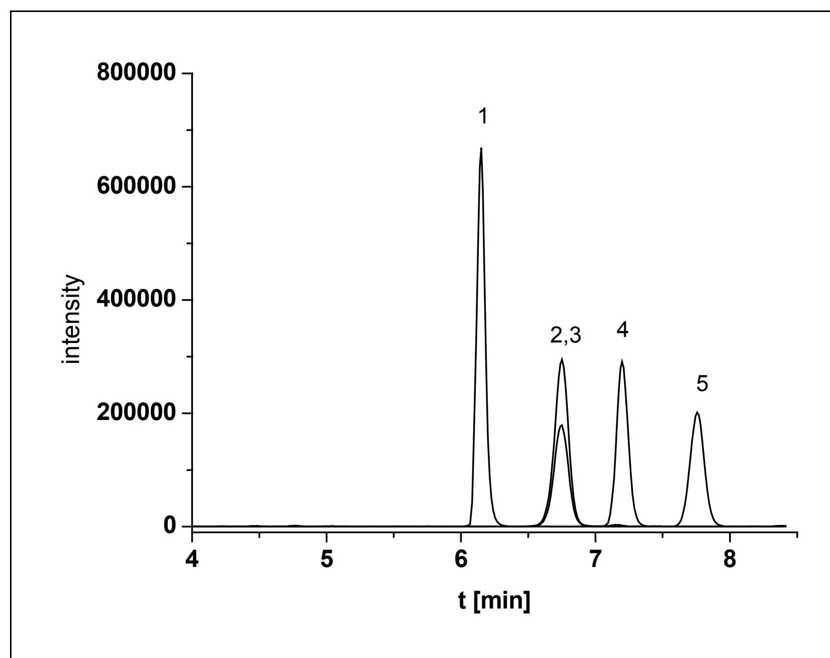

Figure 3: CE-ESI-MS analysis of five Amanita mushroom toxins (bicyclic oligopeptides). $[M-H]^-$ molecular ion masses are in brackets. 1: phalloidin (787.3 m/z), 2: γ-amanitin (901.4 m/z), 3: α-amanitin (917.4 m/z), 4: phallacidin (845.3 m/z), 5: β-amanitin (918.3 m/z)

It is obvious that α- and γ-amanitin could not be separated under the chosen conditions. However, the two comigrating analytes can be easily distinguished by their mass traces.

3.2 Identification

The analysis of intact biomolecules can be achieved by CE-MS experiments with soft ionisation techniques, such as electrospray ionisation (ESI). Furthermore, the utilised ion-trap system is ideally suited for the unambiguous

identification of the migrated oligopeptides at trace levels by recording multidimensional MS-spectra in the auto-MS^n mode. The molecular ion mass peaks of the toxins recorded in methanolic solution reflect the naturally occurring isotope mixture according to their elemental composition [7]. The observed isotopic distribution in the mass spectra of the analysed oligopeptides correlated very well with calculated model spectra and proved to be quite characteristic already in the first mass dimension.

The fragmentation patterns of the investigated amatoxins in the MS^n modes are closely related. Independent of the ionisation mode, in an MS^2-experiment first water of an aliphatic alcohol group of a side chain was cleaved. In addition, in the case of positive ionisation a few fragments were produced by an additional loss of water from a second aliphatic alcohol group. The unusually high stability of the bicyclic structure is the reason why α- and γ-amanitin were not further fragmented in an MS^3-experiment. Only in the negative ionisation mode β-amanitin showed signals of sufficient intensity in such experiments. β-amanitin takes a special status in the negative ionisation mode as its molecular ion stabilises the negative charge much better compared to the two other amatoxins because of the carboxylic acid group that is situated in β-amanitin in addition to the phenolic OH group. The mass difference of 44 *m/z* results from decarboxylation, the further observed mass differences of 17 and 34 *m/z* cannot result from a simple cleavage of side chain substituents, so it must be assumed that a complex reallocation within the molecule takes place, which can only be elucidated by using high resolution mass spectrometry. The MS^n-spectra of β-amanitin in the negative ionisation mode are exemplarily shown in the following figures.

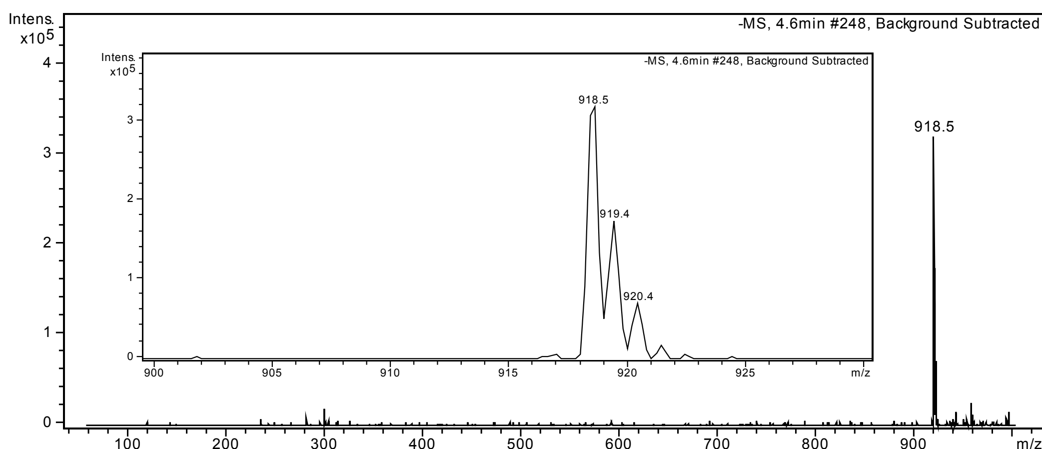

Figure 4: MS [1]-spectrum of β-amanitin, negative detection mode

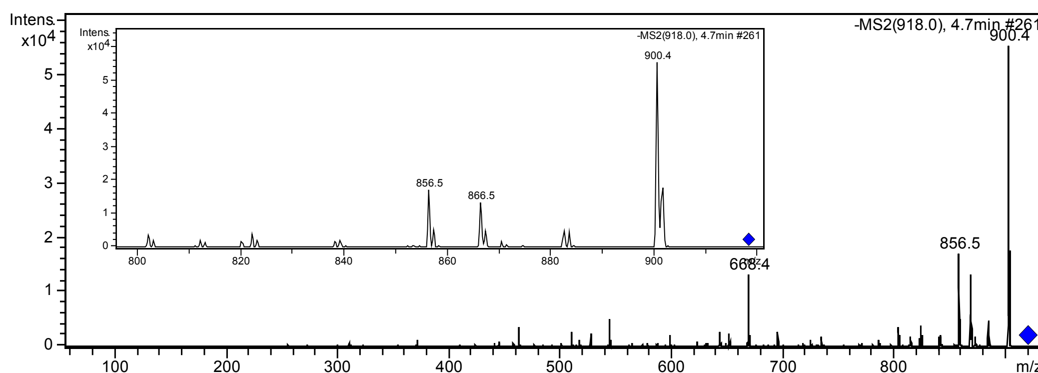

Figure 5: MS ^{2}-spectrum of β-amanitin, negative detection mode

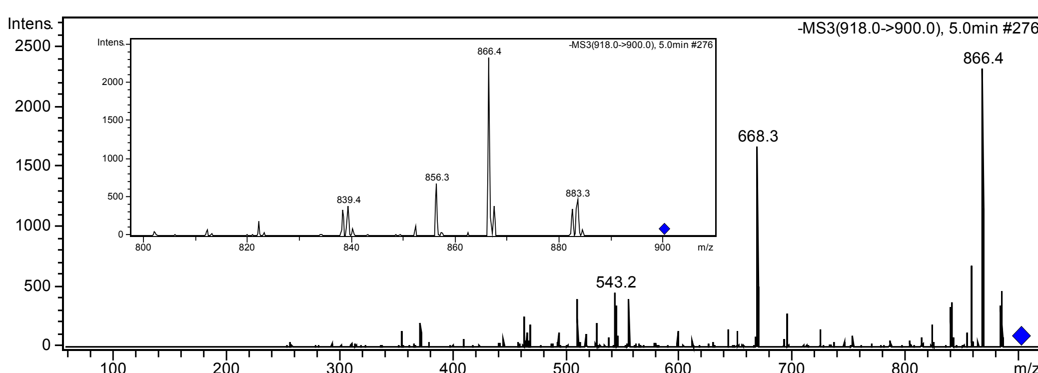

Figure 6: MS ^{3}-spectrum of β-amanitin, negative detection mode

In contrast to the amatoxins the extent of fragmentation of the phallotoxins strongly depends on the ionisation mode. In the case of positive ionisation a high degree of fragmentation was observed already in an MS^{2}-experiment, which indicates that the bicyclic structure was broken up. MS^{3}-experiments with isolated higher mass fragments from the MS^{2} step (resulting from water loss) did not supply signals of sufficient intensity. Negative ionisation generated a different result: In the MS^{2}-experiment only few high mass fragments ions were formed, which points to an intact bicyclic structure. For phallacidin, similarly to β-amanitin, water loss from the aliphatic OH groups took place in the MS^{2}- and MS^{3}-experiments in the negative mode as well as decarboxylation of the carboxylic acid group.

In general the molecule frameworks of the phallotoxins, especially of phalloidin, proved not to be as stable as those of the amatoxins under ESI-MS conditions. It can be concluded, that the auto-MS^{n} functionality of the ion-trap combined with the migration times of the separation step provides an unambiguous identification of the mushroom toxins.

3.3 Limits of detection and repeatability

The figures of merit for the procedure presented here were evaluated. Limits of detection in the low nanomolar region were found for ESI-MS detection in negative polarity mode. Thus, the sensitivity of the procedure is high enough for the reliable detection of the mushroom toxins at trace levels (e.g. hazardous concentrations in poisoned food samples).

Repeatability measurements yielded RSD values between 0.2 and 0.3% for the migration time.

Table 3: Limits of detection and repeatability (N = 6) as RSD in % of phalloidin, phallacidin, α-, β- and γ-amanitin

	concentration [µmol/l]	**3σ limit of detection [nmol/l]**	**[ng/ml]**	**RSD of migration time [%]**
α-amanitin	13.1	15	13	0.3
β-amanitin	13.0	39	36	0.2
γ-amanitin	13.3	44	40	0.3
phalloidin	15.2	14	11	0.2
phallacidin	14.2	28	24	0.2

3.4 Analysis of mushroom real samples

The applicability of the developed CE-ESI-MS^n procedure for the analysis of extracts of fresh and dried mushroom samples was investigated. For each analysis 20-30 mg of an Amanita sample were extracted in methanol by ultrasonication, diluted with running buffer and directly injected into the separation capillary. All analytes were identified via auto-MS/MS experiments.

In Figure 7 an electropherogram of a methanolic mushroom extract of *Amanita phalloides* also known as “destroying angle” is shown.

In this mushroom sample the amatoxins α-, β- and γ-amanitin as well as the phallotoxins phalloidin and phallacidin were identified. In addition one further phallotoxin, phallisacin (861.3 *m/z*) was identified whose fragmentation is similar to that of phallacidin.

Figure 8 shows an electropherogram of a methanolic extract of *Amanita virosa* which is also called “destroying angel”. In this sample α- and γ-amanitin, phalloidin, phallacidin and phallisacin were present. However, as expected from literature, no β-amanitin was found in *Amanita virosa* [8].

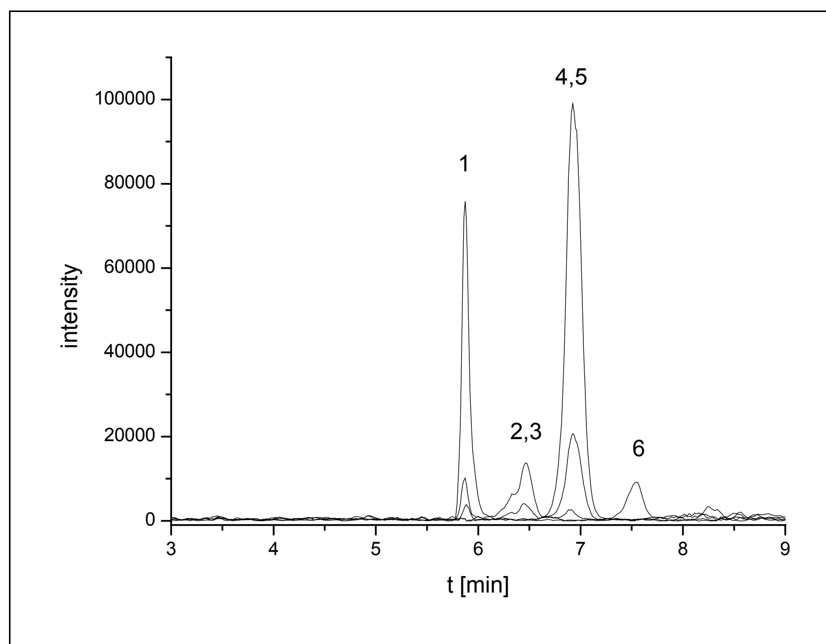

Figure 7: Electropherogram of a methanolic extract of *Amanita phalloides*, $[M\text{-}H]^-$ molecular ion masses are in brackets. 1: phalloidin (787.3 m/z), 2: α-amanitin (917.4 m/z), 3: γ-amanitin (901.4 m/z), 4: phallacidin (845.3 m/z), 5: phallisacin (861.3 m/z), 6: β-amanitin (918.3 m/z)

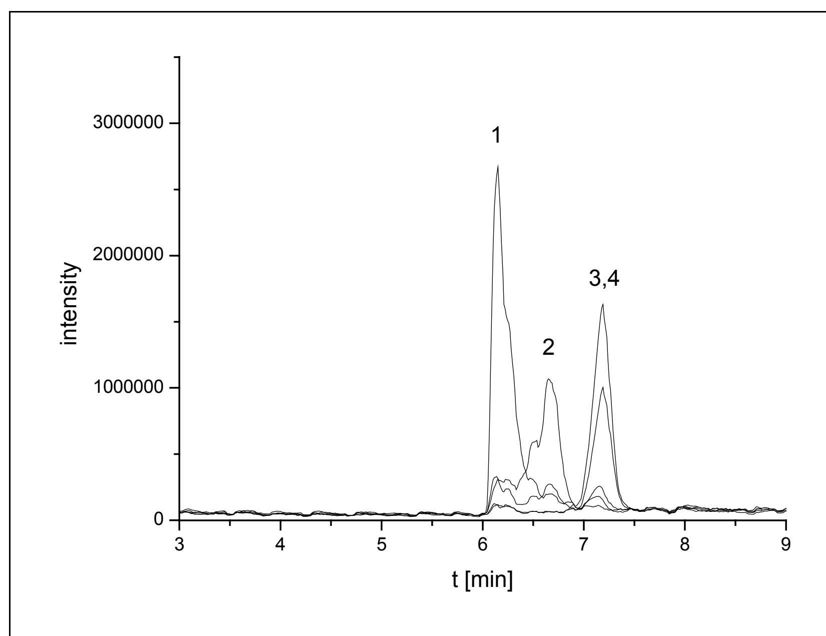

Figure 8: Electropherogram of a methanolic extract of *Amanita virosa*, $[M\text{-}H]^-$ molecular ion masses are in brackets. 1: phalloidin (787.3 m/z), 2: α-amanitin (917.4 m/z), 3: phallacidin (845.3 m/z), 4: phallisacin (861.3 m/z)

In another experiment the methanolic extract of a mushroom was analysed which was identified by a morphological examination as *Amanita verna* ("death cap"). But in this mushroom not even traces of amatoxins or phallotoxins were found. This result correlates with literature reports of finds of *Amanita verna* without any toxins [8].

4. Conclusion

In this work, it was shown that the coupling of CE with ESI-MS is a powerful tool for the analysis of biomolecules. A CE-ESI-MS^n procedure was developed for the analysis of five forensically relevant mushroom toxins. The definite identification of the examined oligopeptides, amatoxins and phallotoxins, was achieved by recording MS-MS spectra in the negative polarity detection mode. This procedure provides detection limits in the low nanomolar range as well as good repeatability of the migration times.

The CE-ESI-MS^n procedure was successfully applied for the identification of amatoxins and phallotoxins in extracts of fresh and dried mushroom samples.

References

[1] Brüggemann O, Meder M, Freitag R (1996) J Chromatogr A 744: 167-176
[2] Maurer HH, Schmitt CJ, Weber AA, Kraemer T (2000) J Chromatogr B 748: 125-135
[3] CD Römpp Chemie Lexikon – Version 1.0 (1995) Georg Thieme Verlag Stuttgart/New York
[4] Ishizaki K, Kinbara S, Miyazawa N, Takeuchi Y, Hirabayashi N, Kasai H, Araki T (1997) Toxicology Letter 90: 29-34
[5] Stutz H (2005) Electrophoresis 26: 1254-1290
[6] Huhn C, Neusüß C, Pelzing M, Pyell U, Mannhardt J, Pütz M (2005) Electrophoresis 26: 1389-1397
[7] Hesse M, Meier H, Zeeh B (2005) Spektroskopische Methoden in der organischen Chemie. Georg Thieme Verlag Stuttgart
[8] Bresinsky A, Besl H (1985) Giftpilze mit einer Einführung in die Pilzbestimmung. Wissenschaftliche Verlagsgesellschaft Stuttgart

Jan Rittgen, Ute Pyell
Philipps-Universität Marburg
Fachbereich Chemie
Hans-Meerwein-Straße
D-35032 Marburg
Email: rittgen@web.de

Michael Pütz
Bundeskriminalamt
Fachbereich KT 34 (Toxikologie)
Thaerstraße 11
D-65193 Wiesbaden
Email: michael.puetz@bka.bund.de