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Forensic Science International 127 (2002) 208–217

Forensic Science International

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Accidental fatal poisoning by *Nicotiana glauca*: identification of anabasine by high performance liquid chromatography/photodiode array/mass spectrometry

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Received 12 January 2002; received in revised form 12 April 2002; accepted 16 April 2002

Abstract

A method, based on reversed phase high performance liquid chromatography (HPLC) was developed for the detection and quantification of anabasine, the toxic alkaloid of *Nicotiana glauca*, in forensic applications. A standard solid phase extraction (SPE) method was used for the extraction of anabasine from viscera, but was optimized for the extraction of this alkaloid from plant material. The careful selection of mobile phase components allowed the direct coupling of electron impact (EI) and Z spray mass selective detector (ZMD) of the HPLC. Under these conditions, anabasine was well separated from nicotine and could be detected on the PDA (limit of detection, LOD = 250 ng/ml), TMD (LOD = 10 μg/ml) and ZMD (LOD = 1 ng/ml) detectors. Three geographically isolated *N. glauca* trees were analyzed for alkaloid content and it was found that both the leaves and the flowers contain anabasine. The optimized HPLC method was used to analyze two viscera samples (the stomach and contents of a mother and child who putatively died from food poisoning) and a flower exhibit. Anabasine was detected in both the viscera samples, supporting the finding that these fatalities were due to the ingestion of *N. glauca* accidentally collected with traditional spinach (marog). The alkaloid profile of the flower exhibit submitted with the viscera samples was similar to those obtained from flowers collected from three different *N. glauca* trees. The results show that anabasine and/or *N. glauca* poisoning can easily be confirmed using the forensic methodology described.

Keywords: *Nicotiana glauca*; Anabasine; HPLC; Mass spectrometry; Poisoning; Forensic

1. Introduction

Nicotiana glauca R.A. Grah (Family Solanaceae), also called wild tobacco or tree tobacco, is a virgate shrub of up to 5 m high with glaucous leaves and tubular yellow flowers. *N. glauca* is indigenous to Argentina and was most likely introduced into Namibia in contaminated horse feed during the German occupation (1884–1914), from where it spread to South Africa. It is closely related to commercial tobacco, *Nicotiana tabacum* L., and is widespread throughout South Africa in places where the natural vegetation has been disturbed, such as roadsides and riverbanks [1].

The smoking of *N. glauca* has been reported [2–4] and the plant has also been used medicinally [2] and in ethno-veterinary medicine [5]. Warmed leaves are applied to the head to relieve headache, on the throat to relieve pain and put in shoes for painful feet [6]. Animal deaths, mainly of ostriches, have also been reported [2]. It has been used as an insecticide [7–9], but its use has been discontinued due to the development of more specific and less toxic insecticides. In humans, accidental ingestion of *N. glauca* does occur [10–14] and usually results in death of the unsuspected user if sufficient plant material is ingested. In southern Africa a traditional meal is prepared consisting of porridge and marog (*Amaranthus hybridus*, Amaranthaceae), but juvenile

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0379-0738/02/$ – see front matter
PII: S0379-0738(02)00123-8

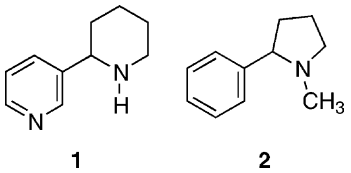

Fig. 1. Chemical structures of anabasine (1) and nicotine (2).

Table 1
The LOD and the LOQ for anabasine (1) with the PDA, TMD and ZMD detectors

Detector	LOD	LOQ
PDA (200–600 nm)	250 ng/ml	500 μg/ml
TMD (50–550 amu)	10 μg/ml	50 μg/ml
ZMD (100–300 amu)	1 ng/ml	50 ng/ml

N. glauca plants are sometimes accidentally collected with the marog (or as marog), resulting in poisoning. In the past, the Forensic Chemistry Laboratory (Johannesburg) of the Department of Health (South Africa) has occasionally been confronted with suspected ''food poisoning'' incidents, often being linked to the ingestion of poisonous plant material, including *N. glauca*. An actual case study will be presented here where an elderly lady and young man died after a supper of porridge and marog. The possible cause of death was ascribed to food poisoning and samples, including viscera, were submitted to our laboratory for chemical analysis.

The toxic principle of *N. glauca,* anabasine (1) (also called neonicotine, Fig. 1), is a small pyridine alkaloid with a molecular mass of 162.23. Anabasine has teratogenic properties and exposure to it may cause reproductive defects. The LD_{50} (gpg, scu) of anabasine is listed as 22 mg/kg [8]. Ingestion of sufficient amounts of plant material containing anabasine will result in increased salivation, vertigo, confusion, disturbed vision and hearing, photophobia, nausea, vomiting, diarrhoea, respiratory depression, spasms, neuromuscular blockage and finally death [7,10].

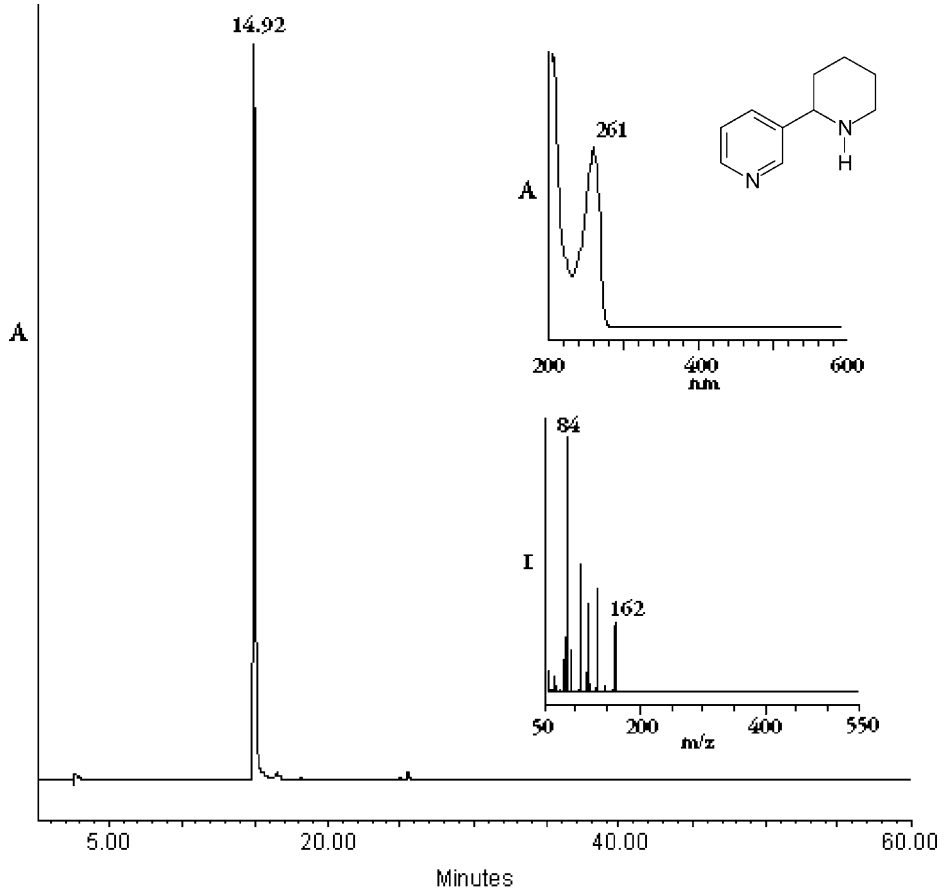

Fig. 2. Chromatogram of Anabasine (1000 μg/ml) at R_t = 14.92 min as detected on the PDA detector. Inserted are the UV (λ_{max} = 261 nm) and TMD (BP = 84 *m/z*) spectra.

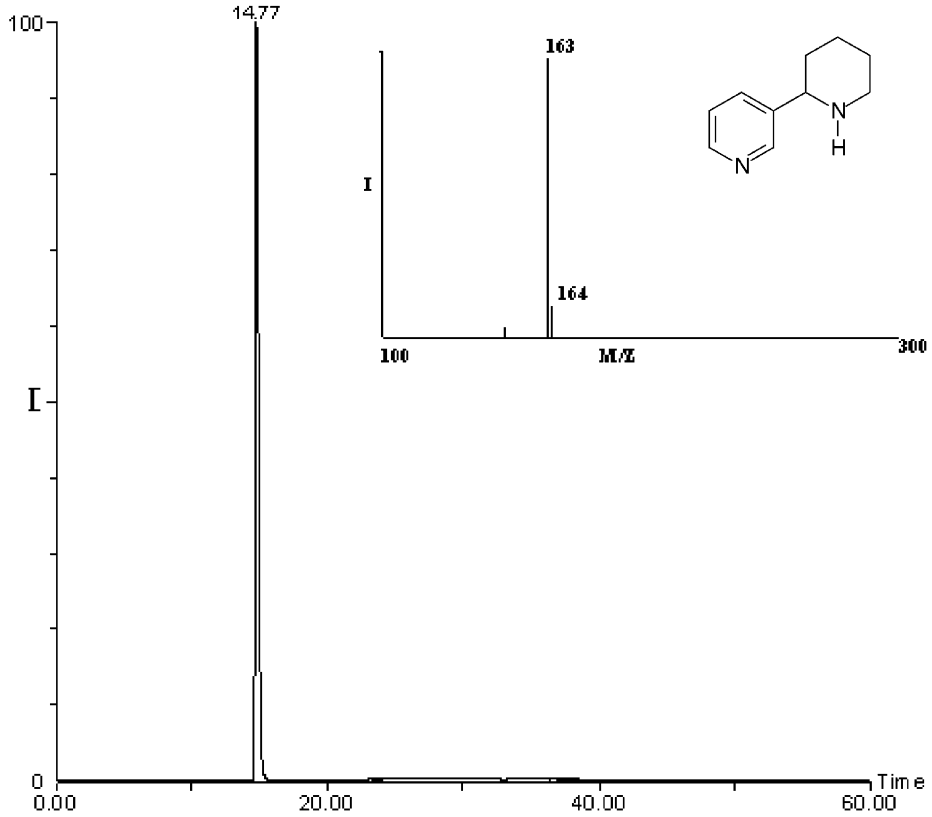

Fig. 3. Chromatogram of anabasine (10 μg/ml) at R_t = 14.77 min as detected on the ZMD detector. Inserted is the positive electrospray spectrum of anabasine (BP = 163 *m/z*).

Table 2
Anabasine contents of vicera and plant samples

Sample (n = 3)	R_t (min) (Anabasine) PDA/TMD	R_t (min) (Anabasine) PDA/ZMD	Percentage match TMD NIST library	Anabasine concentration PDA detector (μg/ml) (μg/g wet weight) (μg/g dry weight)	Anabasine concentration ZMD detector (μg/ml) (μg/g wet weight) (μg/g dry weight)
Stomach—mother	15.20 ± 0.49	14.67 ± 0.06	NM[a]	17.4 ± 0.5 [2.3]	19.1 ± 0.6 [2.6]
Stomach—son	15.11 ± 0.52	14.87 ± 0.08	NM[a]	5.1 ± 0.4 [0.8]	9.3 ± 0.3 [1.0]
Flower exhibit	15.02 ± 0.31	14.76 ± 0.05	85 ± 2	1840 ± 60	1883 ± 95
Witfield tree					
Leaves	14.95 ± 0.11	14.73 ± 0.09	91 ± 1	1991 ± 75	NQ
Flowers	14.99 ± 0.09	14.72 ± 0.08	86 ± 2	1467 ± 58	(Not quantified)
Germiston tree					
Leaves	15.05 ± 0.16	15.62 ± 0.14	83 ± 6	1851 ± 82	NQ
Flowers	15.01 ± 0.14	15.68 ± 0.11	79 ± 7	1408 ± 67	NQ
Rau tree					
Leaves	14.98 ± 0.08	14.71 ± 0.08	90 ± 1	1924 ± 71	NQ
Flowers	15.11 ± 0.11	14.74 ± 0.06	89 ± 2	1517 ± 51	NQ

[a] NM: no match.

In forensic chemical analysis, the forensic analyst is faced with a daunting task of not only detecting and identifying all chemical components in submitted samples and case exhibits, but also to confirm the results with an alternative analytical technique to ensure that no co-elution occurred or that a false positive result was obtained in the first analysis. Although not specified by law, this is an internationally accepted norm, and the use of three-dimensional detectors (PDA and MS) would be a first choice. This prompted our laboratory to develop alternative analytical techniques to those published and/or already in use by the laboratory.

The analysis of anabasine by gas chromatography (GC) or gas chromatography–mass spectrometry (GC–MS) is well established [11–13]. However, the determination of anabasine/nicotine by high performance liquid chromatography (HPLC) has received limited attention. Published HPLC methods for nicotine and related alkaloids utilizing UV detection [15–17] were based on chromatographic conditions that are now known to be less than optimal for basic analytes on reversed phases [18,19] (the use of mobile phases at or near a neutral pH), were time-consuming and involved elaborate sample preparation techniques. Although, reversed phase ion-pairing liquid chromatography has been established as a viable alternative for the analysis of basic analytes on silica-based reversed phase stationary phases [15,19], it is not suitable for interfacing with a mass spectrometer due to the involatile nature of the mobile phase. In a recent review on human poisoning by plant material [20], the authors have developed a general method for the detection of various toxins in whole blood after liquid–liquid extraction, followed by HPLC-(tandem) mass spectrometry. However, the method has not been applied for the analyze of *N. glauca.*

We now want to report an efficient method for the identification of anabasine by HPLC–PDA–MS.

2. Material studied, methods, techniques

2.1. Standards and reagents

(±)-Anabasine (90%), potassium chloride, sodium chloride, potassium dihydrogen phosphate and sodium monohydrogen phosphate (>99.5%) were obtained from Sigma. Ammonium acetate (98%) was obtained from BDH Laboratory Supplies, UK. Sulphuric acid (Suprapur 96%) and ammonia (GR 25%) Pro Analisi was obtained from E-Merck, Darmstadt, Germany. Acetonitrile (gradient quality 190 nm UV cutoff) and methanol (gradient quality 205 nm UV cutoff) were obtained from Romil, UK. HPLC grade water (20 MΩ) was obtained from a Milli-Q/reversed osmosis system (Millipore, USA). Oasis HLB solid phase extraction (SPE) cartridges (60 mg) were obtained from Waters Corporation, Milford, USA. The phosphate-buffered saline solution (PBS) was prepared according to the prescribed method supplied by Waters, Milford, USA [21].

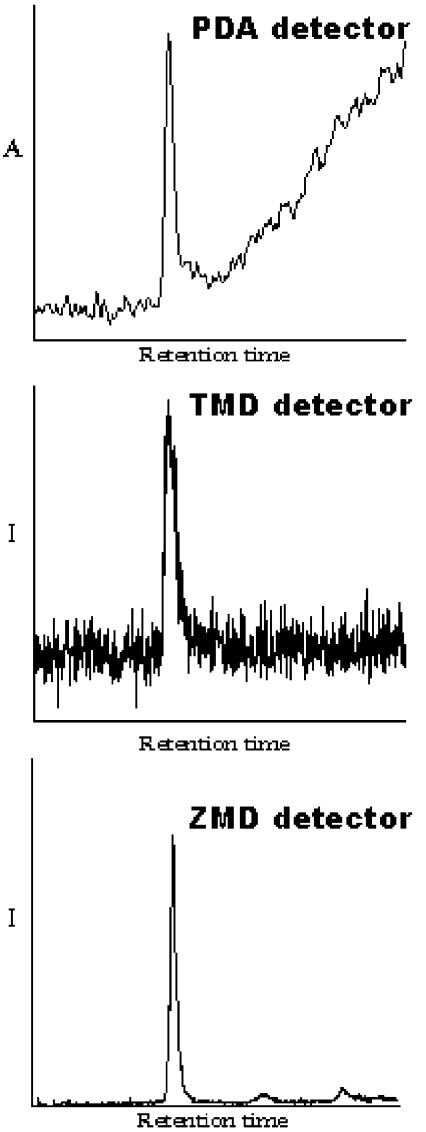

Fig. 4. Chromatograms of the three different detectors depicting the individual LODs for anabasine: PDA detector (250 ng/ml), TMD detector (5 μg/ml) and ZMD detector (10 ng/ml).

2.2. Instruments and conditions

A Waters 2690 HPLC system equipped with a both a 996 photodiode array (PDA) detector and a termabeam mass selective detector (TMD) (electron impact (EI) mode; maximum mass 1000 *m/z*) from Waters was used for initial screening and high concentration studies. A Waters 2690 HPLC system equipped with a 996 PDA detector and a Z spray mass selective detector (ZMD) (Micromass, UK) (electrospray mode; maximum mass 2000 *m/z*) was used for low concentration studies. A Waters Xterra RP C18 HPLC column (150 mm × 2 mm, 5 μm) was used for all experiments. A

Harvard syringe pump (Model 11) was obtained from Harvard Apparatus, Holliston, USA and was used for all direct injection experiments on the ZMD detector.

To ensure the maximum retention of basic alkaloids, the initial chromatographic conditions were 100% water containing 10 mM ammonium acetate, the pH adjusted to 9.5 with ammonia (25%). After injection, the chromatographic conditions were gradually changed through a linear gradient profile to 90% acetonitrile and 10% of the original aqueous mobile phase in 40 min. These conditions were kept stable for 5 min where after the HPLC system re-equilibrated the analytical column to the initial conditions. The flow rate was kept constant at 0.2 ml/min.

The nebulizer of the TMD detector was optimized with a caffeine test solution (100 μg/ml) to ensure efficient nebulization and droplet formation. The electrospray ionization (ESI) probe of the ZMD detector was optimized by direct injection via a syringe pump using an anabasine test solution (10 μg/ml).

2.3. Chemical fingerprinting of N. glauca trees

To investigate the alkaloid variation in *N. glauca*, materials from three isolated trees were collected in Gauteng Province (South Africa). The first tree was found near the Rand Afrikaans University in Hursthill, Johannesburg (called RAU plant); the second tree in an industrial site in

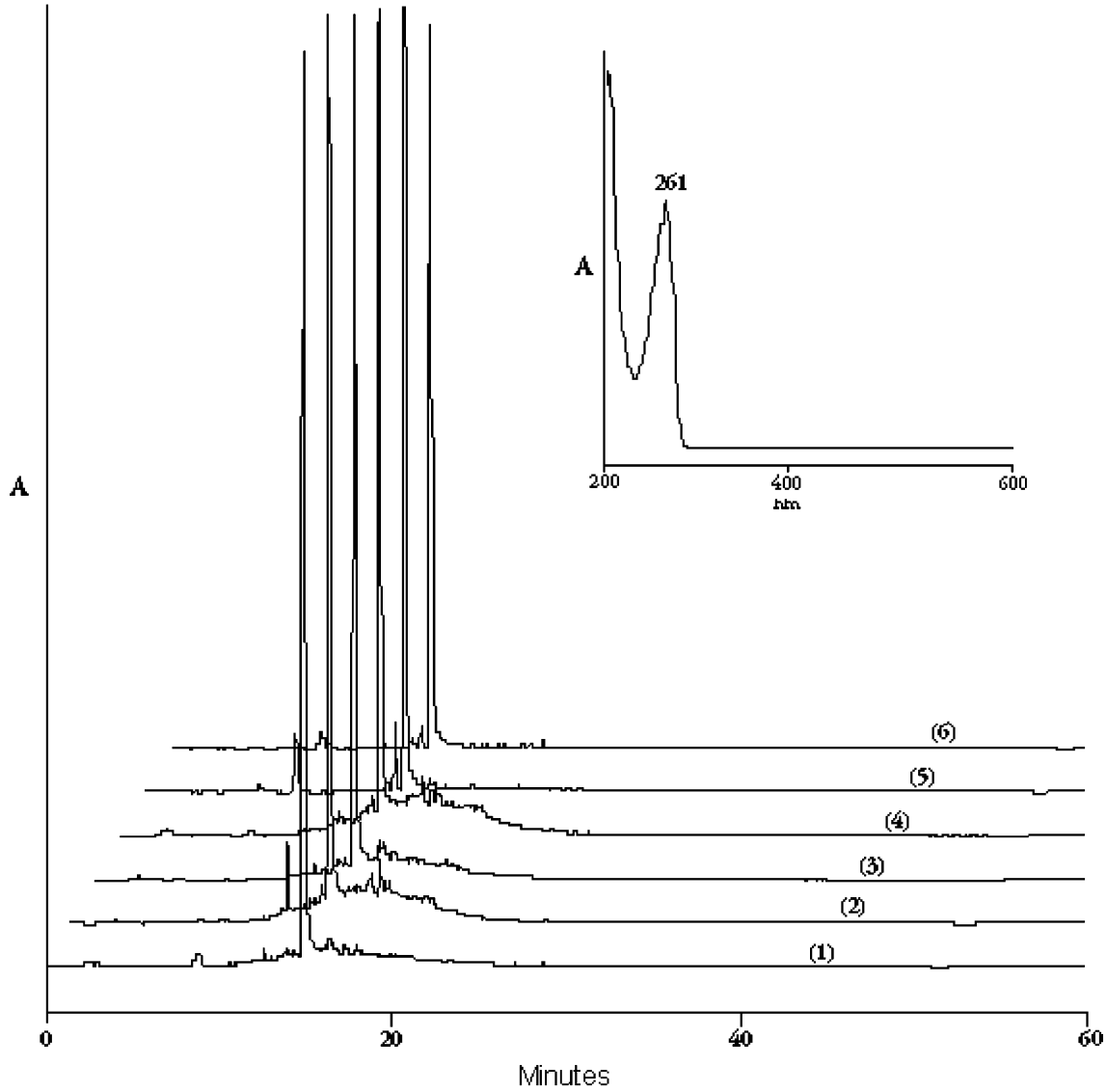

Fig. 5. Comparison of the flower and leave extracts of three *N. glauca* trees. (1) Flowers Witfield; (2) leaves Witfield; (3) flowers RAU; (4) leaves RAU; (5) flowers germiston; (6) leaves germiston. Inserted is the UV spectrum of the main component.

Meadowbrook Extension 8, Germiston (called Germiston plant), and the third tree in an industrial area in Witfield, Boksburg (called Witfield plant). Leaves and flowers were collected from each tree and dried separately at 40 °C to constant weight. The leaves and flowers were finely ground using an Ika Werk A10 water-cooled mill (Janke & Kunkel, Straufen, Germany) and 0.5 g of each specimen was mixed with 25 ml 0.02 M sulphuric acid. The acidified plant material was extracted at 40 °C for 30 min using a shaking water bath. The plant material was filtered off and re-extracted with 25 ml 0.02 M sulphuric acid. The aqueous extracts were combined and the pH adjusted to approximately 9.5 with ammonia solution (25%). The aqueous extract of each plant was passed through an Oasis HLB SPE cartridge and the adsorbed material stripped from the solid phase material with 1.5 ml methanol. The methanol extracts were injected into each HPLC system (2 μl into TMD; 1 μl into ZMD) using the optimized HPLC method described above.

2.4. Chemical extraction and analysis of viscera and case exhibit

As the two fatalities under investigation were submitted to our Laboratory as a possible food poisoning case, a general extraction procedure was followed. An aliquot of liquefied stomach and contents (3 g) was mixed with 20 ml of PBS solution and sonicated for 30 min. After filtration through filter paper (Whatman no. 41) the aqueous phase was passed through an Oasis HLB cartridge and the adsorbed compounds stripped from the solid phase material with 0.4 ml methanol. The methanol solution was injected (10 μl) directly into each HPLC system and analyzed using the optimized HPLC method described.

A part of a flower exhibit (3 g) that was submitted with the case, was treated as described for the viscera samples. The methanol solution, obtained by stripping the bound compounds from the Oasis cartridge, was injected (1 μl) directly into each HPLC system and analyzed using the optimized HPLC method described above.

3. Results and discussion

3.1. Chromatography and detection of anabasine

Anabasine is a diprotic base with a pK_{a2} of 3.21 [8]. At a neutral pH, more than 75% of the anabasine will be in a protonated state, resulting in strong interaction with residual

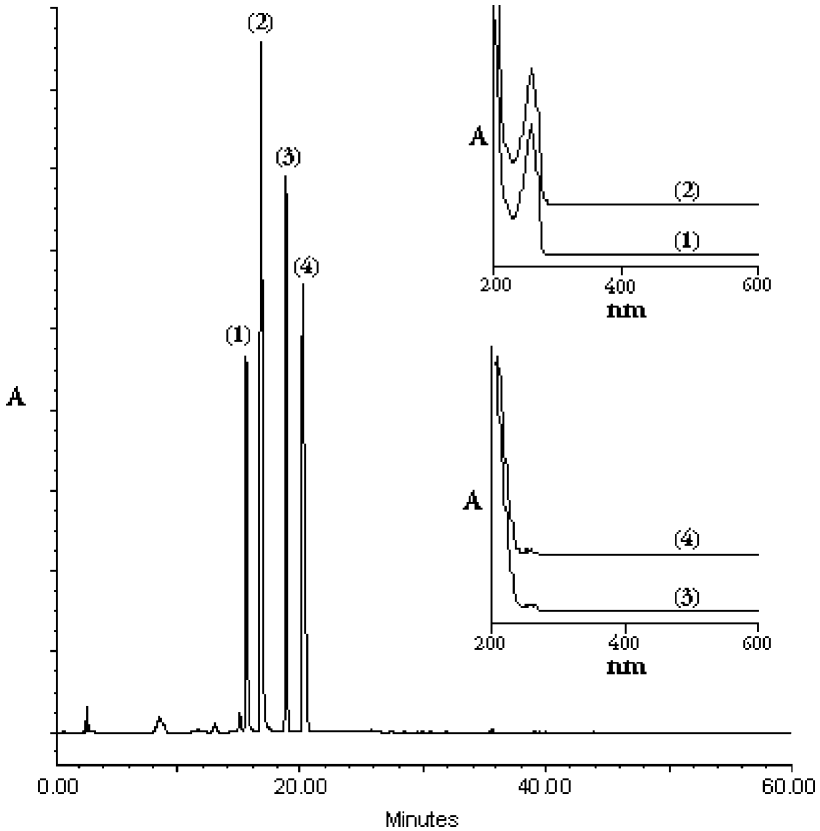

Fig. 6. Chromatogram of an alkaloid mixture containing anabasine (1), nicotine (2), scopolamine (3) and atropine (4) as observed on the PDA detector. Inserted are the UV spectra of the above mentioned alkaloids.

silanol groups that will result in poor peak shape and severe peak tailing. The chromatography can be improved by lowering the pH to 2, but this again will lead to protonation of anabasine, and various other basic alkaloids, thereby becoming unretained on the HPLC column. Alternatively, a base deactivated reversed phase column can be used with the addition of triethylamine (TEA) to the mobile phase [22], but this will lead to unnecessary complications when interfacing the HPLC system to a mass selective detector, especially the ZMD detector. Ion-pairing reagents will also improve the peak shape, reduce the tailing and increase the retention of anabasine and other basic alkaloids [15], but commonly used ion-pairing reagents, such as hexanesulfonic acid or octanesulfonic acid will render any interfaced mass selective detector inoperative due to involatile nature of the eluent components. Ammonium acetate displays ion suppresing characteristics and is also volatile, and can therefore be included in an eluent to be introduced into a mass spectrometer.

A concentration of 10 mM ammonium acetate, adjusted to a pH of 9.5 with ammonia, was found to be sufficient to affect reproducible retention times -14.93 ± 0.03 (TMD system), 14.72 ± 0.03 (ZMD system), but low enough not to interfere with ion formation during nebulization into the mass spectrometers. A typical chromatogram of an anabasine standard (1000 μg/ml) on the TMD HPLC system (Fig. 2) shows anabasine with a retention time of 14.92 min and characteristic UV and mass spectra. Likewise, a typical single ion reaction (SIR) chromatogram of an anabasine standard (10 μg/ml) on the ZMD HPLC system (ESI+ mode, Fig. 3) shows anabasine with a retention time of 14.77 min resulting in an ESI+ ''mass spectrum''. The mass ion observed at 163 *m/z* relates to the $[M+H]^+$ species that normally form under these conditions.

An array of analytical standards was used to calibrate the mass spectrometer and all analytical work was done within the calibration range. On the TMD HPLC system the calibration curve was prepared covering the 100–1000 μg/ml concentration range. A linear curve-fitting algorithm that produced a 0.997 coefficient of determination ($r = 0.998$; $r^2 = 0.997$) was applied. A second calibration curve, covering the 500 ng/ml–10 μg/ml concentration range, produced identical results to the calibration curve at the higher concentration range. On the ZMD HPLC system a calibration curve was prepared covering the 100–10,000 ng/ml concentration range. The coefficient of determination was 0.9997 and was obtained using a second order curve-fitting algorithm ($r = 0.9997$; $r^2 = 0.9994$).

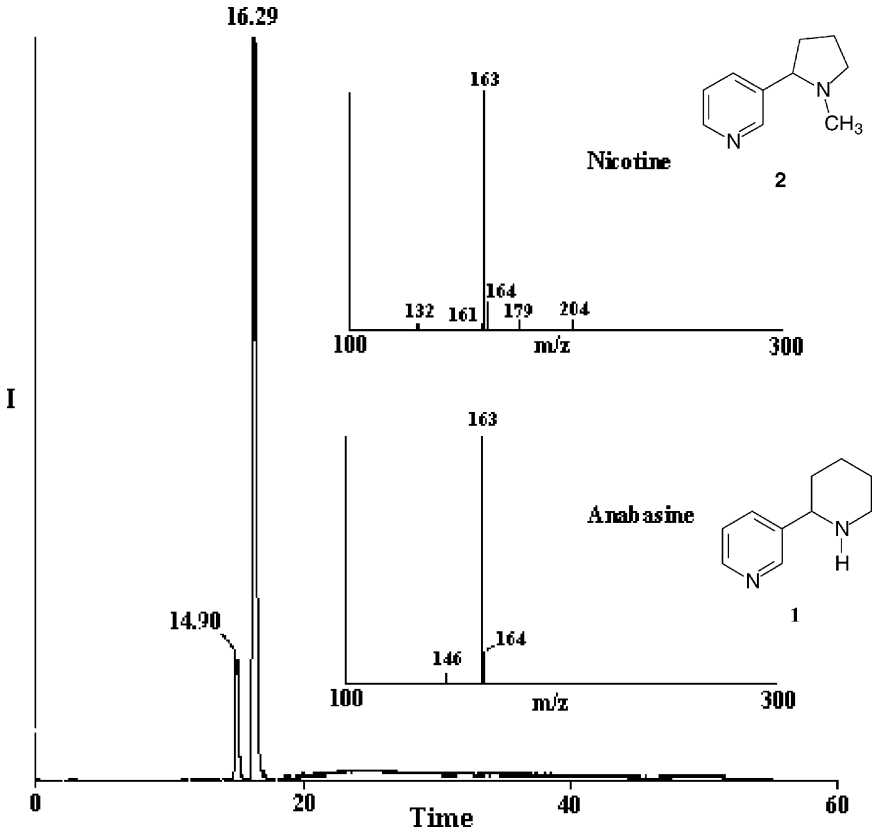

Fig. 7. Chromatogram of the separation of anabasine (**1**) ($R_t = 14.90$) and nicotine (**2**) ($R_t = 16.29$) on the ZMD HPLC system. Inserted the characteristic ESI+ spectra obtained for both compounds under conditions optimized for anabasine.

The limit of detection (LOD) and the limit of quantitation (LOQ) for the three detectors used are summarized in Table 1. The LOD was determined experimentally—the LOD was taken as the concentration that produced a detector signal that could be clearly distinguished from the baseline noise (±3 times baseline noise, Fig. 4) and the LOQ was taken as the concentration that produced a detector signal ±10 times greater than the LOD signal.

3.2. Chemical fingerprinting of N. glauca trees

The extraction procedure used was developed to extract alkaloids selectively from plant material. The chromatograms obtained from the flower and leaf extracts of each plant (Fig. 5) clearly show that anabasine is the main alkaloid extracted from the flowers and leaves of each plant. Some authors [23–25] have reported the presence of nicotine (**2**) in *N. glauca*, but we could not detect nicotine in our samples. It has also been reported in literature [24] that the leaves only contain anabasine, but we found that both leaves and flowers contained anabasine, although the alkaloid concentration were higher in leaves that in flowers (Table 2). The three trees we studied were sufficiently uniform (both qualitatively and quantitatively) in their alkaloid contents to allow a reliable characterization of the species (Fig. 5).

To ensure that nicotine was not overlooked in the extracts analyzed, or that anabasine and nicotine co-eluded, an alkaloid cocktail containing of analytical samples of anabasine, nicotine, scopolamine and atropine was injected into the TMD HPLC system. From the chromatogram obtained (Fig. 6) it was clear that anabasine ($R_t = 15.52$) was well resolved from nicotine ($R_t = 16.72$), scopolamine ($R_t = 18.89$) and atropine ($R_t = 20.25$). A 10 μg/ml nicotine standard, spiked with ±1 μg/ml anabasine, was injected into the ZMD HPLC system. From the chromatogram (Fig. 7) it was again obvious that anabasine ($R_t = 14.98$) was well resolved from nicotine ($R_t = 16.34$). The optimized electrospray conditions for anabasine also produced distinctively different fragmentation/adduct spectra for anabasine and nicotine. To confirm that nicotine, if present, would have been detected, a nicotine sample was subjected to the extraction procedure as described.

3.3. Evaluation of SPE extraction and analysis of viscera and exhibit

The PDA/TMD chromatogram obtained from the 10 μl injections of each stomach homogenate extraction, as well as the 1 μl injection of the extract of the flower exhibit is depicted in Fig. 8. Small amounts of anabasine were detected in both viscera samples and a large amount of anabasine was detected in the flower exhibit (Table 2). The exhibit also displayed an alkaloid profile similar to those obtained from the flowers of the Witfield, RAU and Germiston plants (a major anabasine peak with trace levels of other components).

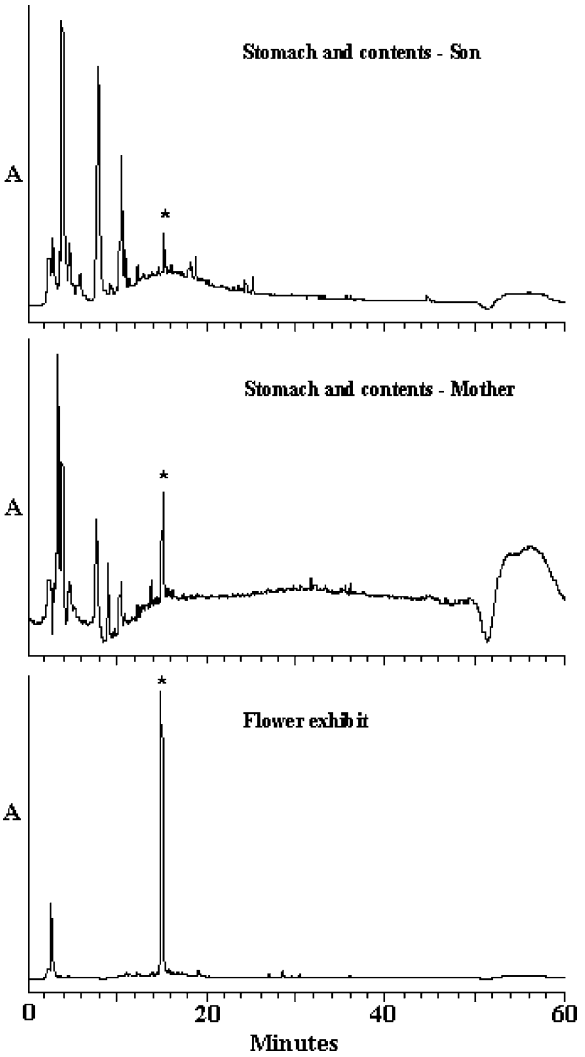

Fig. 8. PDA/TMD chromatograms obtained by injecting 10 μl of the extract of stomach and contents of the son (1) and mother (2). Chromatogram (3) is a 1 μl injection of an extract of the flower case exhibit. The presence of anabasine is indicated (∗).

Fig. 9 is the PDA/ZMD chromatogram obtained from the 10 μl injections of each stomach homogenate extraction and the 1 μl injection of the flower exhibit extract. The results obtained correlated very well with those from the PDA/TMD HPLC system as anabasine was detected in both viscera samples and the flower exhibit. The characteristic ESI+ spectrum of anabasine was observed in all samples analyzed.

Although the anabasine concentration in the stomach (and contents) of both the mother and son appeared to be too low for a fatal poisoning [8], it must be taken into consideration that both the mother and son vomited extensively before they died from a neuromuscular blockade resulting in respiratory suppression.

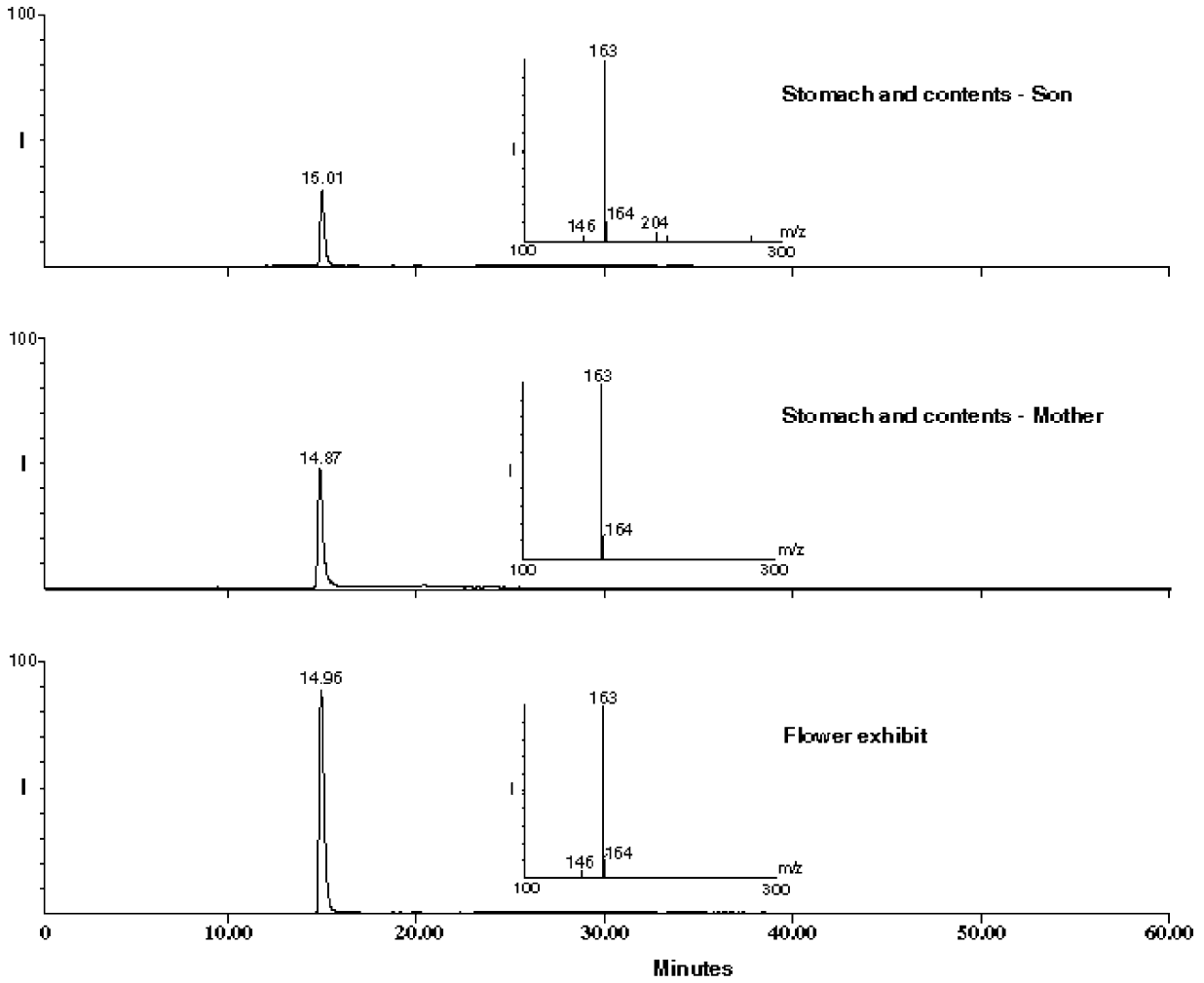

Fig. 9. PDA/ZMD chromatograms obtained by injecting 10 μl stomach and contents of the son (1) and mother (2). Chromatogram (3) is a 1 μl injection of an extract of the flower case exhibit. Inserted the observed ESI+ spectrum of each main component.

It is clear that the coupling of a HPLC–PDA system to a mass selective detector is a viable alternative to GC–MS for the detection, identification and quantification of anabasine in biological samples in a single analysis run. As we have demonstrated, the method is suitable for forensic application.

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