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Strategies to avoid the mis-identification of anatoxin-a using mass spectrometry in the forensic investigation of acute neurotoxic poisoning

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Abstract

Anatoxin-a (AN) is a potent neurotoxin, produced by a number of cyanobacterial species, and consumption of freshwater contaminated with this toxin has led to animal deaths. Forensic investigations of suspected AN poisonings are frequently hampered by difficulties in detecting this toxin in biological matrices due to its rapid decay. In addition, detection of AN using single quadrupole mass spectrometry (MS) is suspect due to the presence of the amino acid, phenylalanine (Phe), since these compounds are isobaric and elute similarly in reversed phase liquid chromatography (LC). Approaches to prevent the misidentification of AN that have been explored in these studies included: (a) fluorimetric LC following derivatisation using 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F); (b) methylation using diazomethane prior to LC–MS determination; (c) multiple tandem MS using a quadrupole ion-trap (LC–MS^3); and (d) hybrid quadrupole time-of-flight (QqTOF). Interference from Phe was not observed in any of procedures, (a)–(c), and the high mass accuracy obtained in method (d), readily distinguished between AN (165.11536) and Phe (165.07898). LC–MS^n was also employed to study the fragmentation pathway of Phe and multi-stage MS spectra provided characteristic fragmentation information that clearly distinguished between AN and Phe. The difficulties associated with the over reliance on low resolution MS without MS/MS data in forensic toxicology are discussed.
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Keywords: Cyanobacteria; Neurotoxins; LC–MS; Quadrupole time-of-flight

1. Introduction

Anatoxin-a (AN) is a neurotoxin, produced by several cyanobacterial genera, including *Anabaena* and *Planktothrix* (formerly *Oscillatoria*) spp. [1–3]. This toxin is a low molecular weight bicyclic secondary amine (Fig. 1A.) and is a potent nicotinic agonist that acts as a post-synaptic, depolarising, neuromuscular blocking agent [4]. Fatal intoxications due to AN have included cattle in Finland [5] and dogs in Scotland and Ireland [6,7]. These toxic incidents are frequently dramatic due to the rapidity of onset of toxic symptoms, which include muscle fasciculation and convulsions, resulting in death within minutes due to respiratory failure [4,8].

Forensic investigations of suspected AN poisonings are often troubled by problems in detecting this toxin in biological and environmental matrices due to its rapid decay. Also, there have been two recent incidents highlighting where possible confusion can occur in forensic investigations of suspected AN poisonings due to the presence of the amino acid, phenylalanine (Phe) (Fig. 1B.). The death of a young adult in the USA in July 2002, following exposure to lake-water, was ascribed to AN poisoning in the coroner's report. This conclusion was reached based mainly on evidence of the identification of AN using liquid chromatography–single quadrupole MS (LC–MS). This identification has since been shown to be incorrect and was the result of a confusion between Phe and AN which are isobaric and have similar LC retention times [9].

In September 2003, an investigation of the fatal intoxication of two dogs in a lake in eastern France found the presence

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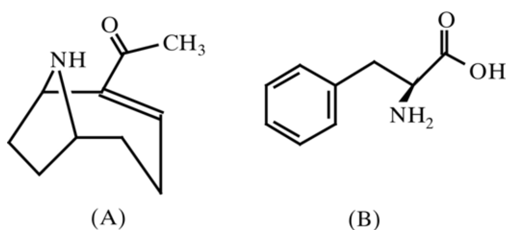

Fig. 1. Structures of: (A) anatoxin-a (AN); (B) phenylalanine (Phe).

of AN in benthic cyanobacteria (*Planktothrix*), found along the shoreline. However, the large quantity of Phe present prevented confirmation and quantification of AN in the stomach and intestine contents of these dogs [10].

Most early studies of freshwaters, contaminated by AN, have been carried out using LC–UV [11,12], but this method does not detect AN degradation products. A highly sensitive fluorimetric LC method, using derivatisation with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), was developed for the simultaneous determination of AN, HMAN and their degradation products [7,13]. Gas chromatography (GC) has also been employed to determine derivatised AN in freshwaters using both MS and electron capture detection [14–18].

A number of liquid chromatography–mass spectrometry (LC–MS) methods have been used for the determination of anatoxins in cyanobacteria [19–22], in food supplements [23] and in fish muscle [24]. A detailed study of the mass fragmentation pathways for AN and HMAN using a quadrupole ion-trap (QIT) instrument, led to the development of sensitive LC–MS^n methods [25]. In this report, strategies to avoid the mis-identification of AN in forensic investigations of acute neurotoxic poisonings are presented.

2. Experimental

2.1. Chemicals

Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) (Aldrich, Gillingham, UK), anatoxin-a hydrochloride (Calbiochem-Novabiochem, Nottingham, UK, λ_{227}; $\log \varepsilon = 4.10$), Phe and 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) (Sigma–Aldrich, Dorset, UK) were purchased. Water, acetonitrile and methanol were of HPLC grade (Labscan, Dublin, Ireland) and trifluoroacetic acid (TFA) was LC-spectrograde (Sigma–Aldrich, Dorset, UK).

2.2. Fluorimetric liquid chromatography

Standards of AN and Phe were derivatised with NBD-F according to the procedure detailed previously [13]. The LC system consisted of a pump (LC-10AD), column oven (CTO-10A) and an RF-551 fluorescence detector (Shimadzu, Duisberg, Germany). The analytical LC column was Ultramex C_{18} (5 μm, 250 mm × 3.2 mm, Phenomenex, Macclesfield), used with the recommended pre-column and operated at 35 °C. The mobile phase was acetonitrile–water (45:55); the flow rate was 0.5 mL/min; fluorimetric detection ($\lambda_{ex} = 470$ nm, $\lambda_{em} = 530$ nm).

2.3. Liquid chromatography–quadrupole ion trap mass spectrometry

The LC system was a Waters 2690 Alliance (Waters Corporation, Milford, MA, USA), which included an autosampler that maintained samples at 4 °C. This was linked to a Finnigan MAT LCQ *Classic* ion-trap mass spectrometer (ThermoElectron, San Jose, CA, USA) using Xcalibur software. Isocratic chromatography was performed using acetonitrile–water (15:85) containing 0.05% TFA, at a flow rate of 400 μL/min. The analytical column (Luna-2 C_{18}, 250 mm × 4.6 mm, 5 μm, Phenomenex, Macclesfield, UK) was operated at 35 °C.

MS analysis was performed at atmospheric pressure using an electrospray ionisation (ESI) source and data were acquired in positive mode as previously reported [25]. Multiple tandem MS produced collision-induced dissociation (CID) spectra and were obtained by trapping the $[M+H]^+$ ion at *m/z* 166 for AN and Phe. Instrument settings and procedures for MS^n experiments were as reported previously [25]. Fragmentation experiments were performed on the product ions to obtain characteristic spectra for each toxin. The mass-acquire window width for ion trapping was 3 amu. Relative collision energies (%RCE) were optimised for each compound and were in the range, 24–32%. Calibration standards obtained with and without Phe indicate that the presence of Phe does not cause any ion supression effects for AN.

2.4. Hybrid quadrupole time-of-flight (QqTOF) mass spectrometry

A hybrid quadrupole time-of-flight mass spectrometer, QqTOF MS (Qstar, Applied Biosystems, Warrington, UK), with a nano-electrospray (ES023C, Proxeon, Denmark) ion source (nanoESI), was used to produce collision induced dissociation spectra of AN and Phe, in positive mode and of Phe in negative ion mode. Standards (1–5 μg/mL) were prepared in acetonitrile and 5–10 μL was loaded into a nanospray capillary (Long NanoES spray capillary, ES381, Proxeon, Denmark) using a 0.5–10 μL eppendorf GELoader tip and a TOMY PMC-060 Capsulefuge to remove air bubbles. While scanning in the range, *m/z* 30–400, the voltages applied during acquisition were as follows: ionspray voltage (IS) = 900 V, declustering potential (DP) = 30 V, focusing potential (FP) = 300 V, declustering potential-2 (DP2) = 15 V, collision gas (CAD) = 2 (arbitrary units). Collision energies of 25–35 V were used during acquisitions. The scan time was 1 s, and the total accumulation time for each spectra was in the range, 10–30 min.

2.5. Methylation of phenylalanine using diazomethane

Diazomethane was prepared in situ using an MNNG diazomethane generator (Sigma–Aldrich). MNNG (1 mmol) was reacted with sodium hydroxide (5N, 0.6 mL) and the diazomethane was collected in ether. The diazomethane solution (200 μL) was added to separate solutions of Phe and AN and to a mixture of the two compounds (100 ng/200 μL acetonitrile) and allowed to stand for 10 min. Hydrochloric acid (100 μL) was added to quench the reaction, solvents were evaporated and the residues were reconstituted in acetonitrile (100 μL).

3. Results and discussion

The mistaken identification of anatoxin-a in a high profile investigation of the first suspected human intoxication involving this neurotoxin has cast doubt on the analytical methods that have been used to monitor anatoxins [9]. In particular, LC–MS, using a single quadrupole instrument, does not readily distinguish between phenylalanine and AN, as they have the same nominal mass (isobaric) together with similar retention times in reversed phase LC. This case and another involving dog fatalities, have revealed that significant problems can be encountered in the forensic investigations of anatoxins, especially in complex matrices [10]. The following approaches to prevent the misidentification of AN have been explored in these studies including: (a) fluorimetric LC following derivatisation using 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F); (b) multiple tandem MS using a quadrupole ion-trap (LC–MS^3); (c) hybrid quadrupole time-of-flight MS; and (d) methylation using diazomethane prior to LC–MS determination.

3.1. Fluorimetric LC of anatoxin-a with phenylalanine

A previously developed fluorimetric LC method for the simultaneous determination of AN, HMAN and their degradation products, was examined to determine if Phe was a potential interferant. The derivatisation conditions using NBD-F were followed as described [7]. It was found that NBD-Phe eluted just after the reagent peaks in reversed phase LC and such early elution was expected since there remains a free carboxylic acid moiety after derivatisation of Phe at the nitrogen. Therefore, there was no interference with NBD-AN, which eluted at 14.5 min (Fig. 2), nor were there any interferences with AN and HMAN degradation products (not shown). These studies confirm that the presence of Phe should not prevent the sensitive determination of anatoxins using this fluorimetric LC method.

3.2. Mass fragmentation pathways of phenylalanine and anatoxin-a

A comparison of the mass fragmentation pathways of AN and Phe was undertaken to identify characteristic product

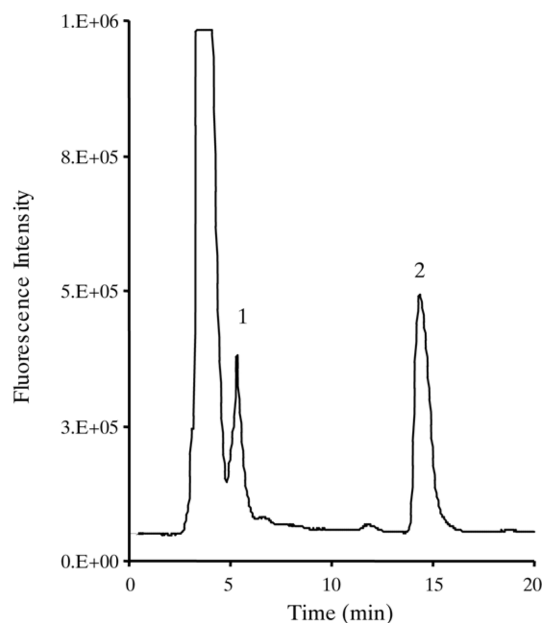

Fig. 2. Chromatogram of a water sample spiked with AN and Phe. Derivatision with NBD-F was used with fluorimetric detection: NBD-Phe (1) and NBD-AN (2). Chromatographic conditions: Ultremex column (C_{18}, 5 μm, 250 mm × 3.2 mm, 35 °C); 20 μL injection; mobile phase was acetonitrile–water (45/55); flow rate = 0.5 mL/min; fluorimetric detection, λ_{ex} = 470 nm, λ_{em} = 530 nm.

ions and fragmentation processes and the main findings are summarised in Fig. 3. Collision-induced dissociation spectra were obtained by trapping and fragmenting the molecular ions and product ions for AN and Phe in multiple tandem MS experiments using a quadrupole ion-trap instrument. One of the major product ions in the MS^2 spectrum of AN is due to the loss of ammonia and was observed at *m/z* 149, $[M+H-NH_3]^+$. The MS^3 spectrum (Fig. 4B) showed product ions at *m/z* 131 $[M+H-NH_3-H_2O]^+$, *m/z* 121, 107, 105, 91, 81 and 79. MS^4 experiments, targeting *m/z* 131, gave product ions at *m/z* 116 and 91 and the fragmentation of the ion at *m/z* 107 produced a single product ion at *m/z* 79. The MS^2 spectrum of Phe showed a major product ion, due to the loss of formic acid, at *m/z* 120 $[M+H-HCO_2H]^+$, and a less abundant ion at *m/z* 131 $[M+H-NH_3-H_2O]^+$. The MS^3 spectrum, targeting *m/z* 120, showed product ions at *m/z* 103, 93 and 79 (Fig. 4C).

3.3. LC–MS^3 determination of anatoxin-a in the presence of phenylalanine

The direct combination of LC with MS reduces the emphasis on chromatographic separations due to the mass selectivity and capability of applying distinctive fragmentation patterns. Although AN and Phe have similar retention characteristics in reversed phase LC [9], Fig. 4A shows that, using our previously published LC conditions [25], the separation of AN (7.47 min) and Phe (7.95 min) is possible. However, the prox-

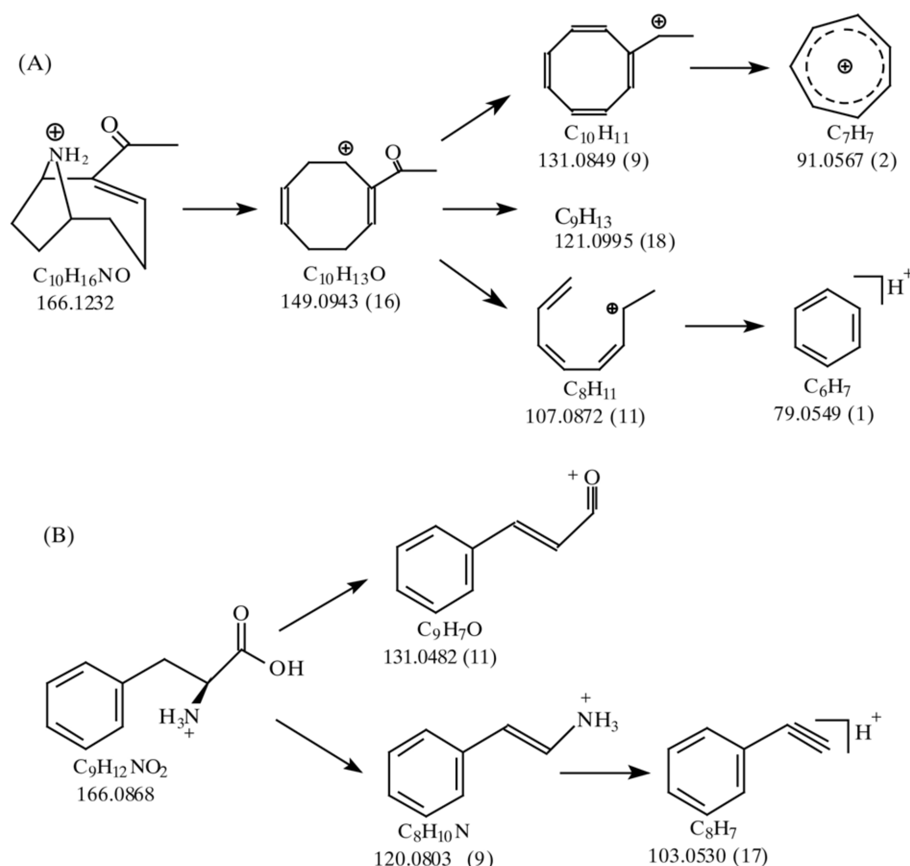

Fig. 3. Proposed fragmentation pathways for: (A) AN and (B) Phe elucidated by multiple tandem MS experiments using QIT MS, showing the typical losses and structures of product ions. High mass accuracy data from QqTOF MS are presented to support the assignments with error values (ppm) in parenthesis.

imity of retention times indicates that this should not be relied upon as the basis of a robust analytical method. Using the selected ion trapping process, QIT MS^n allows an unambiguous assignment of precursor/product sequences and signals are remarkably free from interferences typically present in complex biological matrices.

Previously, LC–MS^n methods were developed for the rapid and sensitive determination of AN in cyanobacteria and drinking water [25]. Fragmentatation studies showed that AN has a greater range of product ions than Phe. These data confirm that LC–MS^3 is preferred for the unambiguous identification of AN since in MS^1 they have isobaric precursor ions, and in MS^2 there are common ions, albeit with different intensities. A feature that is unique to QIT MS is that there is usually an improvement in detection sensitivity in multiple MS modes. This is attributed to the reduction in background noise (N) being more dramatic than the decline in analyte signal (S) giving an improved S/N in MS^2 and MS^3 stages [26,27]. The calibration data for standard AN, spiked into lake water containing Phe, were linear; $r^2 = 0.998$ (2.5–10 μg/mL), with a relative standard deviation (%RSD) ≤ 8.0 ($N = 5$). The detection limit for AN was 0.1 μg/mL (S/N = 3).

3.4. Hybrid quadrupole time-of-flight mass spectrometry

A nano-electrospray source on a QqTOF MS instrument was used to obtain mass spectra of AN and Phe. Although high mass accuracy MS data was obtained for both AN and Phe in positive mode (Fig. 5), only Phe produced significant MS data in negative mode. The high resolution of this instrument is such that the isobaric precursor ions, are partially resolved (Fig. 6A) because of the small differences in their accurate masses, $[M + H]^+$, AN (*m*/*z* 166.1232) and Phe (*m*/*z* 166.0868). The product ion spectra obtained by MS/MS on the QqTOF are much superior to those generated by in-source fragmentation on the single-stage TOF instrument. This is a consequence of the high specificity of fragmentation that is achieved through the selection of a narrow precursor *m*/*z* range of ions in the quadrupole [28].

One of the obvious drawbacks of QIT MS is the lack of signals in the lower mass region, due to the low mass cutoff which amounts to approximately 30% of the precursor ion mass/charge value. Interestingly, using QqTOF MS, only a few product ions below *m*/*z* 77 were observed for Phe, whereas AN showed several product ions in the low mass

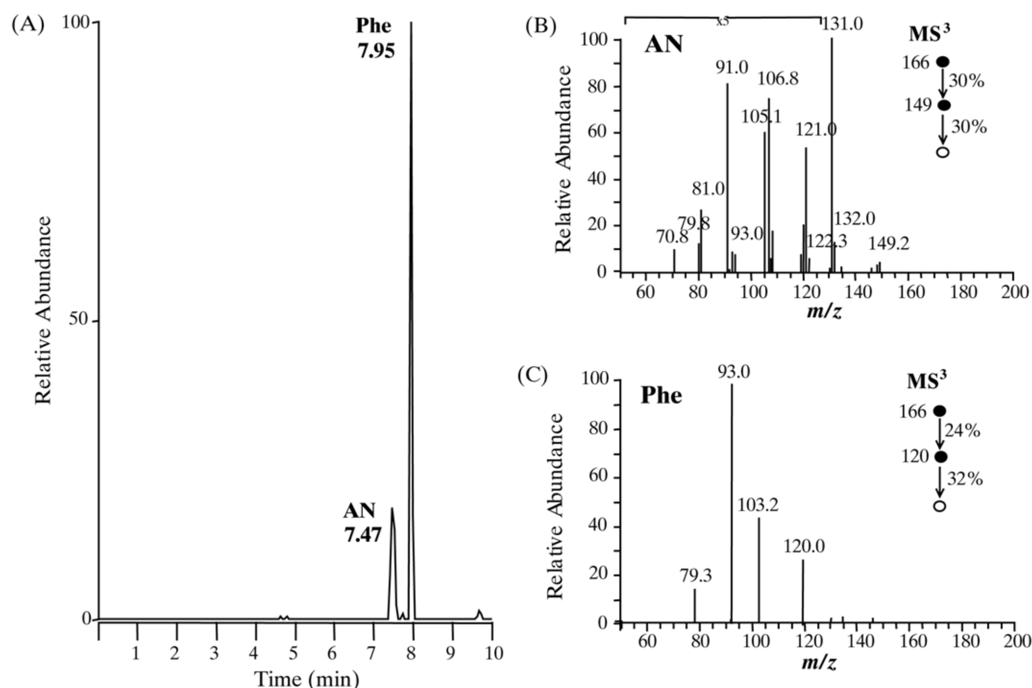

Fig. 4. LC–MS^3 chromatogram of a water sample containing standard AN (7.47 min.) and Phe (7.95 min.) obtained using QIT MS detection. The selected ions and relative collision energies for fragmentation at each MS stage are shown as inserts. Chromatographic conditions: Luna column (C_{18}, 5 μm, 250 × 3.2 mm, 35 °C); 5 μl injection; mobile phase was acetonitrile–water (15/85); flow rate = 0.4 mL/min. Positive CID product ion spectra of MS^3 for (A) AN-a and (B) Phe. The selected ions for trapping and fragmentation, together with the optimised RCE are shown as inserts.

region. The base peak in the AN spectrum (Fig. 5A) was at *m/z* 43, $[CH_3CO]^+$, and this is an important diagnostic ion for anatoxins since it is formed from side-chain fragmentation. This ion is not observed in the mass spectra of HMAN and analogues, as they produce the homologous ion, *m/z* 57, $[CH_3CH_2CO]^+$. Another abundant ion in the AN spectrum (Fig. 5A) was present at *m/z* 149 $[M + H\text{-}NH_3]^+$ but this was not observed in the Phe spectrum (Fig. 5B). MS/MS data were used to confirm formulae assignments of the major ions (Fig. 3), especially those that are isobaric and are indistinguishable in QIT MS where only unit resolution is possible. The error values, related to the differences between the calculated and observed masses, were less than 20 ppm, which is within instrument specification.

3.5. Limitations of low-resolution MS^2

Analysis of micro-organic contaminants in environmental samples presents a range of problems, one of which is due to interferences from non-target compounds that can create an artificially high background [29]. A study involving the analysis of pharmaceuticals in sewage effluent using different MS analysers revealed large variations in quantitation and, in particular, accurate mass analysis gave a concentration that was lower by a factor of five when compared to analysis using a single quadrupole instrument. Another problem is that the carbon-13 isotope of a large co-eluting compound, one mass unit less than the target analyte, can interfere with both SIM and MRM determinations [29].

However, in light of the fact that many laboratories do not have ready access to a range of multiple tandem and high-resolution MS instruments, we examined the possibility of improving the reliability of measurements using single quadrupole MS. A distinguishing feature was the observation of sodium adduct ions in Phe spectra but no such adduct ions were observed in AN spectra. Therefore, a simple modification to a SIM protocol for AN determination is to include measurements of both *m/z* 166 $[M + H]^+$ and 188 $[M + Na]^+$ signals, since a low value for the latter indicates the lack of Phe interference.

However, a more reliable method that would improve the reliability of single quadrupole MS in monitoring programmes is to perform a simple sample pre-treatment that clearly distinguishes AN and Phe. The method that was selected was methylation, using diazomethane, since only Phe was changed by this treatment. In addition, the excess reagent was readily destroyed allowing the products to be analysed following solvent evaporation. The effect of this methylation step is shown on the precursor ion, $[M + H]^+$, signals of AN and Phe using nano-electrospray QqTOF MS. Fig. 6A shows the partially resolved signals for AN and Phe and, after methylation, Fig. 6B shows that the Phe signal is removed. This facile step to the formation of the methyl ester of Phe results in a mass change of 14 Da, and therefore, Phe interference will be removed when using low resolution SIM, targeting the *m/z* 166 ion. This treatment had no effect on AN, as shown in QqTOF MS. Although the methyl ester of Phe and HMAN are isobaric only the latter is observed in the

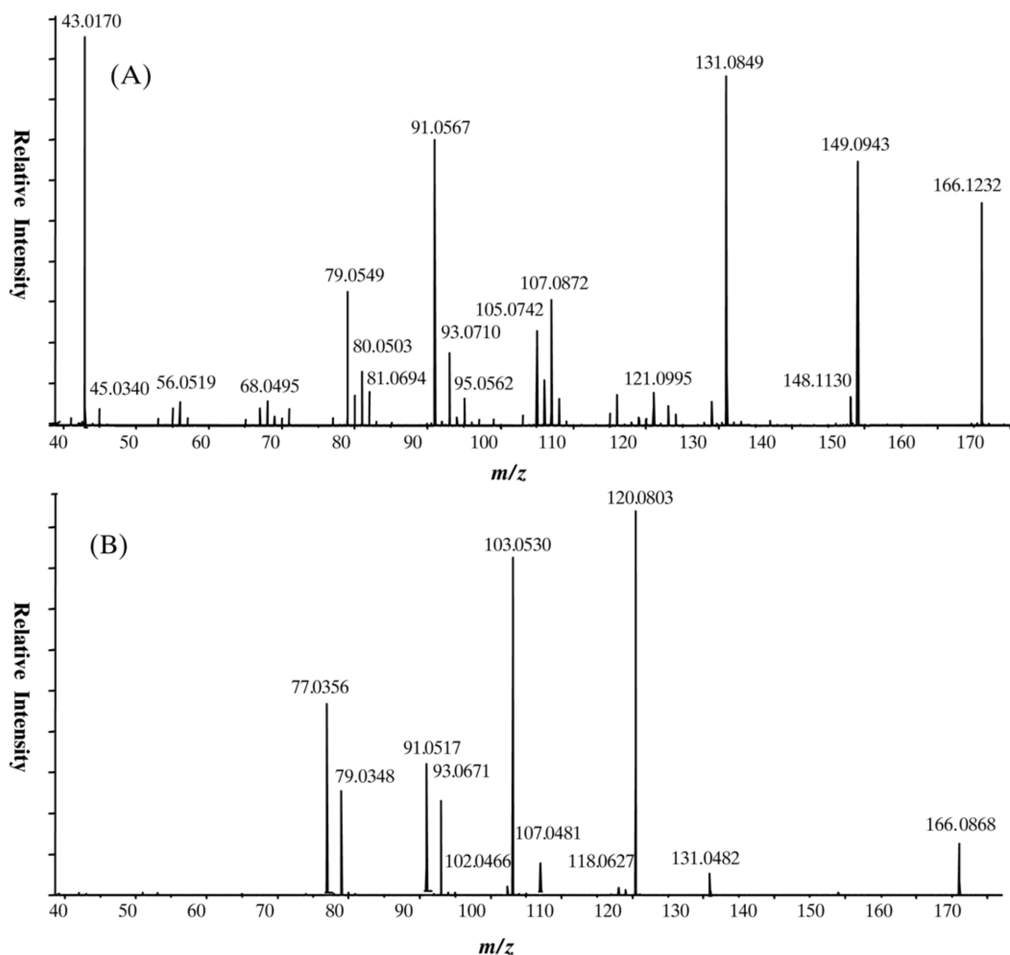

Fig. 5. Spectra of (A) AN and (B) Phe, obtained using positive nano-electrospray with a hybrid quadrupole time-of-flight (QqTOF) mass spectrometer.

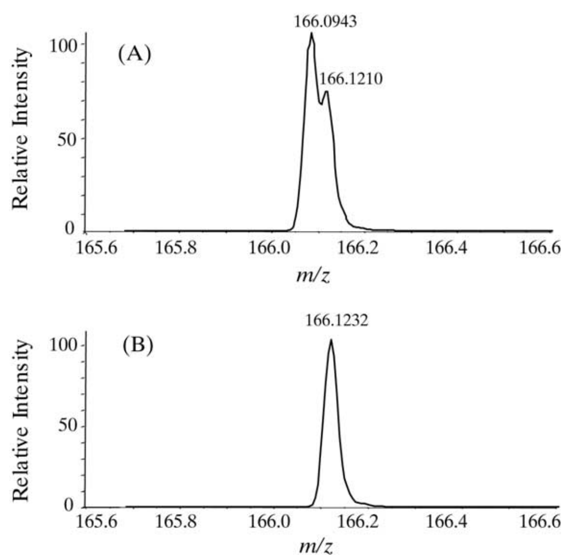

Fig. 6. High resolution MS of an extract containing a mixture of AN and Phe, obtained using nano-electrospray QqTOF MS, in positive mode: (A) without treatment with diazomethane, Phe (*m/z* 166.0943), AN (*m/z* 166.1210); (B) after treatment of sample with diazomethane.

chromatogram using the elution conditions described. Phe and HMAN may coelute but the Phe methyl ester is strongly retained.

4. Conclusions

A number of methods have been developed to prevent the misidentification of AN and to avoid confusion with Phe in the forensic investigations of neurotoxin poisonings.

- Fluorimetric LC determination of AN, following derivatisation using NBD-F, was not affected by Phe.
- Multiple tandem MS (MS^n) studies were performed to identify characteristic product ions for AN and Phe which led to the development of an LC–MS^3 method.
- The high-resolution capability of hybrid quadrupole time-of-flight MS allows the isobaric protonated ions of AN and Phe to be distinguished.
- Methylation using diazomethane prior to LC–MS determination removes Phe interference.

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