Analytical documentation of an Arabian horse fatality related to Oenanthe crocata poisoning

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Analytical documentation of an Arabian horse fatality related to *Oenanthe crocata* poisoning

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Abstract

Analytical detection of *Oenanthe crocata* toxins in biological samples is challenging because of their instability, the lack of commercially available standards and the exceptionally low detection of these molecules using mass spectrometry. This work aims to report the used analytical methods that allowed identification of the main plant toxins in biological samples from an equid (an Arabian horse) fatality related to hemlock water dropwort (*Oenanthe crocata Linnaeus*) intake. Using both LC-DAD and LC-HRMS methods allowed identification (i) of oenanthotoxin in roots found on the site, root fragments found in the stomach, stomach content, kidney, and liver, and (ii) of the hydrogenated metabolite of oenanthotoxin (2,3-dihydro-oenanthotoxin) in roots found on the site, root fragments found in the stomach, stomach content, kidney, liver and spleen. Reported analytical data about *Oenanthe crocata* toxins can be useful for identification of the ingested plant and for supporting a poisoning diagnosis in such cases.

Keywords

Oenanthe crocata poisoning, horse, oenanthotoxin, 2,3-dihydro-oenanthotoxin, LC-HRMS, LC-DAD

1. Introduction

In France, three species of the *Apiaceae* family can be the sources of severe neurological poisoning: the cowbane (*Cicuta virosa Linnaeus*), the big hemlock (*Conium maculatum Linnaeus*) and the hemlock water dropwort *(Oenantha crocata Linnaeus)*. The first isolation of oenanthotoxin ((2E,8E,10E,14R)-heptadeca-2,8,10-trien-4,6-diyne-1,14-diol) (OT), the main toxin of hemlock water dropwort, was achieved by Clarke et al. in 1949 [1] . An OT fluorimetric determination method was proposed in 1980 [2] and a successful detection (using liquid chromatography coupled UV detector (LC-DAD)) of OT was reported in 1985 by King *et al.* in a human poisoning case (detection in residual plant material and stomach content) [3]. In 2006, Kite *et al.* reported a liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method used for identification of OT together with a toxic hydrogenated metabolite (2,3-dihydro-oenanthotoxin; 2,3DHOT) in ingested roots and stomach content in a suspected poisoning case of a pony [4]. Lately, a human poisoning fatal case was reported: OT and 2,3DHOT were detected using liquid chromatography coupled with high resolution mass spectrometry (LC-HRMS) in vegetal and stomach content [5]. Nevertheless, analytical literature data remain sparse and only concern *Oenantha crocata*-related toxin detection in vegetal or in human or animal stomach contents.

Several human or equine poisoning cases related to *Oenantha crocata* were already well described in literature, but this intoxication remains uncommon [3-8]. In this situation, we report a fatal equine intoxication case related to this plant which is, for the first time, documented by identification of OT and 2,3DHOT in several biological samples using both LC-DAD and LC-HRMS methods.

2. Case history

A 7-year-old Arabian horse was submitted to the School of Veterinary Medicine of Nantes (France) in January 2021 for a necropsy. The owner suspected an accidental poisoning by a neighbour who admitted to have fed the horse with some roots found in his plot of land. The horse was found dead within one hour after the ingestion; a necropsy was performed the following day. It has been highlighted the presence of several parts of vegetal roots (measuring up to 5 cm x 1 cm x 0.4 cm) in the stomach. Section of the roots found on site showed hexagonal subdivisions structures and a yellowish liquid; these findings were consistent with Hemlock Water Dropwort (*Oenantha crocata Linnaeus*) identification.

Microscopic evaluation of several organs (including the brain) was unremarkable. Necropsy samples (vegetal roots fragments found in the stomach of the horse, stomach content, liver, kidney, spleen), together with samples of roots found on the site (identified as roots of *Oenantha crocata Linnaeus* by an experienced botanist) (Figure 1), were sent to the toxicology laboratory of university hospital of Lille (France).

3. Material and methods

3.1 Chemicals

Methanol absolute UPLC/MS quality as well as acetonitrile were provided by BioSolve, France. Ammonium formate, methyl-clonazepam and β- OH- ethyltheophyllin (purity > 98%) were provided by Sigma-Aldrich Chimie Sarl, France and formic acid of analytical grade was provided by Prolabo, France. Ultra-pure water was generated using a Milli-Q Plus (Millipore, Molsheim, France).

3.2 Sample preparation

The sample preparation was carried out using methanol extraction following the previously proposed method by Kite *et al.* [4]. Necropsy samples were carried and stored at -20°C before analysis, while the samples of roots found on the site were carried and stored at room temperature. After milling, 1 g of crushed roots as well as stomach content (2.5 g), were extracted using methanol (5 mL), vortex mixed for 30 seconds and kept at 4°C for 2 hours. Similarly, 10 g of liver, kidney, and spleen samples were crushed, mixed with 5 mL of methanol, vortex mixed for 5 min and kept at 4°C for 24 hours. Theses methanolic suspensions were twice centrifuged: (i) firstly at 2500 g for 10 min, the first supernatant was transferred to another tube and (ii) secondly centrifugated at 8000 g for 14 min. One mL of supernatant of liver, kidney, and spleen samples was dried under nitrogen and retake with 100 µL of methanol.

3.3 LC-DAD analysis

LC-DAD analysis was performed using an Acquity class I ultra-high-performance liquid chromatography with photodiode array detection device (Waters, Milford, MA, USA) [9]. Briefly, 100 µL of supernatants were mixed with 50 µL of the internal standard solution (methyl-clonazepam at 200 mg/L) in order (i) to check that the chromatographic and detection conditions are correct, and (ii) to obtain an additional criterium for identification [relative retention time (rRT)]: 15 µL were injected in the column (Acquity BEH C18; 150 × 2.1 mm × 1.7 µm) (Waters, Milford, MA, USA) maintained at 50°C. Mobile phases involved 5 mM ammonium formiate water solution with 0.1 % formic acid (A) and acetonitrile (B). The flow

rate was set at 0.45 mL/min and the mobile phase gradient was as follows: 0-1.4 min: 15% B; 1.4-2.8 min: 15 to 25% B; 2.8-11.0 min: 25 to 79% B; 11.0-11.2 min: 79% B; 11.2-12 min: decrease from 79 to 15% B; and a final column equilibration (15% B) for 3 min. Spectre scan was performed between 200 to 400 nm and data reprocessing was performed using Empower 3 software (Waters, Milford, MA, USA). It is of note that the use of LC-DAD in the process of identification of OT is recommended as it allows for separation of OT with cicutoxin, an isomer of OT, with UV spectra of cicutoxin different to OT [10].

3.4 LC–HRMS analysis

LC-HRMS analysis was performed using an Acquity class I ultra-high-performance liquid chromatographic system with quadripole time-of-flight (QTOF) mass spectrometry detection device (XEVO G2-XS QTOF, Waters, Milford, MA, USA) [11]. Briefly, 50 µL of supernatant were mixed with 100 µL of mobile phase A (5 mM ammonium formiate water solution and 0.1 % formic acid) and 50 µL of the internal standard solution (β- OH- ethyltheophyllin at 800 µg/L and methyl-clonazepam at 62.5 µg/L). Fifteen µL were injected and chromatographic separation was achieved using an Acquity HSS C18 column (150 × 2.1 mm × 1.8 µm) (Waters, Milford, MA, USA) maintained at 50°C. Mobile phases were 5 mM ammonium formiate water solution with 0.1 % formic acid (A) and 0.1% formic acid in acetonitrile (B). The flow rate was set at 0.4 mL/min. The mobile phase gradient was programmed as follows (flow rate was 0.4 mL/min): 0-0.5 min: 13% B; 0.5-10 min: 13 to 50% B; 10 – 10.25 min: 50 to 95% B; 10.25-12.25 min: 95% B; 12.25-12.5 min: decrease from 95 to 13% B; and a final column equilibration (13% B) for 2.5 min. Detection was performed using electrospray positive ionization (ESI) mode: spray ion voltage was 20V; source and desolvatation temperatures were 120°C and 450°C, respectively; nitrogen was used as desolvatation gas and argon as collision gas. It is of note that ESI in negative mode, or the use of atmospheric chemical ionization (APCI) in positive mode, were not carried out in this study in accordance with previous results of Kite *et al* [4]. A mass scan was performed using Data-Independent Acquisition (DIA) by MSe acquisition mode between *m/z* 100 and 1000 for molecular ions research (function 1), and between *m/z* 50 and 1000 with a collision energy ramp ranging from 10 to 40 eV for mass fragments research (function 2). Data reprocessing was performed using Unify software (Waters, Milford, MA, USA). In addition, OT and 2,3DHOT predicted fragmentation was performed using Progenesis QI software (Waters, Milford, MA, USA). The mass precision of 12 ppm for molecular ion identification and 20 ppm for fragment identification was applied.

4. Results

Firstly, samples of roots found on the site (identified as roots of *Oenantha crocata Linnaeus*) were analysed in order to identify OT and 2,3DHOT (time retention and UV spectra) (Figure 2) using literature data provided by Kite *et al.* [4], *i.e.* UV spectra related to 2,3DHOT and OT. This identification was performed using the standard LC-DAD screening conditions routinely applied in laboratory [9]. It is of note that the use of water/acetonitrile gradient was the most efficient choice for chromatographic separation of 2,3DHOT and OT joining King *et al.* [3] and Kite *et al.* [4] in using this gradient. Two chromatographic peaks exhibited UV spectra similar to already reported ones [4]: chromatographic peak at 8.07 min (rRT: 1.16) had a specific UV absorbance of 2,3DHOT with λ max at 226, 235 and 293 nm, and the one at 8.2 min (rRT: 1.18) had a specific UV absorbance of OT with λ max at 251, 266, 296, 315 and 336 nm (Figure 2).

LC-HRMS analysis allowed identification of two chromatographic peaks (Figure 3): at 11.41 min (rRT from the closer internal standard, methyl-clonazepam: 1.18) [main ion pattern: *m/z* 241.1587; 259.1693; 281.1513; 297.1253] and 11.33 min (rRT: 1.17) [main ion pattern: *m/z* 261.1850]. At 11.41 min, the predominant ion (*m/z* 259.1692) was the protonated form of OT ([M+H]+); *m/z* 281.1513 was a sodium adduct ([M+Na]+); *m/z* 297.1253 was a potassium adduct ([M+K]+); and *m/z* 241.1587 was a dehydrated form of protonated OT ([(M+H)-H_2O]+). At 11.33 min, *m/z* 243.1743 and *m/z* 261.1850 were dehydrated form of protonated ([(M+H)-H_2O]+) and protonated form ([M+H]+) of 2,3DHOT, respectively. In is of note that dehydration position could be the hydroxy group on the C1, or the one on the C14.

Analysis of fragmentation spectra was performed using Progenesis QI software: (i) for OT (11.41 min; Figure 3), the most abundant fragment ions were *m/z* 173.0961 ([(M+H)-C_4H_9O]+); 145.0648 ([(M+H)-$C_7H_{14}O$]+); 115.0542 ([(M+H)-$C_8H_{16}O_2$]+), and (ii) for 2,3DHOT (11.33 min; Figure 3), the most abundant fragment ions were *m/z* 175.1119 ([(M+H)-C_4H_9O]+); 143.0700 ([(M+H)-$C_6H_{13}O_2$]+); 117.0698 ([(M+H)-$C_8H_{16}O_2$]+). Previous published data of a fragmentation spectra analysis realised by LC - MS was reported by Kite *et al.* [4] for OT and 2,3DHOT and a fragmentation spectrum obtained by LC-HRMS was reported by Martinez *et al.* [5] for 2,3DHOT.

As a second step, these analytical data were successfully applied to the analysis of the samples from the equine necropsy: using LC-DAD method, OT and 2,3DHOT were detected in vegetal roots found in the stomach and stomach content; using LC-HRMS method, OT was detected in vegetal roots found in the stomach, stomach content, kidney, and liver, and

2,3DHOT was detected in vegetal roots found in the stomach, stomach content, kidney, liver, and spleen, as reported in Table 1.

5. Discussion-conclusion

The hemlock water dropwort is well-known as “navet de diable” and “oenanthe safranée” in France, or “dead man’s finger’s”, “horsebane”, “dead tongue”, “five-fingered root”, “water lovage”, or “yellow water dropwort” in England [12]. Poisoning cases can occur due to the possible confusion with wild carrot (*Daucus carota*), pignut (*Conopodium majus*), sweet flag (*Acorus calamus*), watercress (*Narsturtium officinale*), wild celery (*Apium graveolens*), wild ginseng (*Panax ginseng*), or parsnip (*Pastinaca sativa*) roots [8]. *Oenanthe crocata* tuberous roots (4 to 6 cm long), the most poisonous part, are composed of 5 or more fleshy cylindrical tubers. Their structure is a multichambered system of hexagonal shape and a yellowish liquid can be observed when cutting these vegetal roots [8]. This yellow liquid gave the name of *Crocata* to this plant, in reference with the colour of saffron [13]. OT and 2,3DHOT act as non-competitive antagonists of GABA receptors. Horses are more susceptible to *Oenantha crocata* poisoning than cattle and pigs. Clinical signs in horses (non-vomiting animals) are dominated by hypersalivation, mydriasis, muscle tremors, respiratory distress and convulsions but sometimes only sudden death. In humans, the clinical signs are completed/supplemented by ataxia, grinding/gnashing of teeth, hallucinations, nausea and vomiting seizures; they occur between 15 minutes and 10 hours after ingestion [8]. The lethal dose (LD50) for OT via intra peritoneal administration in rats and mice was reported to be 2.94 mg/kg of body weight [8, 14]. Literature data in human intoxications report that the OT potentially lethal oral dose may be as low as 10 to 20 mg, which is contained in about 20 grams of the *Oenanthe crocata* root [6]. Between 2012 and 2018, 21 of human symptomatic poisoning cases with *Oenanthe crocata* were reported in France [7].

Analytical detection of *Oenanthe crocata* toxins in biological samples remains a challenge due to their instability (*e.g.* OT and 2,3DHOT stabilities in *post-mortem* samples is unknown), the lack of commercially available analytical standards and the exceptionally low response of related substances using mass spectrometry detection. To date, an analytical approach was proposed by Martinez *et al.* [5] with a combination of a genetic nrDNA ITS2 sequence investigation in the extracted vegetal and a 2,3DHOT detection in gastric content using LC-HRMS. Nevertheless, identification of OT and 2,3DHOT can be performed using a combination of LC-DAD and LC-HRMS (for enhanced detection in organs *e.g.* liver)

methods, in order to support poisoning diagnosis, and we hope that the reported analytical data can be helpful in this way.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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Figures and table legends

Table 1: Detection of oenanthotoxin and 2,3-dihydro-oenanthotoxin in vegetal roots found in stomach and *post-mortem* samples of the horse by LC-DAD and LC-HRMS (*nd: not detected*).

Figure 1: Hemlock Water Dropwort (*Oenantha crocata Linnaeus*) roots found on the site.

Figure 2: LC-DAD chromatograms (C18 column, water/acetonitrile mobile phase gradient, spectral absorbance sum) and UV spectra of 2,3-dihydro-oenanthotoxin (2,3DHOT) [λ max: 226, 235 and 293 nm] and oenanthotoxin (OT) [λ max: 251, 266, 296, 315 and 336 nm] of a methanol extract of *Oenanthe crocata* roots.

Figure 3: LC-HRMS extracted single ion [*m/z* 259.1692 for oenanthotoxin (OT), and m/z 261.183 for 2,3-dihydro-oenanthotoxin (2,3DHOT)] chromatogram (C18 column and water/acetonitrile mobile phase gradient) of a methanol extract of *Oenanthe crocata* roots, together with chemical structures, and fragmentation patterns of OT and 2,3DHOT.

Table 1

	vegetal roots found in stomach	stomach content	kidney	liver	spleen
LC-DAD	OT 2,3DHOT	OT 2,3DHOT	*nd*	*nd*	*nd*
LC-HRMS	OT 2,3DHOT	OT 2,3DHOT	OT 2,3DHOT	OT 2,3DHOT	*(OT: nd)* 2,3DHOT

FIGURE 1

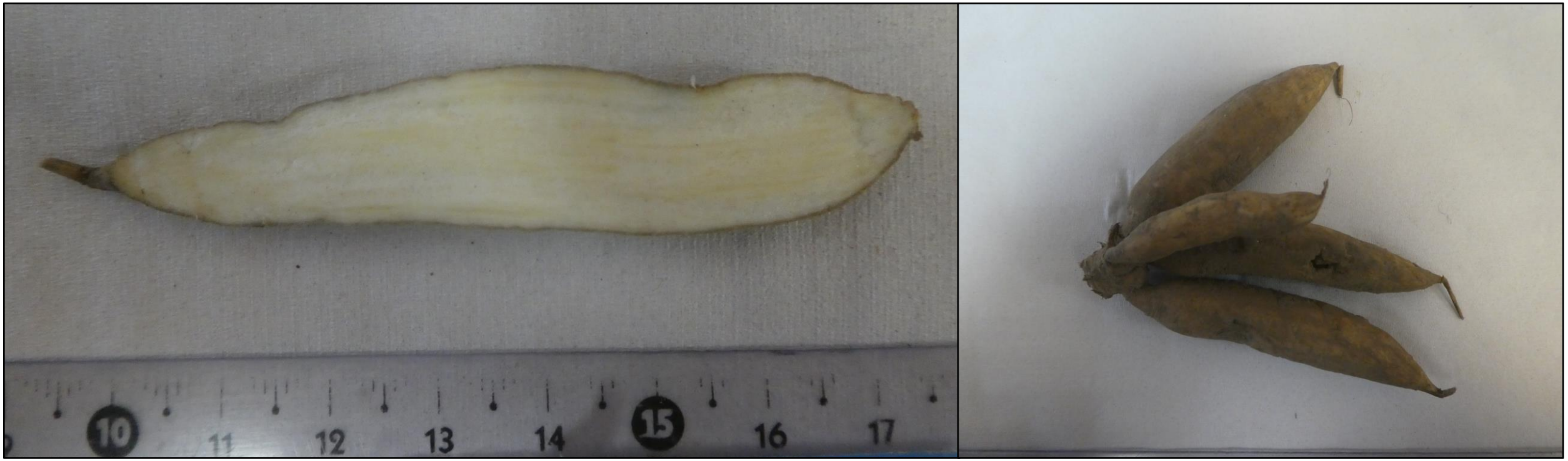


FIGURE 2

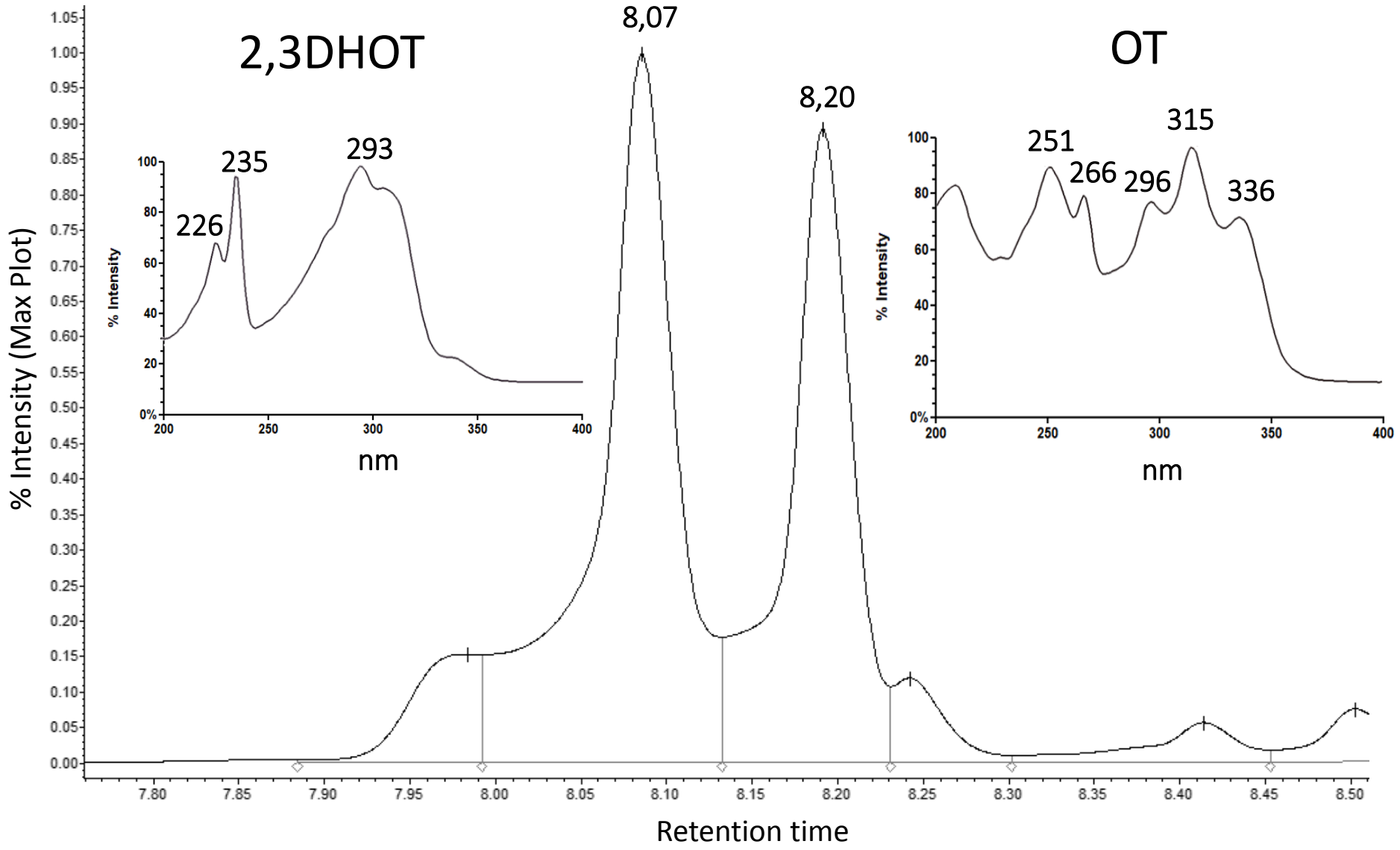
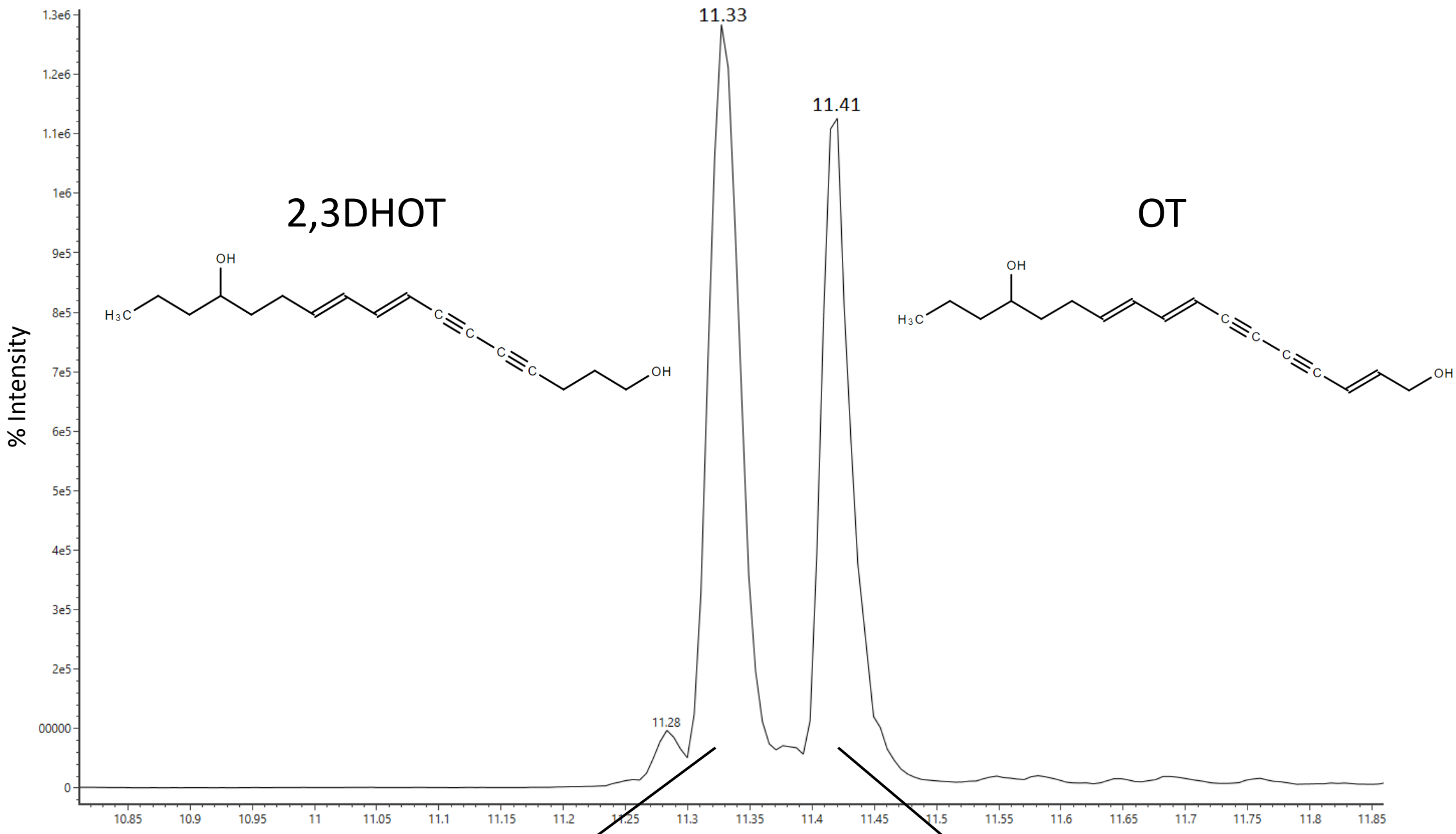

2,3DHOT
226
235
293
% Intensity
nm
8,07
8,20
OT
251
266
296
315
336
% Intensity
nm
% Intensity (Max Plot)
Retention time

FIGURE 3


2,3DHOT
OT
11.33
11.41
11.28
% Intensity
Retention time
1.3e6
1.2e6
1.1e6
1e6
9e5
8e5
7e5
6e5
5e5
4e5
3e5
2e5
00000
0
10.85
10.9
10.95
11
11.05
11.1
11.15
11.2
11.25
11.3
11.35
11.4
11.45
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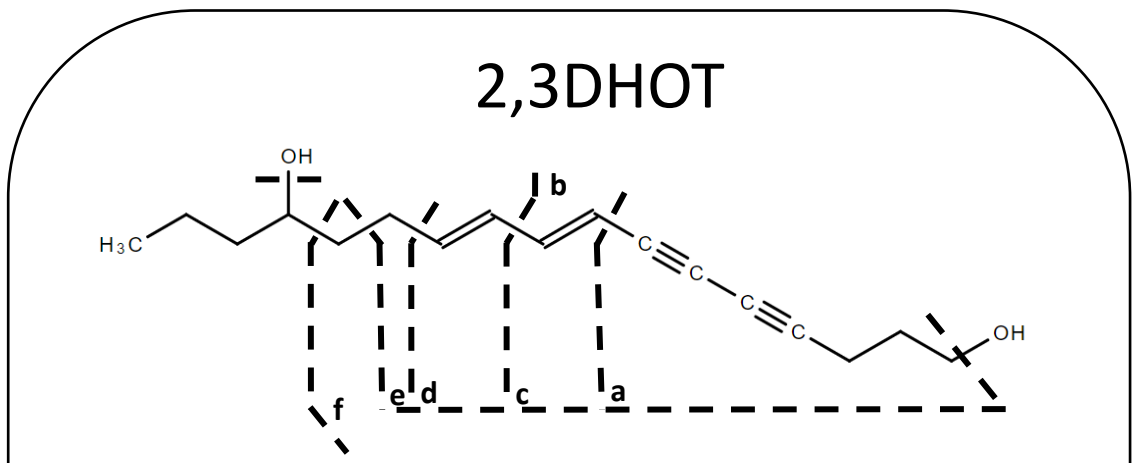
11.75
11.8
11.85
OH
H3C
OH


2,3DHOT
OH
H3C
OH
a
b
c
d
e
f
100%
50%
0%
% Intensity
m/z, Da
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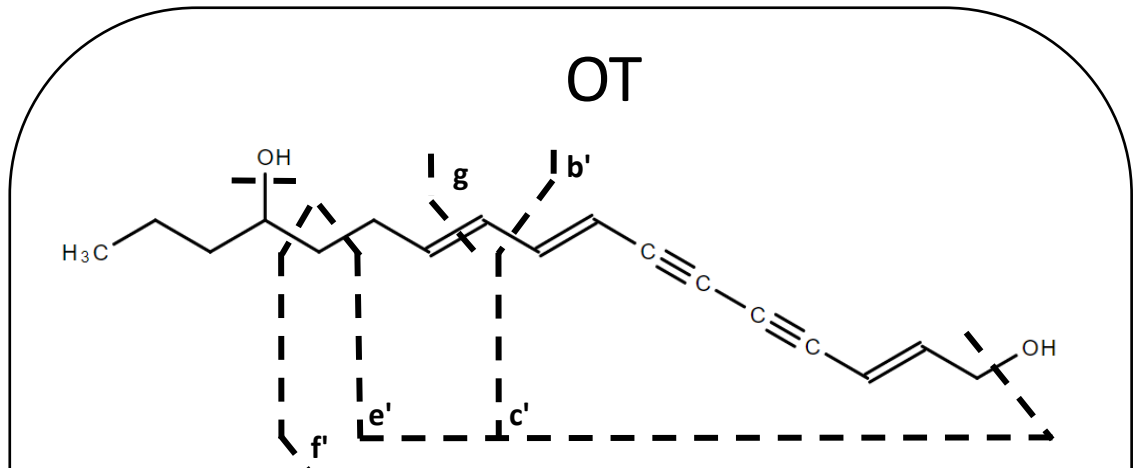
175.1119
243.1753
261.1850
100
150
200
250


OT
OH
H3C
OH
g
b'
e'
c'
f'
100
50%
0%
% Intensity
m/z, Da
115.0541
133.0648
145.1014
173.0965
241.1587
259.1692
281.1513
297.1253
100
150
200
250
300

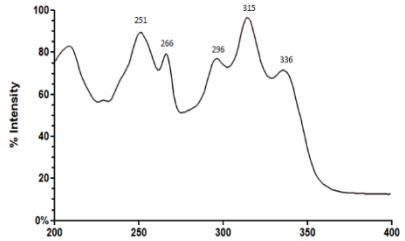
Analytical documentation of an Arabian horse fatality related to *Oenanthe crocata* poisoning

horse found dead within one hour after ingestion

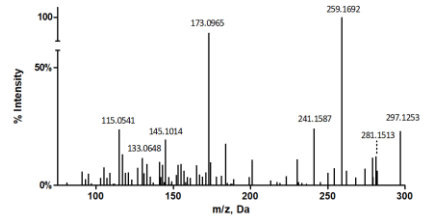
Necropsy samples

Oenantha crocata Linnaeus roots

LC -DAD



LC -HRMS



Identification of oenanthotoxin and 2,3-dihydro-oenanthotoxin in biological samples