ANATOXIN-A IN SEA FIGS ASSOCIATED WITH HUMAN FOOD POISONINGS IN FRANCE

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Contamination of sea figs with ATX  
2022/03/20
1 — Symptomatology of intoxication related to the consumption of sea figs
Poisoning cases involving sea figs

- Sea figs of the genus Microcosmus, fished in the Mediterranean. Products highly prized for their iodized taste
- 20 poisoning cases between January 2011 and January 2020
- 30 people involved
- 20 women and 10 men
- Aged 17 to 80 years

**Symptoms**

- Dizziness: 54%
- Vomiting: 50%
- Diplopia, visual disturbances: 58%
- Difficulty concentrating: 4%
- Diarrhea: 19%
- Muscle cramps: 19%
- Abdominal cramps: 19%
- Headaches: 15%
- Nausea: 8%
- Paresthesia: 8%
- Tremors: 4%
- Ringing in the ears: 4%
- Ataxia: 23%
- Asthenia: 23%

**Schmitt et al. (2019). Cerebellar syndrome associated with ingestion of Mediterranean Microcosmus: a French case series**

**Sometimes fortuitous nature of the reports!!!**

Case of a patient who took an oral medication intended for vaginal administration

Symptoms actually due to consumption of sea figs!!

Contamination of sea figs with ATX
2 — Preliminary investigations
Protocol implemented

Analysis of regulated toxins
- Lipophilic toxins
- Domoic acid
- Saxitoxins
- Hemolytic activity

Complementary investigations
- Non-targeted analysis (high resolution mass spectrometry – LC-HRMS)

Contamination of sea figs with ATX

2022/03/20
Non-targeted analysis - LC-HRMS (I. Dom PhD thesis)

1. Non discriminating extraction (lipophilic)

2. LC-HRMS analysis
   - suspect screening (screening of a list of toxins)
   - looking for unknown compounds (unbiased analysis)

Contamination of sea figs with ATX
List of 820 toxins (marine and freshwater toxins)

- Suspicion of anatoxin-a (ATX) but
  - Chromatographic method not adapted (co-elution of ATX-a and Phe, isobaric compounds)
  - ATX found in 1 sample but for 1 replicate only (out of 3)
  - unexpected result (freshwater cyanotoxin in a marine organism)

Need for additional analyses
- Method adapted for hydrophilic toxins (cyanotoxins) → HILIC
3 — Complementary investigations
Contamination of sea figs with ATX

Protocol implemented

Aqueous solvent

Grinding

Test portion

Homogenate

SPE

Extract

LCMS analyses in low and high resolution (HILIC-MS)

TSQ Vantage (low resolution)

API 5600 Qtof (high resolution)

Low (LR) versus high resolution (HR)

Hydrophilic toxins

Contamination of sea figs with ATX
Targeted analyses: HILIC-MS/MS (low resolution)

Analytical method developed by the national reference laboratory for marine biotoxins as part of the monitoring of emerging compounds in France (EmergTox)

Chromatogram of a mixed solution of toxins after HILIC-LRMS analysis

ATX: 165.11536 Da

Phenylalanine (Phe): 165.07898 Da

In low resolution, no mass distinction (165.1 Da)!

→ Prerequisite: need for good chromatographic separation ATX / Phe

Contamination of sea figs with ATX

2022/03/20
Verification of the presence of ATX in the sea fig extracts

- Presence of a single peak at the retention time of ATX
- Increase in ATX-a peak intensity correlated with the amount of ATX added to the sea fig extract
Targeted analysis - HILIC-MS/MS (low resolution)

ATX concentrations found in the sea fig samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>ATX concentration (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP-1-2011</td>
<td>194</td>
</tr>
<tr>
<td>FP-6-2012</td>
<td>1240</td>
</tr>
<tr>
<td>FP-17-2018</td>
<td>1133</td>
</tr>
<tr>
<td>Sea fig Control</td>
<td>22</td>
</tr>
<tr>
<td>Mussel control</td>
<td>&lt; LOD*</td>
</tr>
</tbody>
</table>

(*) LOD = limit of detection (8 µg/kg)
Confirmatory analyses: HILIC-MS/MS (high resolution)

Contamination of sea figs with ATX

ATX standard

Sea fig sample

Same retention time

Same MS/MS spectrum

Same exact mass (error < 5 ppm)
Confirmatory analyses: HILIC-MS/MS (high resolution)

List of ATX analogues searched in the sea fig samples

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Formula</th>
<th>Mass (Da)</th>
<th>Extraction Mass [M + H]^+ (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATX-a</td>
<td>C10H15NO</td>
<td>165.11536</td>
<td>166.12264</td>
</tr>
<tr>
<td>hATX-a</td>
<td>C11H17NO</td>
<td>179.13101</td>
<td>180.13829</td>
</tr>
<tr>
<td>Carboxy ATX-a</td>
<td>C11H15NO3</td>
<td>209.10519</td>
<td>210.11247</td>
</tr>
<tr>
<td>Carboxy hATX-a</td>
<td>C12H17NO3</td>
<td>223.12084</td>
<td>224.12812</td>
</tr>
<tr>
<td>Carboxy dihydroATX-a</td>
<td>C11H17NO3</td>
<td>211.12084</td>
<td>212.12812</td>
</tr>
<tr>
<td>N-methyl ATX a</td>
<td>C11H17NO</td>
<td>179.13101</td>
<td>180.13829</td>
</tr>
<tr>
<td>(10S)-ATX alcohol</td>
<td>C10H17NO</td>
<td>167.13101</td>
<td>168.13829</td>
</tr>
<tr>
<td>(10R)-ATX alcohol</td>
<td>C10H17NO</td>
<td>167.13101</td>
<td>168.13829</td>
</tr>
<tr>
<td>nor ATX-a</td>
<td>C9H13NO</td>
<td>151.09971</td>
<td>152.10699</td>
</tr>
<tr>
<td>Dihydro ATX-a</td>
<td>C10H17NO</td>
<td>167.13101</td>
<td>168.13829</td>
</tr>
<tr>
<td>Dihydro hATX-a</td>
<td>C11H19NO</td>
<td>181.14666</td>
<td>182.15394</td>
</tr>
<tr>
<td>Epoxy ATX-a</td>
<td>C10H15NO2</td>
<td>181.11028</td>
<td>182.11756</td>
</tr>
<tr>
<td>Epoxy hATX-a</td>
<td>C11H17NO2</td>
<td>195.12593</td>
<td>196.13321</td>
</tr>
<tr>
<td>ATX-(a)s</td>
<td>C7H17N4O4P</td>
<td>252.09874</td>
<td>253.10602</td>
</tr>
<tr>
<td>Phe</td>
<td>C9H11NO2</td>
<td>165.07898</td>
<td>166.08626</td>
</tr>
</tbody>
</table>

None of the ATX analogues were found in the sea fig samples (non-targeted analyses in "suspect screening" mode)
4 — Study of the variability of contamination levels in sea figs
Samples analyzed

- *Microcosmus sulcatus*
  - Area: FAO 37.2.1, Adriatic Sea (Croatia)
  - Sample related to a food poisoning in the Gard department (jan. 2020) → 3 peoples (2×W 54 y/o, W 17 y/o). Important quantities of sea figs eaten by the two 54-y/o women

- *Microcosmus sulcatus*
  - Area: FAO 37.2, Central Mediterranean Sea
  - Sample coming from a store in the Var department (Géant Casino of Hyères), not related to any food poisoning

- *Microcosmus sp.*
  - Thau lagoon
  - Sample bought from a fisherman, not related to any food poisoning
Samples analyzed

Sample preparation
- Cleaning
- Shucking
- Weighing of each individual (flesh versus liquid/exudate)

- 9 individuals
  - Total weight of the animals 12.4 – 38.4 g
  - Mean = 24.7 g, SD = 9.0 g
  - Flesh/liquid ratio: 0.5 – 3.3

- 12 individuals
  - Total weight of the animals 20.7 – 86.6 g
  - Mean = 49.0 g, SD = 21.9 g
  - Flesh/liquid ratio: 0.4 – 1.2

- 5 individuals
  - Total weight of the animals 15.3 – 59.0 g
  - Mean = 32.9 g, SD = 17.7 g
  - Flesh/liquid ratio: 0.5 – 0.8

Contamination of sea figs with ATX
Analysis in low resolution (HILIC-MS)

Protocol implemented

Contamination of sea figs with ATX
Contamination of sea figs with ATX
Conclusion: These preliminary results show that it is not possible to generalize about the existence or not of a correlation between the size of the sea figs and their contamination level. Several factors come into play.
Suspicion of the presence of hATX in 1/5 sea fig of the Thau lagoon (LC-MS low resolution)

- Specific transitions of hATX
- But RT < that of the hATX standard

- Different HRMS2 fragmentation spectra for the hATX standard and the violet
- However, some low mass fragments are common → not hATX but related compound?
Conclusions and perspectives

- Evidence of ATX-a in violets involved in TIAC cases (low and high resolution)
- High variability of contamination of samples (194 - 1240 µg/kg)
- High variability of contamination between individuals, but no correlation between size and contamination level
- Need for further investigations to know if ATX-a is indeed responsible for the intoxication cases
- What about contamination of other marine organisms?
First Evidence of the Presence of Anatoxin-A in Sea Figs Associated with Human Food Poisonings in France

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