Quantification of emerging micropollutants in an amphipod crustacean by nanoliquid chromatography coupled to mass spectrometry using multiple reaction monitoring cubed mode

Martin SORDET, Alexandra BERLIOZ-BARBIER, Audrey BULETE, Jeanne GARRIC and Emmanuelle VULLIET
Overview

- Introduction
  - Context
  - Objectives
  - Substances of interest
  - Organism of interest

- Analytical Strategy

- Analytical method
  - MicroQuEChERS
  - NanoLC analysis
  - MRM³ mode

- Application to real samples
  - Exposure experiment
  - Results

- Conclusion & Outlook
Source of pharmaceutical contamination:

- Pharmaceutical manufacturer
- Agriculture Run-off
- Household effluent
- Hospital effluent
- Wastewater treatment effluent
- Waste landfill
- Aquaculture

Aquatic ecosystems

What impact on the aquatic ecosystem?
Objectives

Analytical objectives:

- Quantification of three substances émergentes in the amphipod crustacean *Gammarus fossarum*
  - Carbamazepine
  - Oxazepam
  - Testosterone

- Complex matrix of small size: *Gammarus fossarum*
  - Miniaturisation and optimisation of the sample preparation
  - Extraction and analysis at individual scale

- Optimisation of the analysis by nano liquid chromatography (NanoLC) coupled to mass spectrometry using MRM³ mode

Application to an exposure experiment in lab in order to:

- Evaluation the kinetics of accumulation and depuration of these molecules by *G. fossarum*

- Evaluation the inter-individual variabilities for bioaccumulation
### Substances of interest

<table>
<thead>
<tr>
<th>Substance</th>
<th>Molecular Structure</th>
<th>pKa</th>
<th>Log P</th>
<th>MM (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbamazepine</td>
<td><img src="image" alt="Carbamazepine" /></td>
<td>13.9</td>
<td>2.45</td>
<td>236.3</td>
</tr>
<tr>
<td>Anticonvulsant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxazepam</td>
<td><img src="image" alt="Oxazepam" /></td>
<td>10.6</td>
<td>2.24</td>
<td>286.7</td>
</tr>
<tr>
<td>Anxiolytic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td><img src="image" alt="Testosterone" /></td>
<td></td>
<td>3.32</td>
<td>288.4</td>
</tr>
<tr>
<td>Steroid hormone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Gammarus fossarum

- Amphipod crustacean
- High density in rivers
- Invasive specie
- Acts as a natural filter for many substances
- Size ≈ 1 cm
- Wet weight ≈ 20 mg ⇔ ≈ 6 mg dry weight

Miniaturiation of the analytical method
Analytical strategy

QuEChERS
- Liquid-Liquid extraction assisted by salt
- Purification using dispersive phase
- On-line preconcentration
- Separation
- Detection using MRM$^3$ mode

• Quantification using matrix-match calibration
• Validation according ICH recommendations
Miniaturisation and optimisation of QuEChERS methodology

Quick
Easy
Cheap
Effective
Rugged
Safe

Extraction and purification at individual scale
Miniaturisation and optimisation of QuEChERS methodology

Conditions tested:
- Nature of the dispersive phase:
  - PSA
  - PSA/C18
  - Z-SEP (based on zirconia dioxyde)
- Mass of the dispersive phase

Average recoveries of targeted compounds (50 ng/g) after extraction and clean-up using 80 mg of three different sorbents (n=2)

Average recoveries of targeted compounds (50 ng/g) after extraction and clean-up using different masses of PSA/C18 (n=2)
NanoLC: why use it?

Decrease of internal diameter of the column:
- Decrease of the injection volume
- Decrease of the optimal flow
  - Increase of the ionisation recovery associated with electrospray source
  - Increase of the sensitivity in detection
  - Increase of the sensitivity in detection
- Decrease of solvent consumption

However:

Decrease of the injection volume:
- Decrease of the quantity available for the detection
- Hence the need to use a larger injection volume technique
NanoLC analysis

Step 1: On-line preconcentration
- Injection volume: 1µL
- µ-precolumn: C18 Acclaim PepMap 150 0.3 x 5 mm, 5 µm
- Optimized using an experimental design

Final conditions:

<table>
<thead>
<tr>
<th>Loading time</th>
<th>Loading Flow</th>
<th>Loading solvent</th>
<th>Sample composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 min</td>
<td>30 µL/min</td>
<td>H₂O/MeOH 98/2 + 0.05% acetic acid</td>
<td>H₂O/MeOH 90/10</td>
</tr>
</tbody>
</table>

Step 2: Elution and separation
- Nano-column: C18 Acclaim PepMap 150 75 µm x 15 cm, 3 µm
- Mobile phases:
  - A: H₂O + 0.05% acetic acid
  - B: MeOH + 0.05% acetic acid
- Flow: 400 nL/min
- Analysis time: 30 minutes
Detection by MRM$^3$ mode

Q1
Selection of a precursor ion

q2
Collision-induced dissociation

Hybrid Q3/LIT
Selection of a product ion
Formation of sub-product ions
Resonant excitation

MRM mode
MRM$^3$ mode

In complex matrices:
- Increase of sensibility
- Increase of specificity

Fortin et al., Anal. Chem. 81 (2009)

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Optimisation of MRM$^3$ mode

- Parameters common to both MRM and MRM$^3$ mode:
  - Declustering potential (DP)
  - Entrance potential in collision cell (EP)
  - Collision energy (CE)

- Specific parameters of MRM$^3$
  - Accumulation time in the linear ion trap
  - Excitation time
  - Excitation energy (AF2)

Exemple of testosterone:

\[
\begin{align*}
\text{Precursor ion (m/z)} & \quad 289.4 \\
\text{Product ion (m/z)} & \quad 109.1 \\
\text{Sub-product ion (m/z)} & \quad 79 \\
\text{DP (V)} & \quad 126 \\
\text{EP (V)} & \quad 10 \\
\text{CE (V)} & \quad 31 \\
\text{AF2 (V)} & \quad 0.12
\end{align*}
\]

Influence of excitation energy on intensity of fragmentation in MRM$^3$ mode with the transition 189.4/109.1/79.0 m/z of the testosterone.
Performance of the analytical method

Comparison between MRM and MRM$^3$ mode:

<table>
<thead>
<tr>
<th></th>
<th>LOQ MRM (ng/g ww)</th>
<th>LOQ MRM$^3$ (ng/g ww)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbamazepine</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Testosterone</td>
<td>4.7</td>
<td>2.2</td>
</tr>
</tbody>
</table>
Application to real sample: exposure experiment

- Exposure solution: 200 ng/L of a mix of carbamazepine, oxazepam and testosterone
- One beaker per sampling time:
  - 12h-24h-48h-72h-7 days-14 days (bioaccumulation)
  - 24h-48h (depuration)
- The exposure water is renewed everyday
- The gammarids were fed with alder leaves
- Each beaker contained 15 gammarids
- 10 gammarids were sampled per beaker

Thermostated bath: 12°C
Kinetics of bioconcentration of carbamazepine and oxazepam in *G. fossarum* exposed at 200 ng/L

### Concentrations in water (*C*<sub>w</sub>) and in gammarids at bioconcentration steady state (*C*<sub>org</sub>) and bioconcentration factor (BCF)

<table>
<thead>
<tr>
<th>Time of exposure</th>
<th>Carbamazepine (RSD,%)</th>
<th>Oxazepam (RSD,%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12h</td>
<td>&lt;LOQ</td>
<td>23.4</td>
</tr>
<tr>
<td>24h</td>
<td>&lt;LOQ</td>
<td>37.4</td>
</tr>
<tr>
<td>Day 2</td>
<td>NC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.1</td>
</tr>
<tr>
<td>Day 3</td>
<td>8.0</td>
<td>18.1</td>
</tr>
<tr>
<td>Day 7</td>
<td>12.9</td>
<td>23.1</td>
</tr>
<tr>
<td>Day 14</td>
<td>18.6</td>
<td>10.9</td>
</tr>
<tr>
<td>24h of depuration</td>
<td>&lt;LOQ</td>
<td>NC&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>48h of depuration</td>
<td>&lt;LOQ</td>
<td>27.1</td>
</tr>
</tbody>
</table>

### Evaluation of the inter-individual variability of gammarids exposed to carbamazepine and oxazepam

\[
BCF = \frac{C_{org}}{C_w}
\]

- "<LOQ" indicates below the limit of quantification.
- "NC" indicates not calculated.

<sup>a</sup>measured on days 3-7-14
<sup>b</sup>measured on days 7 and 14
<sup>c</sup>mean *C*<sub>org</sub>/*C*<sub>w</sub> ± 3SD
<sup>d</sup>measured in water of exposure
Conclusion and outlooks

- Quantification of three pharmaceuticals in the amphipod crustacean *Gammarus fossarum*
  - Carbamazepine
  - Oxazepam
  - Testosterone

- Miniaturisation and optimisation of the sample preparation by microQuEChERS methodology

- First application of the nanoLC coupled to mass spectrometry using MRM³ mode for the analysis of small molecules:
  - Increase of the sensitivity
  - Increase of the specificity

- Application to real sample:
  - Evaluation the kinetics of accumulation and depuration of these molecules by *G. fossarum*
  - Evaluation the inter-individual variabilities for bioaccumulation

Outlooks:

- Discriminate the experimental variability and the real inter-individual variability

- Evaluation of the kinetics of apparition of potential metabolites
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