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

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5 Alexandr Gish¹, Cynthia Robveille², Thomas Gicquel³, Delphine Allorge^{1,4}, Gilbert Gault⁵,
6 Jean-michel Gaulier^{1,4*}

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¹ CHU Lille, Unité Fonctionnelle de Toxicologie, F-59037 Lille, France

9 ² Ecole Nationale Vétérinaire, Agroalimentaire et de l'Alimentation Nantes-Atlantique -
10 ONIRIS, Nantes, France

11 ³ CHU de Rennes, Laboratoire de toxicologie biologique et médicolégale, Rennes, France

12 ⁴ Univ. Lille, ULR 4483 - IMPECS - IMPact de l'Environnement Chimique sur la Santé
13 humaine, Lille, France

14 ⁵ Ecole Nationale Vétérinaire de Lyon - ENVL, Lyon, France

15
16

17 *Correspondence to Jean-Michel Gaulier

18 Laboratory of Toxicology – CHU

19 Bd du Professeur Jules Leclercq

20 CS 70 001

21 59037 LILLE Cedex

22 France

23 Email: jean-michel.gaulier@chru-lille.fr

24 Fax : +33 320444960

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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

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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 (*Oenanthe 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 *Oenanthe crocata*-related toxin detection in vegetal or in human or animal stomach contents.

Several human or equine poisoning cases related to *Oenanthe 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 (*Oenanthe crocata* Linnaeus) identification.

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

6

7 **3. Material and methods**

8 **3.1 Chemicals**

9 Methanol absolute UPLC/MS quality as well as acetonitrile were provided by BioSolve,
10 France. Ammonium formate, methyl-clonazepam and β -OH-ethyltheophyllin (purity >
11 98%) were provided by Sigma-Aldrich Chimie Sarl, France and formic acid of analytical
12 grade was provided by Prolabo, France. Ultra-pure water was generated using a Milli-Q Plus
13 (Millipore, Molsheim, France).

14 **3.2 Sample preparation**

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

26 **3.3 LC-DAD analysis**

27 LC-DAD analysis was performed using an Acquity class I ultra-high-performance liquid
28 chromatography with photodiode array detection device (Waters, Milford, MA, USA) [9].
29 Briefly, 100 μ L of supernatants were mixed with 50 μ L of the internal standard solution
30 (methyl-clonazepam at 200 mg/L) in order (i) to check that the chromatographic and detection
31 conditions are correct, and (ii) to obtain an additional criterium for identification [relative
32 retention time (rRT)]: 15 μ L were injected in the column (Acquity BEH C18; 150 \times 2.1 mm \times
33 1.7 μ m) (Waters, Milford, MA, USA) maintained at 50°C. Mobile phases involved 5 mM
34 ammonium formiate water solution with 0.1 % formic acid (A) and acetonitrile (B). The flow

1 rate was set at 0.45 mL/min and the mobile phase gradient was as follows: 0-1.4 min: 15% B;
2 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:
3 decrease from 79 to 15% B; and a final column equilibration (15% B) for 3 min. Spectre scan
4 was performed between 200 to 400 nm and data reprocessing was performed using Empower
5 3 software (Waters, Milford, MA, USA). It is of note that the use of LC-DAD in the process
6 of identification of OT is recommended as it allows for separation of OT with cicutoxin, an
7 isomer of OT, with UV spectra of cicutoxin different to OT [10].

8 **3.4 LC–HRMS analysis**

9 LC-HRMS analysis was performed using an Acquity class I ultra-high-performance liquid
10 chromatographic system with quadripole time-of-flight (QTOF) mass spectrometry detection
11 device (XEVO G2-XS QTOF, Waters, Milford, MA, USA) [11]. Briefly, 50 µL of
12 supernatant were mixed with 100 µL of mobile phase A (5 mM ammonium formiate water
13 solution and 0.1 % formic acid) and 50 µL of the internal standard solution
14 (β - OH- ethyltheophyllin at 800 µg/L and methyl-clonazepam at 62.5 µg/L). Fifteen µL were
15 injected and chromatographic separation was achieved using an Acquity HSS C18 column
16 (150 × 2.1 mm × 1.8 µm) (Waters, Milford, MA, USA) maintained at 50°C. Mobile phases
17 were 5 mM ammonium formiate water solution with 0.1 % formic acid (A) and 0.1% formic
18 acid in acetonitrile (B). The flow rate was set at 0.4 mL/min. The mobile phase gradient was
19 programmed as follows (flow rate was 0.4 mL/min): 0-0.5 min: 13% B; 0.5-10 min: 13 to
20 50% B; 10 – 10.25 min: 50 to 95% B; 10.25-12.25 min: 95% B; 12.25-12.5 min: decrease
21 from 95 to 13% B; and a final column equilibration (13% B) for 2.5 min. Detection was
22 performed using electrospray positive ionization (ESI) mode: spray ion voltage was 20V;
23 source and desolvation temperatures were 120°C and 450°C, respectively; nitrogen was
24 used as desolvation gas and argon as collision gas. It is of note that ESI in negative mode, or
25 the use of atmospheric chemical ionization (APCI) in positive mode, were not carried out in
26 this study in accordance with previous results of Kite *et al* [4]. A mass scan was performed
27 using Data-Independent Acquisition (DIA) by MSe acquisition mode between m/z 100 and
28 1000 for molecular ions research (function 1), and between m/z 50 and 1000 with a collision
29 energy ramp ranging from 10 to 40 eV for mass fragments research (function 2). Data
30 reprocessing was performed using Unify software (Waters, Milford, MA, USA). In addition,
31 OT and 2,3DHOT predicted fragmentation was performed using Progenesis QI software
32 (Waters, Milford, MA, USA). The mass precision of 12 ppm for molecular ion identification
33 and 20 ppm for fragment identification was applied.

34

1 4. Results

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

13 LC-HRMS analysis allowed identification of two chromatographic peaks (Figure 3): at 11.41
14 min (rRT from the closer internal standard, methyl-clonazepam: 1.18) [main ion pattern: m/z
15 241.1587; 259.1693; 281.1513; 297.1253] and 11.33 min (rRT: 1.17) [main ion pattern: m/z
16 261.1850]. At 11.41 min, the predominant ion (m/z 259.1692) was the protonated form of OT
17 ($[M+H]^+$); m/z 281.1513 was a sodium adduct ($[M+Na]^+$); m/z 297.1253 was a potassium
18 adduct ($[M+K]^+$); and m/z 241.1587 was a dehydrated form of protonated OT ($[(M+H)-$
19 $H_2O]^+$). At 11.33 min, m/z 243.1743 and m/z 261.1850 were dehydrated form of protonated
20 ($[(M+H)-H_2O]^+$) and protonated form ($[M+H]^+$) of 2,3DHOT, respectively. In is of note that
21 dehydration position could be the hydroxy group on the C1, or the one on the C14.

22 Analysis of fragmentation spectra was performed using Progenesis QI software: (i) for OT
23 (11.41 min; Figure 3), the most abundant fragment ions were m/z 173.0961 ($[(M+H)-$
24 $C_4H_9O]^+$); 145.0648 ($[(M+H)-C_7H_{14}O]^+$); 115.0542 ($[(M+H)-C_8H_{16}O_2]^+$), and (ii) for
25 2,3DHOT (11.33 min; Figure 3), the most abundant fragment ions were m/z 175.1119
26 ($[(M+H)-C_4H_9O]^+$); 143.0700 ($[(M+H)-C_6H_{13}O_2]^+$); 117.0698 ($[(M+H)-C_8H_{16}O_2]^+$).
27 Previous published data of a fragmentation spectra analysis realised by LC - MS was reported
28 by Kite *et al.* [4] for OT and 2,3DHOT and a fragmentation spectrum obtained by LC-HRMS
29 was reported by Martinez *et al.* [5] for 2,3DHOT.

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

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

3

4 **5. Discussion-conclusion**

5 The hemlock water dropwort is well-known as “navet de diable” and “oenanthe safranée” in
6 France, or “dead man’s finger’s”, “horsebane”, “dead tongue”, “five-fingered root”, “water
7 lovage”, or “yellow water dropwort” in England [12]. Poisoning cases can occur due to the
8 possible confusion with wild carrot (*Daucus carota*), pignut (*Conopodium majus*), sweet flag
9 (*Acorus calamus*), watercress (*Narsturtium officinale*), wild celery (*Apium graveolens*), wild
10 ginseng (*Panax ginseng*), or parsnip (*Pastinaca sativa*) roots [8]. *Oenanthe crocata* tuberous
11 roots (4 to 6 cm long), the most poisonous part, are composed of 5 or more fleshy cylindrical
12 tubers. Their structure is a multichambered system of hexagonal shape and a yellowish liquid
13 can be observed when cutting these vegetal roots [8]. This yellow liquid gave the name of
14 *Crocata* to this plant, in reference with the colour of saffron [13]. OT and 2,3DHOT act as
15 non-competitive antagonists of GABA receptors. Horses are more susceptible to *Oenanthe*
16 *crocata* poisoning than cattle and pigs. Clinical signs in horses (non-vomiting animals) are
17 dominated by hypersalivation, mydriasis, muscle tremors, respiratory distress and convulsions
18 but sometimes only sudden death. In humans, the clinical signs are completed/supplemented
19 by ataxia, grinding/gnashing of teeth, hallucinations, nausea and vomiting seizures; they occur
20 between 15 minutes and 10 hours after ingestion [8]. The lethal dose (LD50) for OT via intra
21 peritoneal administration in rats and mice was reported to be 2.94 mg/kg of body weight [8,
22 14]. Literature data in human intoxications report that the OT potentially lethal oral dose may
23 be as low as 10 to 20 mg, which is contained in about 20 grams of the *Oenanthe crocata* root
24 [6]. Between 2012 and 2018, 21 of human symptomatic poisoning cases with *Oenanthe*
25 *crocata* were reported in France [7].

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

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

3

4 **Disclosure of interest**

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

6

7

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1 **Figures and table legends**

2

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

6

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

8

9 **Figure 2:** LC-DAD chromatograms (C18 column, water/acetonitrile mobile phase gradient,
10 spectral absorbance sum) and UV spectra of 2,3-dihydro-oenanthotoxin (2,3DHOT) [λ max:
11 226, 235 and 293 nm] and oenanthotoxin (OT) [λ max: 251, 266, 296, 315 and 336 nm] of a
12 methanol extract of *Oenanthe crocata* roots.

13

14 **Figure 3:** LC-HRMS extracted single ion [m/z 259.1692 for oenanthotoxin (OT), and m/z
15 261.183 for 2,3-dihydro-oenanthotoxin (2,3DHOT)] chromatogram (C18 column and
16 water/acetonitrile mobile phase gradient) of a methanol extract of *Oenanthe crocata* roots,
17 together with chemical structures, and fragmentation patterns of OT and 2,3DHOT.

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19

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21

1

2 **Table 1**

3

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

4

5

FIGURE 1



FIGURE 2

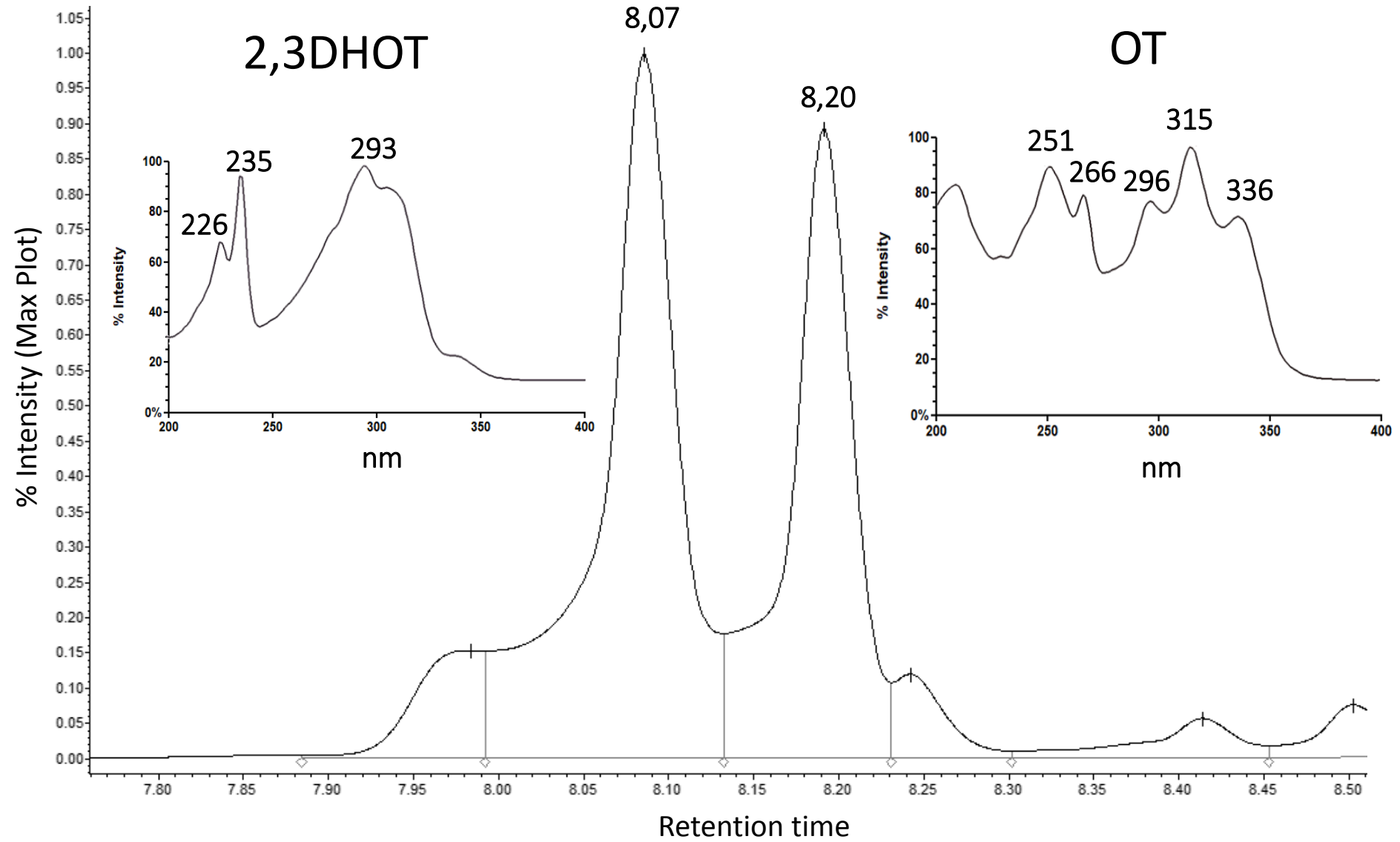
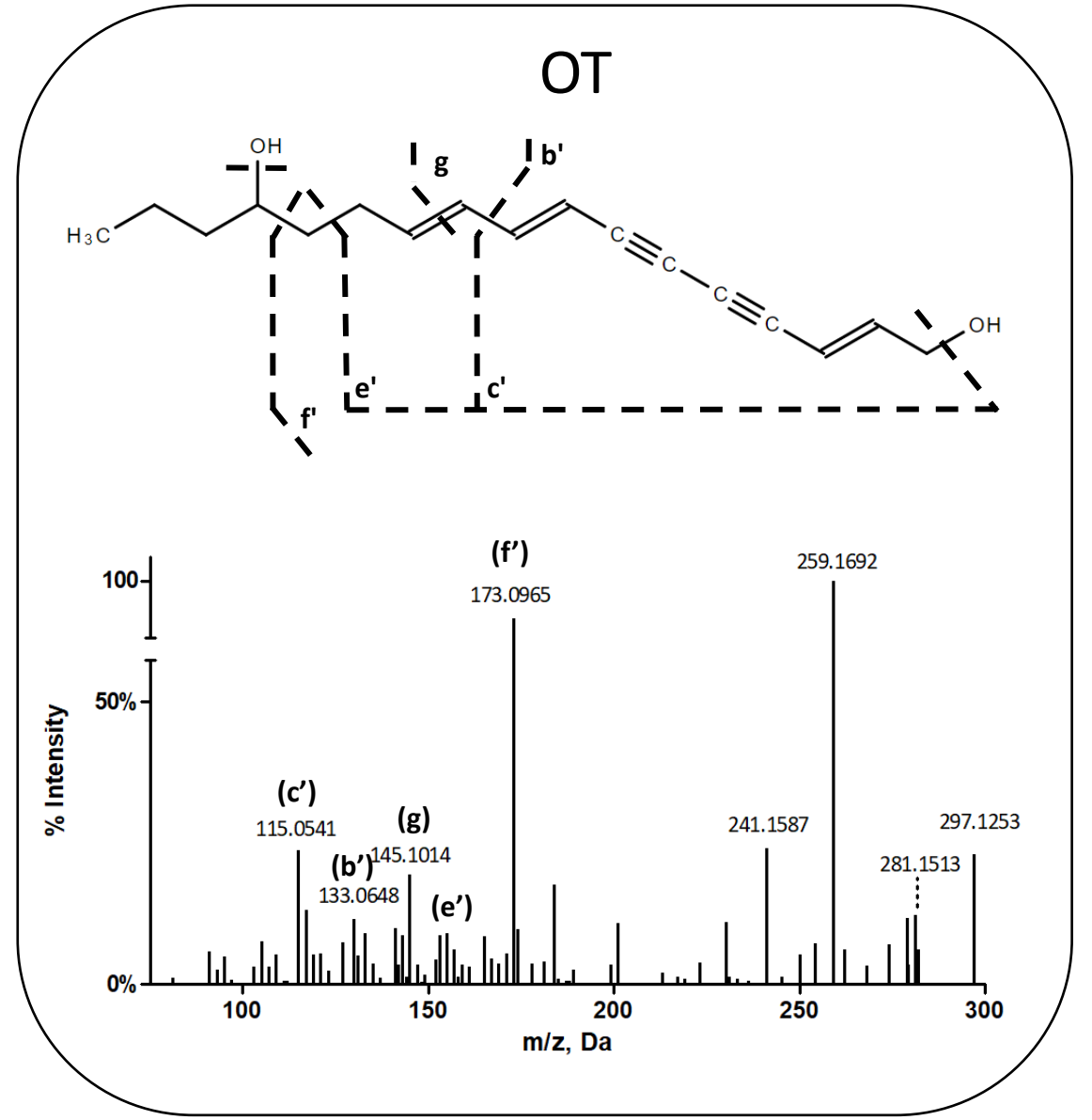
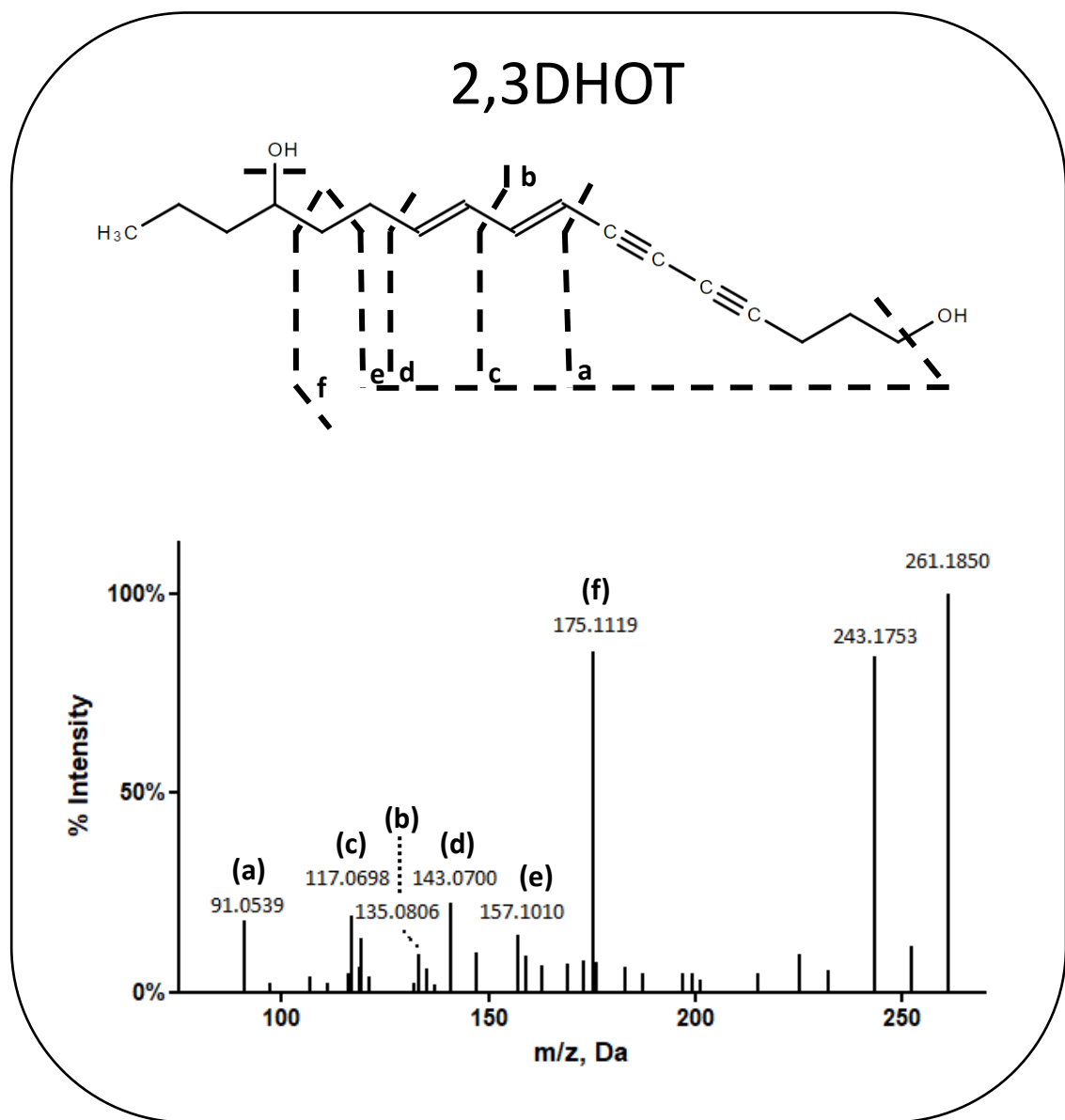
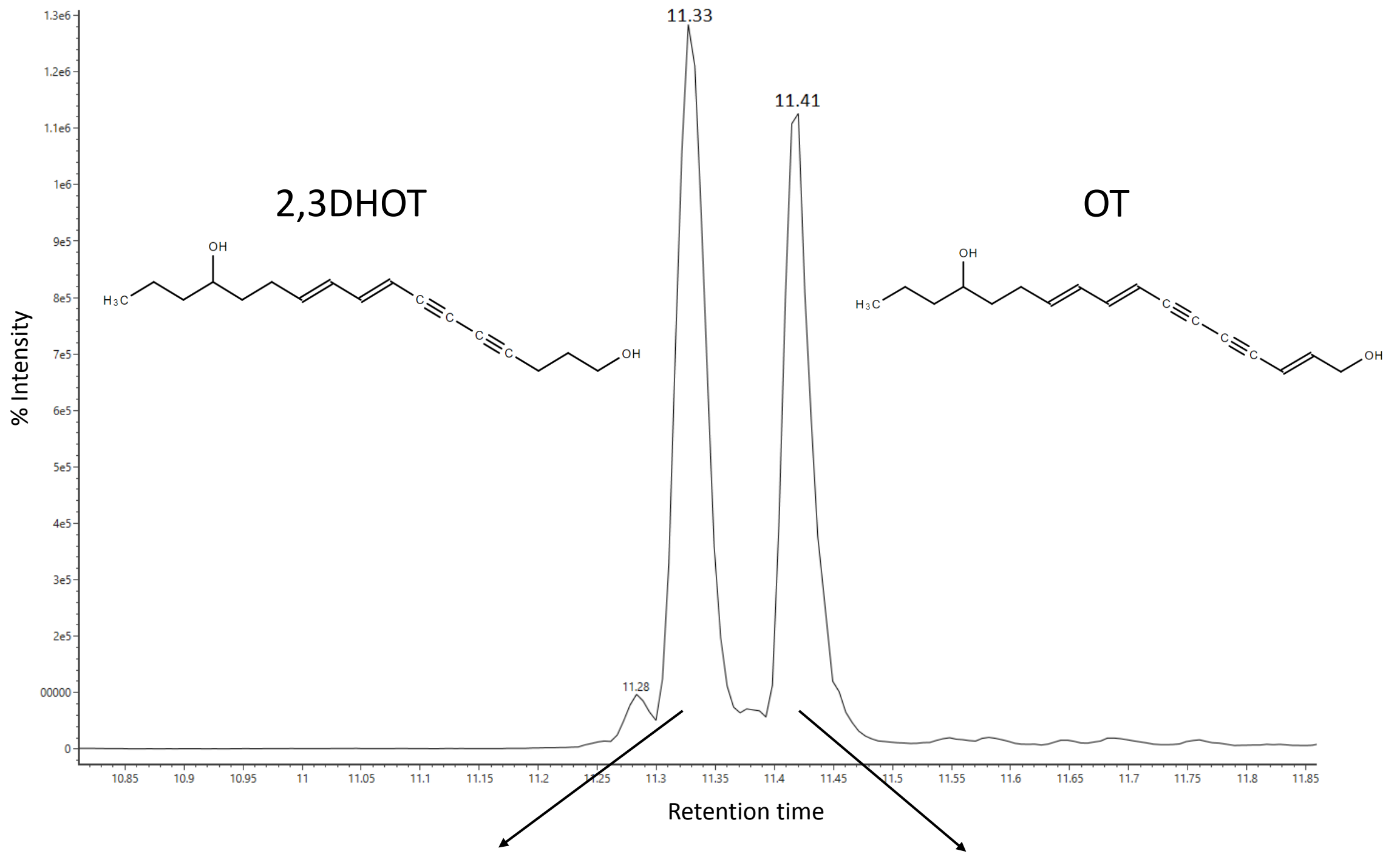


FIGURE 3

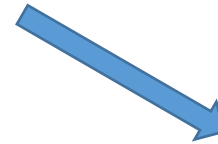
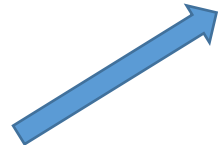


Analytical documentation of an Arabian horse fatality related to *Oenanthe crocata* poisoning



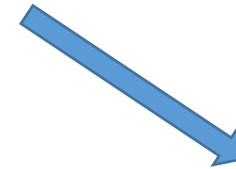
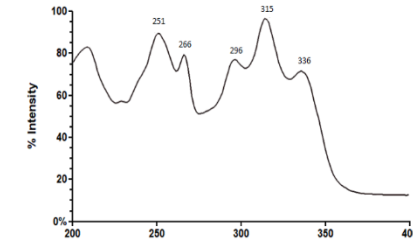
horse found dead within one hour after ingestion

Necropsy samples



Oenanthe crocata
Linnaeus roots

LC -DAD



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



LC -HRMS

