Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 838 (2006) 63–70
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Application of liquid chromatography–mass spectrometry to the investigation of poisoning by *Oenanthe crocata*

Geoffrey C. Kite [a,*], Charlotte A. Stoneham [a], Nigel C. Veitch [a], Bridget K. Stein [b], Katherine E. Whitwell [c]

[a] *Royal Botanic Gardens Kew, Richmond, Surrey TW9 3AB, UK*
[b] *EPSRC National Mass Spectrometry Service Centre, University of Wales Swansea, Swansea SA2 8PP, UK*
[c] *Pathology Section, Animal Health Trust, Lanwades Park, Kennett, Newmarket, Suffolk CB8 7UU, UK*

Received 24 February 2006; accepted 28 April 2006
Available online 26 May 2006

Abstract

Liquid chromatography–mass spectrometry (LC–MS) analysis of methanol extracts of *Oenanthe crocata* roots revealed that oenanthotoxin co-eluted with another major polyalkyne, 2,3-dihydro-oenanthotoxin, using the existing high performance liquid chromatography (HPLC) method (isocratic elution from C18 with aqueous methanol) for investigating *Oenanthe* poisoning. Positive ES or APCI gave $[(M+H)–H_2O]^+$ and its methanol adduct as major ion species for oenanthotoxin, whereas 2,3-dihydro-oenanthotoxin formed $[M+H]^+$ and its methanol adduct. The two polyalkynes could be chromatographically resolved on C18 by gradient elution with aqueous acetonitrile. This provides superior analysis for oenanthotoxin using HPLC with photodiode array (PDA) detection alone, but for LC–MS/MS aqueous acetonitrile was less suitable due to poor ionisation and, with APCI, an increase in the relative abundance of a $[M-1]^+$ species, which could confuse compound assignment. HPLC–PDA and LC–MS/MS methods using an aqueous acetonitrile or aqueous methanol mobile phase, respectively, were successful when applied to the analysis of the stomach contents of a pony suspected to have eaten *O. crocata*. Relevant product ion spectra, by ion trap MS/MS, accurate mass data and complete sets of ^1H and ^{13}C NMR spectral assignments are given for the two compounds.

Keywords: *Oenanthe crocata*; Oenanthotoxin; Poisoning; Liquid chromatography–mass spectrometry

1. Introduction

Oenanthe crocata L. (Apiaceae) is one of the most poisonous plants native to the British Isles [1,2]. Commonly known as hemlock water dropwort, the species is locally abundant in western and southern Britain where it usually grows in wet areas such as along the banks of rivers and canals, in drainage ditches or in wet meadows. The most poisonous parts of the plant are the tuberous roots, which resemble a bunch of small parsnips. Their swollen spindle-like form also gives rise to the plant's alternative common name of dead men's fingers. It is the misidentification of *O. crocata* as an edible species of Apiaceae, such as wild parsnip (*Pastinaca sativa* L.), that is responsible for most cases of poisoning in humans [3,4,5]. In livestock, poisoning is most likely to occur when the roots are exposed during land drainage or ditch clearance work [6].

The toxic principle of *O. crocata* was crystallized by Clarke et al. [7], and later Anet et al. [8] structurally characterised it as the C_{17} polyalkyne oenanthotoxin (**2**, Fig. 1), having isolated the compound from roots harvested in autumn. Roots harvested in spring yielded little oenanthotoxin; instead oenanthetol (the 14-deoxy derivative of oenanthotoxin) and oenanthetone (the 14-ketone of oenanthotoxin) were obtained, with the former compound being found to be less toxic than oenanthotoxin [8]. Subsequently, Bohlmann and Rode [9] isolated over 30 related C_{15} or C_{17} polyalkynes from roots of *O. crocata*, with oenanthotoxin and 2,3-dihydro-oenanthotoxin (**1**, Fig. 1) being obtained in greatest yields.

Oenanthotoxin has an intense and highly characteristic UV absorption spectrum which can be observed even by direct UV spectrophotometry of crude methanol or ether extracts of

* Corresponding author. Tel.: +44 208 332 5368; fax: +44 208 332 5310.
E-mail address: g.kite@kew.org (G.C. Kite).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.04.047

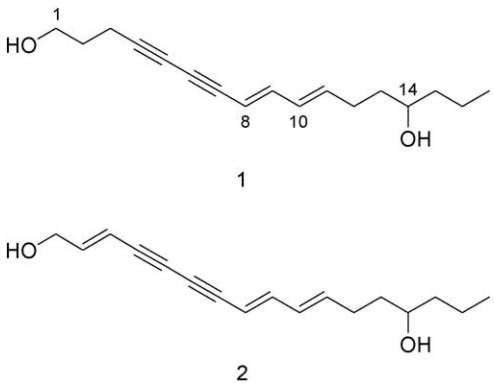


Fig. 1. Structures of 2,3-dihydro-oenanthotoxin (**1**) and oenanthotoxin (**2**).

O. crocata roots [3,10]. Methanolic extracts of *O. crocata* have also been analysed by high performance liquid chromatography (HPLC) with UV detection at 313 nm and the major chromatographic peak observed has been assigned to oenanthotoxin by comparison with an authentic standard [10]. In cases of poisoning in humans where consumption of *O. crocata* was suspected, both HPLC and UV spectrophotometry have been used to detect oenanthotoxin, either from the analysis of residual plant material recovered from the incident or the victim's stomach contents [10]. The potential of fluorimetric determination of oenanthotoxin has also been assessed [11].

We were recently asked to investigate the sudden death of a pony that was associated with the consumption of roots of *O. crocata*. Cases of horse poisoning by *O. crocata* are rare since the plant is readily removed from pastures [12]. Nevertheless, this case presented an opportunity to investigate the application of liquid chromatography–mass spectrometry (LC–MS) to the detection of oenanthotoxin. Because of the potential selectivity of this technique, it may be useful for detecting the toxin in relatively complex matrices, such as extracts from stomach contents, in which the characteristic UV absorbance spectrum of oenanthotoxin could be masked. Transferring the established HPLC separation directly to LC–MS resulted in some initial confusion in mass spectral interpretation. This mainly resulted from the co-elution of oenanthotoxin with another major polyalkyne, making the existing method unsatisfactory for quantitative analysis by HPLC–photodiode array (PDA) detection. Here we report on the analytical challenges and develop alternative chromatographic separations to facilitate the use of LC–MS or HPLC–PDA in the investigation of *O. crocata* poisoning.

2. Experimental

2.1. Chemicals

Methanol, acetonitrile, acetic acid and formic acid (all HPLC grade) were obtained from Fisher Scientific UK (Loughborough, UK). Deionised water (Millipore 'Milli-Q', Billerica, MA, USA) was produced in-house.

2.2. Sample material and preparation

Roots of *O. crocata* were harvested from plants growing wild at Slepe Farm, Poole, Dorset, UK, on 12 June 2005 and stored at −20 °C. Stomach contents of a pony that had died following suspected consumption of *O. crocata* roots were removed during the post mortem and also stored at −20 °C; a sample of roots gathered from the field in which the pony had been grazing was also supplied. For LC–MS analysis, 2 g of frozen roots were ground in a pestle and mortar in methanol and left to extract in 10 ml methanol for 2 h at 4 °C in the dark. A random 64 g sample of frozen stomach contents was allowed to thaw in 200 ml of methanol for 2 h in the dark. After removing the solid residue by filtration, the solutions were dried by rotary evaporation and the extracted material was dissolved in methanol (1 ml per 1 g fresh weight material extracted), with dilutions being made as necessary. Prior to the preparation of the extract of the stomach contents, 200 ml of methanol was processed in the same manner through the equipment to provide a control blank. Re-extraction of the root residue with methanol for 24 h recovered a further 10% of the oenanthotoxin and was deemed unnecessary in circumstances where confirmation of the presence of oenanthotoxin is required rapidly. A sample of mixed pasture was gathered at random from a field near Maidenhead, Berkshire, UK, in which horses were grazing, and 10 g fresh weight was extracted (in 50 ml methanol) and processed as above. The same method of sample preparation was used to prepare an extract of *O. crocata* roots for semi-preparative HPLC, with 19 g of root being initially extracted in 80 ml methanol and the extracted material being finally dissolved in 6 ml methanol.

2.3. LC–MS

Samples were analysed using two LC–MS systems. System 1 consisted of a Thermo-Finnigan Surveyor autosampler, pumps and PDA detector and a Thermo-Finnigan LCQ 'Classic' ion-trap mass spectrometer (San Jose, CA, USA). Chromatography was performed at 30 °C on a Luna C18(2) column (150 mm × 4.6 mm i.d., 5 μm) protected by a Security Guard C18 cartridge (4 mm × 3 mm i.d., Phenomenex UK, Macclesfield, UK). The mobile phase was either isocratic methanol/water (3:1) [10], or gradients of either aqueous methanol or aqueous acetonitrile with or without 1% acetic acid. In all cases the flow rate was 1 ml/min and the gradients passed linearly from 90% aqueous (acetonitrile gradient) or 80% aqueous (methanol gradient) to 0% aqueous in 20 min followed by 5 min isocratic elution in organic solvent and return to initial conditions. The column was equilibrated in the initial solvent mixture for 7 min before injecting the sample; the injection volume was 10 μl. The PDA was set to a wavelength range of 210–420 nm at 1 nm resolution. The ion-trap mass spectrometer was fitted with either a dedicated electrospray (ES) or atmospheric chemical ionisation (APCI) source operated under the following conditions. ES; source voltage 4.5 kV, heated capillary temperature 220 °C: APCI; vaporiser temperature 450 °C, needle current 5 μA, heated capillary temperature 150 °C. For both sources the sheath and auxiliary nitrogen gas pressures were 80

and 20 psi, respectively, and the tube lens offset voltages were 10 V in positive mode and −20 V in negative mode. The solvent flow into the APCI source was the full 1 ml/min from the HPLC while the flow to the ES source was reduced to 200 μl/min by a splitter. Sources were operated in positive and negative modes in separate analyses and the ion-trap analyser was set to survey ions in the range *m/z* 125–1000 and subject the most abundant ions to MS/MS analysis using an ion isolation window of 3 Th and a scaled collision energy of 45%.

System 2 consisted of a Waters Alliance 2695 autosampler and pumps, a Waters 2996 PDA detector and a Waters Micromass ZQ 4000 single quadrupole mass spectrometer (Milford, MA, USA). Chromatography was performed at 30 °C on a Zorbax SB-C18 column (150 mm × 3 mm i.d., 5 μm, Agilent Technologies, Palo Alto, CA, USA) protected by a Security Guard C18 cartridge. Mobile phase gradients were as described above except that the flow rate was 0.5 ml/min and 1% formic acid was used in acidified mobile phase gradients. The PDA settings were also as described for System 1. The mass spectrometer was fitted with a Waters Micromass 'ESCi' multi-mode ionisation source capable of ionisation in ES and APCI modes in both positive and negative polarities in a single analysis. The ESCi conditions for both positive and negative polarities were as follows: ES capillary voltage ± 4 kV, APCI needle current 5 μA, cone voltage (both modes) ±20 V, source temperature 120 °C, desolvation temperature 450 °C, desolvation nitrogen flow 500 l/h, cone nitrogen flow 50 l/h. For each of the four modes, the mass range was *m/z* 125–1000 scanned over 0.4 s with an interscan delay of 0.2 s, giving a duty cycle of 2.4 s.

2.4. Semi-preparative HPLC

Standards of **1** and **2** were obtained by semi-preparative HPLC of a methanol extract of *O. crocata* roots. The HPLC system consisted of Waters LC 600 pumps and 996 PDA detector (Milford, MA, USA) fitted with a LiChrosphere 100RP-18 column (250 mm × 10 mm i.d., 5 μm, Merck, Darmstadt, Germany). The mobile phase was 4.5 ml/min of 50% aqueous acetonitrile and fractions were collected manually after each 100 μl injection of the extract. This process was repeated until milligram quantities of the compounds of interest were obtained for analysis by NMR.

2.5. Probe EI–MS and CI–MS

Characterisation spectra were obtained on isolated fractions using electron ionisation (EI) and ammonia chemical ionisation (CI) in positive ionisation mode. A Micromass Quattro II triple quadrupole low-resolution mass spectrometer was used with a desorption probe for sample insertion. Samples were applied to the probe after dissolution in dichloromethane and dilution into methanol, and dried by evaporation before insertion into the spectrometer. Probe heating rate: 0 mA for 60 s, then 500 mA for 30 s, then 1500 mA for 120 s. MS conditions: EI source temperature 200 °C, electron energy 70 eV; CI source temperature 170 °C, electron energy 50 eV.

2.6. Accurate mass measurement

Accurate mass measurements were performed on a Finnigan MAT900 mass spectrometer in positive ES ionisation mode. Samples were loop injected into a stream of water/methanol (1:1) at a flow rate of 50 μl per minute and ionised with the assistance of nitrogen as sheath gas. Accurate mass measurements were performed on single molecular species by computer-assisted "peak matching" at resolution 8000 (10% valley definition). The reference compound used was polyethyleneimine. Mass spectrometer conditions: accelerating voltage 5 kV, capillary temperature 230 °C, spray voltage +3.0 kV.

2.7. Nuclear magnetic resonance spectroscopy

NMR data were acquired in $CDCl_3$ at 30 °C on a Bruker Avance 400 MHz instrument (Rheinstetten, Germany). Standard pulse sequences and parameters were used to obtain 1D ^{1}H, 1D ^{13}C, COSY, HSQC, and HMBC spectra. Chemical shift values were measured relative to TMS at 0.00 ppm. 2,3-Dihydrooenanthotoxin (**1**): ^{1}H NMR ($CDCl_3$) δ 6.66 (1H, dd, J = 15.5, 10.7 Hz, H-9), 6.12 (1H, dd, J = 15.1, 10.9 Hz, H-10), 5.86 (1H, m, H-11), 5.50 (1H, d, J = 15.5 Hz, H-8), 3.76 (2H, t, J = 6.1 Hz, CH_2-1), 3.61 (1H, m, H-14), 2.47 (2H, t, J = 6.9 Hz, CH_2-3), 2.25 (2H, m, CH_2-12), 1.80 (2H, m, CH_2-2), 1.53 (2H, m, CH_2-13), 1.43 (2H, m, CH_2-15), 1.39 (2H, m, CH_2-16), 0.93 (3H, t, J = 6.6 Hz, CH_3-17); ^{13}C NMR ($CDCl_3$) δ 144.8 (C-9), 138.8 (C-11), 129.9 (C-10), 107.9 (C-8), 84.2 (C-4), 76.0 (C-6), 75.0 (C-7), 71.1 (C-14), 65.9 (C-5), 61.5 (C-1), 39.8 (C-15), 36.5 (C-13), 31.0 (C-2), 29.1 (C-12), 18.8 (C-16), 16.2 (C-3), 14.1 (C-17). Oenanthotoxin (**2**): ^{1}H NMR ($CDCl_3$) δ 6.69 (1H, dd, J = 15.5, 10.8 Hz, H-9), 6.39 (1H, dt, J = 15.8, 5.0 Hz, H-2), 6.14 (1H, dd, J = 15.2, 10.9 Hz, H-10), 5.90 (1H, dd, J = 15.1, 7.1 Hz, H-11), 5.87 (1H, dm, J = 15.9 Hz, H-3), 5.56 (1H, d, J = 15.6 Hz, H-8), 4.25 (2H, dd, J = 5.0, 2.0 Hz, CH_2-1), 3.61 (1H, m, H-14), 2.25 (2H, m, CH_2-12), 1.55 (2H, m, CH_2-13), 1.44 (2H, m, CH_2-15), 1.39 (2H, m, CH_2-16), 0.93 (3H, t, J = 6.5 Hz, CH_3-17); ^{13}C NMR ($CDCl_3$) δ 145.2 (C-9), 144.9 (C-2), 139.4 (C-11), 129.9 (C-10), 109.3 (C-3), 107.7 (C-8), 81.4 (C-7), 80.2 (C-4), 75.7 (C-6), 74.9 (C-5), 71.1 (C-14), 62.8 (C-1), 39.8 (C-15), 36.5 (C-13), 29.1 (C-12), 18.8 (C-16), 14.1 (C-17).

3. Results and discussion

3.1. LC–MS analysis of O. crocata

Analysis of a methanol extract of fresh roots of *O. crocata* by the HPLC method described by King et al. [10] using a methanol/water (3:1) mobile phase on a C18 column produced a UV absorbance chromatogram very similar to the published analysis. The one major chromatographic peak, eluting at 4.8 min, had a UV spectrum similar to that published for oenanthotoxin [10]. Using ionisation by dedicated APCI in positive mode on LC–MS System 1 (see Section 2.3), this chromatographic peak presented an array of ions with the most abundant at *m/z* 241, 261, 273 and 293. The expected *m/z* value of protonated oenanthotoxin is 259 but an ion at this value was only

Table 1
Relative abundances (determined from peak areas in single ion chromatograms) of ion species generated for 2,3-dihydro-oenanthotoxin (**1**) and oenanthotoxin (**2**) following LC–MS analysis of a methanol extract of *Oenanthe crocata* roots using various mobile phases and mass spectrometers equipped with either dedicated or multimode ion sources

Source	Column mobile phase[a]	Rt **1** (min)	Rt **2** (min)	Relative ion abundance **1** (%)						Relative ion abundance **2** (%)				
Dedicated	Luna C18			*m/z* 261	*m/z* 259	*m/z* 283	*m/z* 293	*m/z* 299	*m/z* 302	*m/z* 241	*m/z* 257	*m/z* 259	*m/z* 273	*m/z* 282
APCI	aq MeOH + 1 % HOAc	17.5	17.5	100[b]	14[c]	–	56	–	–	32	–	14[c]	45	–
APCI	aq MeOH	16.8	16.8	67	14[c]	–	55	–	–	27	–	14[c]	47	–
APCI	aq ACN + 1 % HOAc	13.7	14.0	+	+	–	–	–	+	+	1	+	–	1
APCI	aq ACN	13.3	13.5	3	5	–	–	–	3	3	5	+	–	5
ESI	aq MeOH + 1 % HOAc	17.5	17.5	5	–	–	+	–	–	1	–	–	–	–
ESI	aq ACN + 1 % HOAc	13.7	14.0	3	–	–	–	–	+	1	–	–	–	+
Source	Column mobile phase[a]	Rt **1** (min)	Rt **2** (min)	Relative ion abundance **1** (%)						Relative ion abundance **2** (%)				
Multimode	Zorbax SB-C18			*m/z* 261	*m/z* 259	*m/z* 283	*m/z* 293	*m/z* 299	*m/z* 302	*m/z* 241	*m/z* 257	*m/z* 259	*m/z* 273	*m/z* 282
APCI	aq MeOH + 1 % HCOOH	18.6	18.6	12	+[c]	1	1	–	–	2	–	+[c]	1	–
APCI	aq MeOH	18.6	18.6	8	+[c]	–	–	–	–	2	–	+[c]	1	–
APCI	aq ACN + 1 % HCOOH	14.0	14.2	4	1	–	–	–	+	1	+	+	–	+
APCI	aq ACN	14.0	14.2	4	–	–	–	–	+	1	–	–	–	1
ESI	aq MeOH + 1 % HCOOH	18.6	18.6	86	–	47	–	–	–	20	–	–	9	–
ESI	aq MeOH	18.6	18.6	–	–	15	–	82	–	–	–	–	–	–
ESI	aq ACN + 1 % HCOOH	14.0	14.2	100[d]	+	–	–	–	+	34	–	3	–	26
ESI	aq ACN	14.0	14.2	1	–	+	–	4	–	–	–	–	–	–

Relative abundances are not comparable between instruments (+, present at a relative abundance of 0.1–1%). Ion assignments: 2,3-dihydro-oenanthotoxin (**1**): *m/z* 261 $[M+H]^+$, *m/z* 259 [M-1]$^+$, *m/z* 283 $[M+Na]^+$, *m/z* 293 $[(M+H)+MeOH]^+$, *m/z* 299 $[M+K]^+$, *m/z* 302 $[(M+H)+ACN]^+$. Oenanthotoxin (**2**): *m/z* 241 $[(M+H)–H_2O]^+$, *m/z* 257 [M-1]$^+$, *m/z* 259 $[M+H]^+$, *m/z* 273 $[(M+H)–H_2O+MeOH]^+$, *m/z* 282 $[(M+H)–H_2O+ACN]^+$.

[a] For details of column and mobile phase gradient see Section 2.3.

[b] Corresponds to a maximum ion count of 1.3×10^8 and a signal/noise ratio of 500:1.

[c] Assignment to **1** or **2** not possible due to co-elution.

[d] Corresponds to a maximum ion count of 6.5×10^7 and a signal/noise ratio of 325:1.

present at about 19% of the intensity of the base ion (*m/z* 261) of the oenanthotoxin peak.

Since an ion at *m/z* 293 was difficult to rationalise as an ion species of oenanthotoxin, it was decided to investigate further the chromatographic peak ascribed to oenanthotoxin by chromatography of the extract using aqueous methanol or acetonitrile mobile phases under neutral and acidic conditions and using a single quadrupole MS instrument equipped with a multimode ion source (System 2), in addition to a PDA detector. Using an acidified methanol gradient, the oenanthotoxin peak again generated ions at *m/z* 241, 261, 273, and 293 under positive APCI mode, albeit at much lower abundance than with the dedicated APCI source of System 1 (Table 1). An ion at *m/z* 283 was also formed which showed increased abundance in the positive ES mode. No ionisation of the peak was observed under negative ES or APCI modes. Using an acidified acetonitrile gradient the constitution of the oenanthotoxin peak became clearer, since it was resolved into two peaks of approximately equal intensity in the total absorbance UV chromatogram (Fig. 2). The later eluting component (**2**) exhibited the same UV spectrum as oenanthotoxin (λ_{max} recorded at 252, 268, 298, 316, 338 nm) whereas the UV spectrum of the earlier component (**1**) had absorbance maxima at 228, 237 and 295 and 308 nm. With either positive APCI or positive ES modes, **1** gave an ion species at *m/z* 261, whereas ions at *m/z* 241 and 282 were generated from **2**. The two compounds were also resolved using an aqueous acetonitrile gradient without the addition of acid, but coeluted using a water/methanol gradient. Under the latter conditions, positive ES generated an abundant ion at *m/z* 299 in the co-eluting peak. This ion was present at low intensity in component **1** of the resolved peaks in a water/acetonitrile gradient.

In order to confirm the identity and relative molecular masses of components **1** and **2**, they were isolated by semi-preparative HPLC. The probe EI spectrum of **2** was very similar to that published for oenanthotoxin [10] and showed M^+ at *m/z* 258. Thus, the ion species observed following ES or APCI were assigned to $[(M+H)–H_2O]^+ = m/z$ 241 and $[(M+H)–H_2O+\text{solvent}]^+ = m/z$ 273 (for methanol) or *m/z* 282 (for acetonitrile). CI–MS confirmed the proposed molecular mass, showing an abundant ion at *m/z* 259 ($[M+H]^+$) and base peak at *m/z* 276 ($[M+NH_4]^+$),

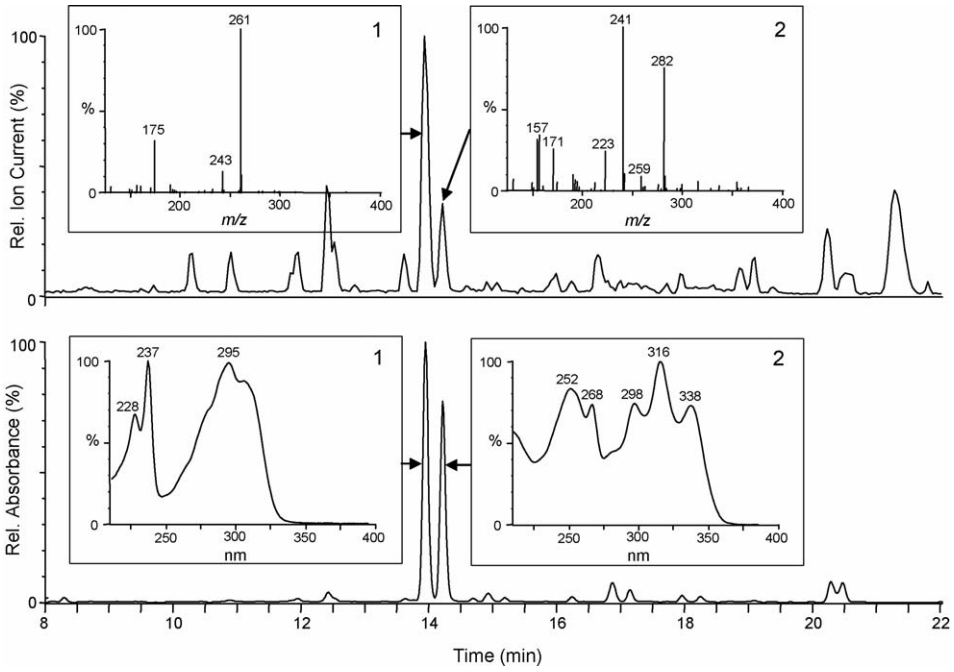


Fig. 2. Base ion (top) and total absorbance (bottom) chromatograms of a methanol extract of *O. crocata* roots using a C18 column and an acidified aqueous acetonitrile mobile phase gradient (see Section 2.3 for details). Insets: positive ion ES mass spectra (ESCi source) and UV absorption spectra of the two major polyalkynes, 2,3-dihydro-oenanthotoxin (**1**) and oenanthotoxin (**2**).

and accurate mass measurement confirmed the proposed molecular formula: HRESIMS *m/z* 276.1957 $[M+NH_4]^+$ (calculated for $[C_{17}H_{26}NO_2]^+$ *m/z* 276.1958).

Probe EI gave M^+ for **1** at *m/z* 260 and CI–MS gave additional confirmation, showing abundant ions at *m/z* 261 and 278 (corresponding to $[M+H]^+$ and $[M+NH_4]^+$, respectively). Thus ion species observed following ES and APCI were assigned to $[M+H]^+ = m/z$ 261, $[M+Na]^+ = 283$, $[M+K]^+ = m/z$ 299, and $[(M+H)+\text{solvent}]^+ = m/z$ 293 (for methanol) and *m/z* 302 (for acetonitrile). In their study of the polyacetylenes of *O. crocata*, Bohlmann and Rode [9] isolated both oenanthotoxin and 2,3-dihydro-oenanthotoxin (M_r 260) in substantially greater amounts than other polyacetylenes. The UV absorption maxima and EI mass spectrum of **1** correspond closely to the data given for 2,3-dihydro-oenanthotoxin [9] and accurate mass measurement was in accordance with the molecular formula of this compound: HRESIMS *m/z* 261.1849 $[M+H]^+$ (calculated for $[C_{17}H_{25}O_2]^+$ *m/z* 261.1849).

Confirmation of the identities of **1** and **2** as 2,3-dihydro-oenanthotoxin and oenanthotoxin, respectively, was obtained by NMR spectroscopy. The ^1H NMR data acquired in $CDCl_3$ were in good agreement with the earlier results of Bohlmann and Rode [9]. However, specific assignments of some methylene and alkenyl protons were lacking from the latter study due to the instrumental limitations of the period. Full sets of both ^1H and ^{13}C NMR spectral resonance assignments for 2,3-dihydro-oenanthotoxin (**1**) and oenanthotoxin (**2**) are given here for the first time (see Section 2.7). The assignments were confirmed by correlations detected in COSY, HSQC, and HMBC experiments.

Following chromatography in acidified acetonitrile/water, a low abundance ion species at *m/z* 259 was generated from oenanthotoxin by the dedicated APCI source of the ion trap instrument allowing it to be studied by MS/MS. The product ion spectrum of protonated oenanthotoxin was dominated by a prominent base ion at *m/z* 241 (loss of water) in accordance with the readiness with which the compound dehydrated during ionisation (Fig. 3A). Two homologous series of ions could then be identified among the less abundant ions in the spectrum that agreed with fragmentation following protonation of either the OH group at C-14 or protonation of a double or triple bond (Scheme 1). An ion at *m/z* 223 would agree with protonation of the OH group at C-1 followed by loss of two water molecules. The product ion at *m/z* 241 produced the same spectrum (not shown) when subjected to MS/MS as the ion at *m/z* 241 generated in the source (Fig. 3B). The most abundant product ions in this spectrum were essentially those predicted from the *m/z* 241 ion species resulting from dehydration of protonated oenanthotoxin shown in Scheme 1.

In recording the above spectra using dedicated APCI and an acidified aqueous acetonitrile mobile phase, a further point of confusion in the analysis was noted in that 2,3-dihydro-oenanthotoxin generated an ion at *m/z* 259 and oenanthotoxin generated an ion at *m/z* 257. These $[M-1]^+$ species, resulting from either hydride abstraction $[M-H]^+$, or dehydrogenation of the protonated molecule $[(M+H)–H_2]^+$, were the most abundant ions generated from the respective compounds by the APCI source in aqueous acetonitrile without the addition of acid. This could lead to the $[M-1]^+$ ion of 2,3-dihydro-oenanthotoxin being incorrectly assigned to the $[M+H]^+$ ion of oenanthotoxin, since both ions have the same *m/z* value. The product ion spectrum of the $[M-1]^+$ ion at *m/z* 259 for 2,3-dihydro-oenanthotoxin (Fig. 3C) shows a similar array of fragments to the product ion spectrum of protonated oenanthotoxin but the relative intensity of the ion resulting from the loss of water is much lower. This dif-

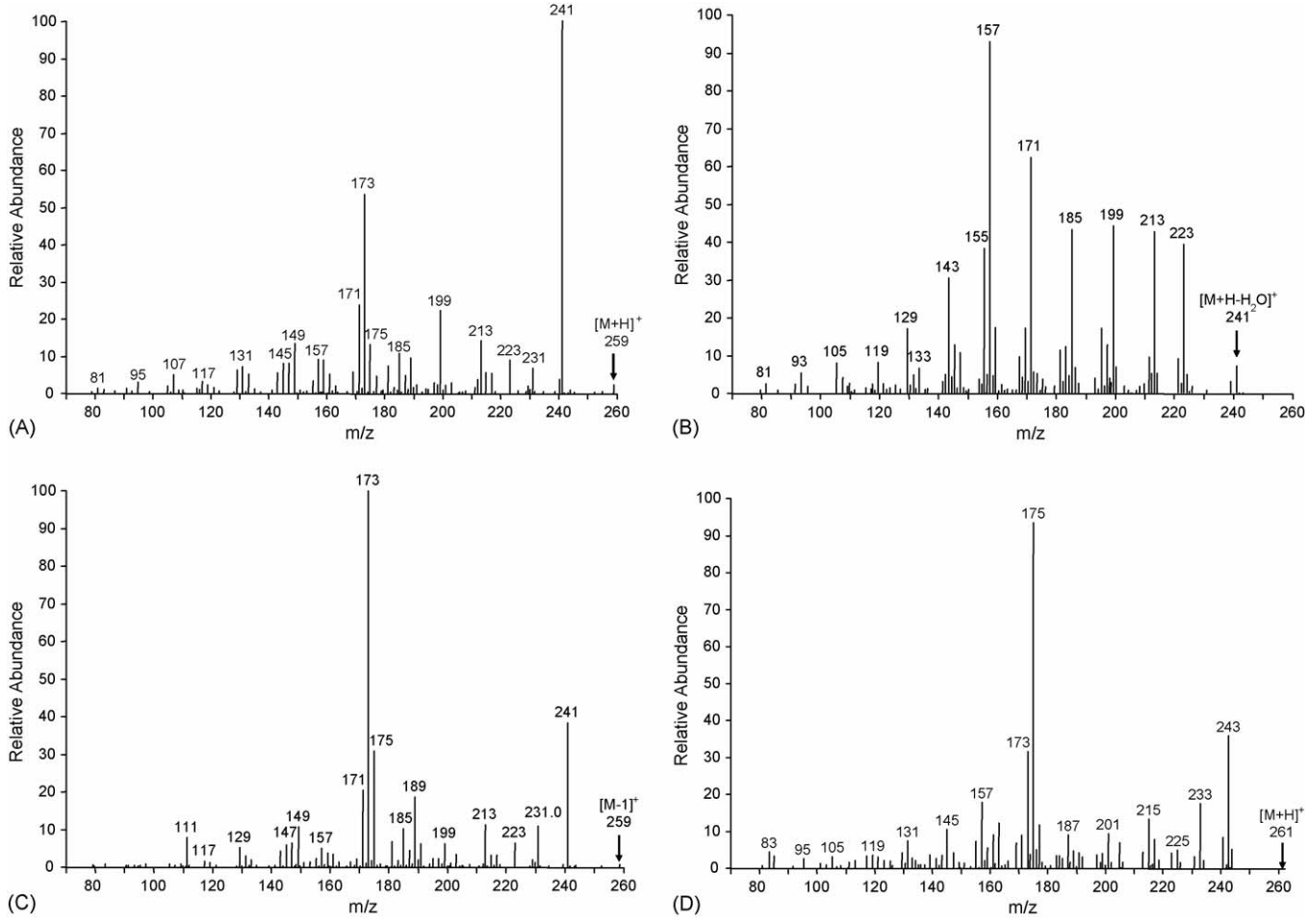
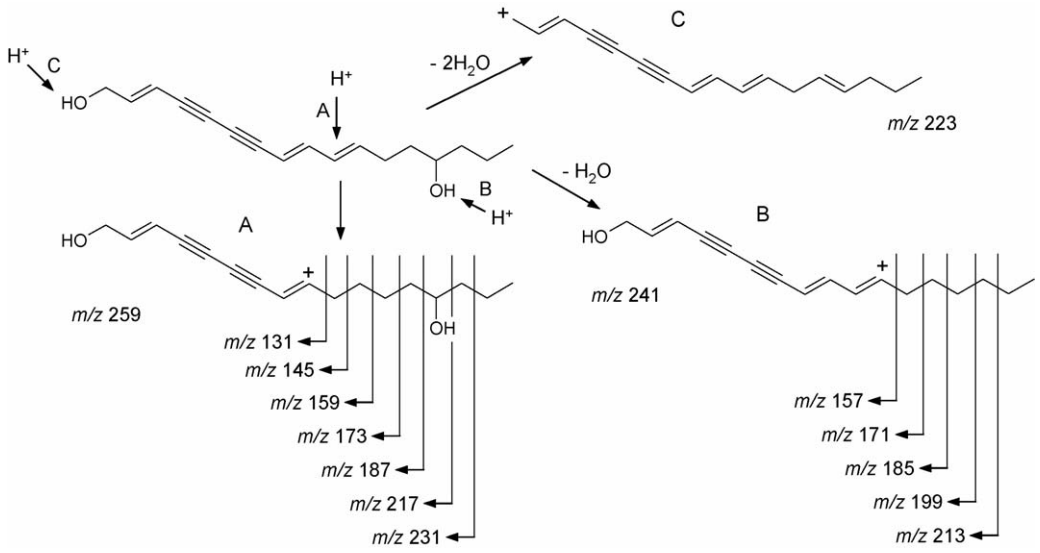


Fig. 3. Product ion spectra (by ion trap MS/MS) of (A) $[M+H]^+$ oenanthotoxin, (B) $[(M+H)–H_2O]^+$ oenanthotoxin, (C) $[M-1]^+$ 2,3-dihydro-oenanthotoxin, and (D) $[M+H]^+$ 2,3-dihydro-oenanthotoxin.



Scheme 1. Suggested fragmentation of $[M+H]^+$ of oenanthotoxin following protonation of either a double or triple bond (A), the OH group at C-14 (B), or the OH group at C-1 (C).

ference is useful in the correct assignment of the oenanthotoxin peak.

The product ion spectrum of protonated 2,3-dihydro-oenanthotoxin (Fig. 3D) showed a similar pattern of ions to that of protonated oenanthotoxin, except the higher value ions were 2 Th greater and the $[(M+H)–H_2O]^+$ ion was at lower relative intensity (in accordance with the compound being less susceptible to dehydration in the source). As noted previously, in aqueous methanol both $[M+H]^+$ of 2,3-dihydro-oenanthotoxin and $[(M+H)–H_2O]^+$ of oenanthotoxin formed abundant methanol adduct ions. The product ion spectrum of the methanol adduct of the $[(M+H)–H_2O]^+$ ion of oenanthotoxin at *m/z* 273 also showed the two ion series indicated in Scheme 1 (i.e. without a methanol adduct) with the ion at *m/z* 187 from series A forming the clear base peak. Additional abundant ions were at *m/z* 255 (loss of water from the precursor ion) and *m/z* 241 (loss of the methanol adduct from the precursor). The product ion spectrum of the methanol adduct of protonated 2,3-dihydro-oenanthtoxin (*m/z* 293) was again complex. Losses of water and methanol were evident but the base ion at *m/z* 273 was due to a combined loss of water and hydrogen (H_2).

3.2. Application to suspected poisoning cases

In the case of the suspected poisoning of a pony by *O. crocata* that was presented to us, oenanthotoxin was readily detected both in a methanol extract of the stomach contents and the sample of roots by HPLC–PDA alone. This could be achieved using either the method of King et al. [10], or an aqueous acetonitrile mobile phase to resolve oenanthotoxin from 2,3-dihydro-oenanthotoxin (Fig. 4). The product ion spectra obtained by LC–MS/MS of both compounds were as described in Section 3.1 and supported the identifications.

In order to assess the usefulness of LC–MS in poisoning cases that may be more challenging analytically, a methanol extract of leaves and roots of grasses and other plant species gathered randomly from grazing pasture was spiked with varying levels of *O. crocata* extract and analysed by LC–PDA–MS/MS. An acidified aqueous methanol gradient was used for chromatography in combination with ionisation by APCI, as this provided maximum sensitivity for oenanthotoxin among the methods tested (Table 1). Oenanthotoxin was monitored by MS/MS of *m/z* 241 $[(M+H)–H_2O]^+$ from which a single ion chromatogram at *m/z* 157 was extracted post acquisition. Using this approach, the on-column detection limit equated to the amount of oenanthotoxin extracted from 2.5 μg of fresh roots of *O. crocata* (summer harvested) present amongst the compounds extracted from 10 mg fresh weight of mixed pasture (Fig. 5). However, at this concentration the co-eluting peak of oenanthotoxin and 2,3-dihydro-oenanthotoxin was also observed with PDA detection as it eluted much later than the major UV-absorbing compounds present in the extract. When this spiked extract was analysed using an aqueous acetonitrile mobile phase gradient, oenanthotoxin (resolved from 2,3-dihydro-oenanthotoxin) was still detected by PDA (S:N at 315 nm = 300:1), but not by APCI–MS. For a pure sample of oenanthotoxin, on-column detection limits were 10 ng for APCI-MS (*m/z* 157 from MS/MS of *m/z* 241) and 200 pg for PDA (315 nm) using an aqueous methanol gradient.

In conclusion, if LC–MS is applied to the investigation of samples from suspected cases of *O. crocata* poisoning, then it

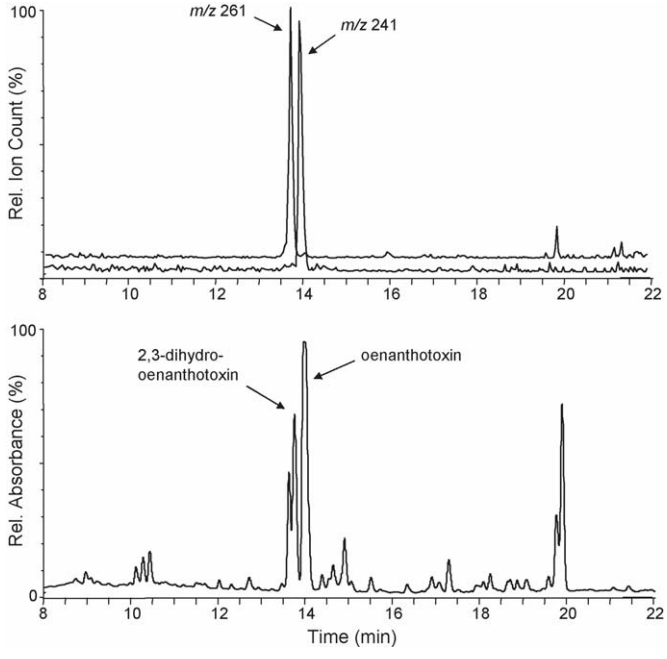


Fig. 4. Extracted single ion chromatograms at *m/z* 261 and *m/z* 241 (above) and UV absorbance chromatogram at 315 nm (below) of a methanol extract of the stomach contents of a pony suspected to have died from the consumption of *O. crocata*. Mobile phase was as in Fig. 2; ionisation was by dedicated APCI. *m/z* 261 = $[M+H]^+$ 2,3-dihydro-oenanthotoxin; *m/z* 241 = $[(M+H)–H_2O]^+$ oenanthotoxin.

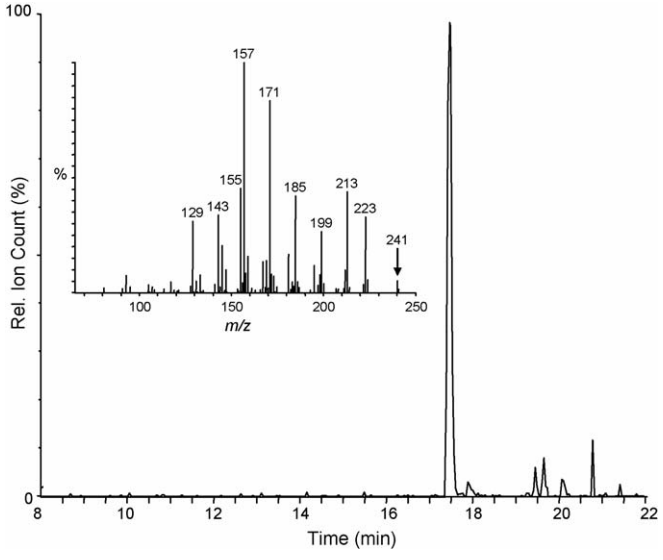


Fig. 5. Single ion chromatogram at *m/z* 157 extracted from product ion spectra of *m/z* 241 recorded continuously during an LC–MS/MS analysis of compounds extracted from leaves and roots of mixed pasture containing 0.025% (fresh weight) *O. crocata* roots (=extract from 2.5 μg fresh weight on-column). Chromatographic separation was achieved on C18 using an acidified aqueous methanol gradient (see Section 2.3). Ionisation was by dedicated APCI (100% = 5×10^5 ions). Inset: product ion spectrum of the main chromatographic peak; cf. MS/MS $[(M+H)–H_2O]^+$ oenanthotoxin, Fig. 3B.

is recommended that dedicated APCI is employed as the ion source and chromatography is undertaken in a gradient of aqueous methanol. On our instrument, the combination of dedicated APCI and ionisation in aqueous methanol achieved the maximum response; however, the co-elution of oenanthotoxin with 2,3-dihydro-oenanthotoxin does require MS/MS capability to obtain confirmatory product ion spectra. Using HPLC with PDA detection alone, use of an acetonitrile gradient provides superior chromatographic separation.

The stability of oenanthotoxin in the gastric environment is not known, thus failure to detect oenanthotoxin in stomach contents does not provide evidence that plant material containing oenanthotoxin had not been ingested. Furthermore, consumption of *O. crocata* roots by horses causes seizuring that need not be fatal. It would be of interest to determine whether oenanthotoxin can be detected in accessible body fluids in non-fatal cases that were exhibiting suspicious signs, such as seizuring, so that remedial action could be taken. HPLC or LC–MS analysis would then present a useful clinical test, if undertaken quickly.

References

[1] M.R. Cooper, A.W. Johnson, Poisonous Plants and Fungi, HMSO, London, 1988.
[2] J. Bruneton, Toxic Plants Dangerous to Humans and Animals, Lavoisier, Paris, 1999.
[3] J.-P. Anger, F. Anger, Y. Chauvel, R.L. Girre, N. Curtes, J.-P. Curtes, Eur. J. Toxicol. 9 (1976) 119.
[4] M.I. Mitchell, P.A. Routledge, Clin. Toxicol. 12 (1978) 417.
[5] C. Downs, J. Phillips, A. Ranger, L. Farrell, Emerg. Med. J. 19 (2002) 472.
[6] C. Van Inzen, S.D. Gunn, Vet. Rec. 125 (1989) 212.
[7] G.G. Clarke, D.E. Kidder, W.D. Robertson, J. Pharm. Pharmacol. 1 (1949) 377.
[8] E.F.L.J. Anet, B. Lythgoe, M.H. Silk, S. Trippet, J. Chem. Soc. 62 (1953) 309.
[9] F. Bohlmann, K.-M. Rode, Chem. Ber. 101 (1968) 1163.
[10] L.A. King, M.J. Lewis, D. Parry, P.J. Twitchett, E.A. Kilner, Hum. Toxicol. 4 (1985) 355.
[11] B. Del Castililo, A. García de Marina, M.P. Martínez-Honduvilla, Ital. J. Biochem. 29 (1980) 233.
[12] J. Milne, Vet. Rec. 3 (1945) 30.