HILIC coupled to mass spectrometry for targeted and non-targeted metabolomics

Marja Lamoree
Why metabolomics for environmental quality assessment?

Limitations of (in vitro) bioassays:

> Focus on one specific endpoint

> Limited sensitivity

Metabolomics

❖ Focus on multiple endpoints (metabolites or sets of metabolites) simultaneously

❖ Includes *in-vivo* metabolism
Aim: study sub-lethal neurotoxic effects in non-target invertebrate species

- Non-targeted metabolomics: method development to investigate changes in (non-target) hydrophilic and hydrophobic metabolites

- Targeted metabolomics: method development for neurotransmitter profiling of *L. stagnalis*

- Apply both approaches to snails exposed to
  - i) imidacloprid
  - ii) a surface water extract
Background - Imidacloprid

- Neonicotinoids - novel generation of neuro-active insecticides
- High water solubility (610 mg/L in 20°C H₂O; log $K_{ow} = 0.57$)
- High environmental occurrence
- Neonicotinoid insecticides probably affect postsynaptic nicotinic acetylcholine receptors (nAChRs)
- Evidence of a connection to honey bee colony collapse
- EU imposed a number of (temporary) use restrictions but its use is still allowed in greenhouses
- Low affinity for the nAChRs of mammals has been shown, but what for invertebrates and aquatic species?

In the Netherlands:

- $> 1 \mu g/L$
- $< 1 \mu g/L$
- $< 0.1 \mu g/L$

Macro-Invertebrate Decline in Surface Water Polluted with Imidacloprid

Tessa C. Van Dijk, Marja A. Van Staalduinen, Jeroen P. Van der Sluijs* PLOSone 2013
**Lymnaea stagnalis** organ dissection

**Sample pretreatment:**
Snap freezing in liquid nitrogen to quench the metabolic activity

16 week old snails, ± 2.85 cm shell length
Metabolomics workflow

Study design
Extraction of metabolites
Chemical analysis

Data analysis
Metabolite screening/identification

Biological interpretation

Study design
Extraction of metabolites
Chemical analysis

Data analysis
Metabolite screening/identification

Biological interpretation
A tissue normalization step was included to normalize data.

A mix of internal standards of different metabolite classes was added to normalize the signal intensities and to align retention times.
Analytical strategy

**Lymnaea stagnalis**
Central nervous system

### Samples
- **MeOH/H₂O fraction**
  - **TARGETED ANALYSIS**
    - Neurotransmitters
    - LC-QQQ
  - **NONTARGETED ANALYSIS**
    - Polar metabolites
    - HILIC - (ESI) TOF
- **Chloroform fraction**
  - **NONTARGETED ANALYSIS**
    - Nonpolar metabolites
    - GC – (APCI) TOF

### SNAIL CNS
- **EXTRACTION**
- **ANALYTICAL STRATEGY**
- **ANALYTICAL METHOD**
Metabolic pathways investigated

- Citrate cycle (TCA cycle)
- Glycolysis / Gluconeogenesis
- Ascorbate and aldarate metabolism
- Purine metabolism
- Pyrimidine metabolism
- Tyrosine metabolism
- Tryptophan metabolism
- Phenylalanine metabolism
- Alanine, aspartate and glutamate metabolism
- Arginine and proline metabolism
- Histidine metabolism
- Biosynthesis of unsaturated fatty acids
- Fatty acid biosynthesis
- Lipid Metabolism
- Amino Acids Metabolism
- Nucleotide metabolism
- Carbohydrate Metabolism
- Other

In total > 70 metabolites covered

Tufi et al., Anal Bioanal Chem 2015
HILIC, RP-LC and GC analysis

Waters **XBridge Amide** column, 150×2.1 mm, 3.5 μm; Amide guard column 10×2.1 mm, 3.5 μm. Gradients of ACN/H2O 30:70 v/v NH4HCOOH 10 mM (A) and ACN/H2O 95:5 v/v ammonium acetate 10 mM (B); for **pH 9.0** adjustment with ammonium hydroxide and for **pH 2.8** with HCOOH to pH 2.8. For both LC approaches, the separation occurred at 30 °C, the flow rate was set to 0.25 ml/min, and the injection volume was 5 μL.

Waters **C18** Symmetry column, 150×2.1 mm, 3.5 μm; C18 guard column 10×2.1 mm, 3.5 μm. Gradient of H2O and ACN with 0.1 % formic acid.

GC-APCI Derivatization of chloroform fractions with 500 μl of a methanolic BF₃ solution, kept for 30 min at 80 °C, with subsequent liquid/liquid extraction with hexane.

HR-TOF-MS on MICROTOF II, Bruker

*Tufi et al., Anal Bioanal Chem 2015*
- Each analytical platform has its own specific performance for the different metabolite classes
- HILIC represents an important chromatographic method for polar metabolites

Tufi et al., Anal Bioanal Chem 2015
Molecular features

**GC-APCI(+) > HILIC-ESI(+) > RPLC-ESI(+)**

*Tufi et al., Anal Bioanal Chem 2015*
Targeted analysis of neurotransmitters, precursors and metabolites

Tufi et al., J. Chromatogr. A 2015
## HILIC columns tested

Physico-chemical parameters of the HILIC columns tested evaluated using a ToF-MS.

<table>
<thead>
<tr>
<th>Column</th>
<th>Provider</th>
<th>Functionality/Support</th>
<th>Size (mm)</th>
<th>Particle size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XBridge HILIC</td>
<td>Waters</td>
<td>Silica/Silica</td>
<td>4.6 x 100</td>
<td>2.5</td>
</tr>
<tr>
<td>XBridge Amide</td>
<td>Waters</td>
<td>Amide/Silica</td>
<td>2.1 x 100</td>
<td>3.5</td>
</tr>
<tr>
<td>TSKgel Amide-80</td>
<td>Tosoh</td>
<td>Amide/Silica</td>
<td>2.0 x 100</td>
<td>5</td>
</tr>
<tr>
<td>Asahipak NH2P-50 2D</td>
<td>Shodex</td>
<td>Amino/Polyvinyl alcohol Amino</td>
<td>2.0 x 150</td>
<td>5</td>
</tr>
<tr>
<td>ZIC-cHILIC</td>
<td>Merck-SEQUANT</td>
<td>Zwitterionic/Silica</td>
<td>2.1 x 150</td>
<td>3</td>
</tr>
<tr>
<td>ZIC-pHILIC</td>
<td>Merck-SEQUANT</td>
<td>Zwitterionic/Polymeric</td>
<td>2.1 x 150</td>
<td>5</td>
</tr>
</tbody>
</table>
Separation factor $k (\log_2)$ distribution
Heat map and hierarchical clustering analysis of peak areas

Tufi et al., J. Chromatogr. A 2015
HILIC-QqQ chromatogram of 20 neurotransmitters, precursors and metabolites

0.3 ml/min; gradient of 100% H₂O and 90:10 ACN:H₂O (v/v)
### Method validation parameters

Tufi et al., J. Chromatogr. A 2015

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Labeled IS</th>
<th>RT</th>
<th>RSD %; RT Intraday</th>
<th>RSD %; RT Interday</th>
<th>Linear range (ng/mL)</th>
<th>$R^2$</th>
<th>LOD (ng/mL)</th>
<th>LOQ (ng/mL)</th>
<th>$R^%$</th>
<th>RSD % Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-MT</td>
<td>3-MT-d$_4$</td>
<td>7.7</td>
<td>1.0</td>
<td>6.2</td>
<td>5–3000</td>
<td>0.996</td>
<td>1.11</td>
<td>3.3</td>
<td>95</td>
<td>2.2</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>5-HIAA-d$_5$</td>
<td>3.4</td>
<td>1.6</td>
<td>4.1</td>
<td>10–2500</td>
<td>0.990</td>
<td>3.85</td>
<td>12</td>
<td>90</td>
<td>2.3</td>
</tr>
<tr>
<td>5-Hydroxy-L-tryptophan</td>
<td>5-Hydroxy-L-tryptophan-d$_4$</td>
<td>12.2</td>
<td>1.0</td>
<td>2.6</td>
<td>20–3000</td>
<td>0.975</td>
<td>3.68</td>
<td>11</td>
<td>73</td>
<td>6.8</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>Acetylcholine-d$_4$</td>
<td>3.0</td>
<td>0.5</td>
<td>4.8</td>
<td>0.05–1000</td>
<td>0.998</td>
<td>0.02</td>
<td>0.1</td>
<td>92</td>
<td>1.1</td>
</tr>
<tr>
<td>Choline</td>
<td>Choline-d$_{13}$</td>
<td>6.3</td>
<td>0.9</td>
<td>5.4</td>
<td>1–1000</td>
<td>0.990</td>
<td>0.35</td>
<td>1.0</td>
<td>85</td>
<td>2.1</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>DOPA-d$_3$</td>
<td>13.3</td>
<td>0.5</td>
<td>4.7</td>
<td>100–3000</td>
<td>0.902</td>
<td>19.5</td>
<td>58</td>
<td>84</td>
<td>9.4</td>
</tr>
<tr>
<td>Dopamine</td>
<td>Dopamine-d$_4$</td>
<td>11.4</td>
<td>0.4</td>
<td>3.8</td>
<td>20–2500</td>
<td>0.995</td>
<td>5.50</td>
<td>17</td>
<td>86</td>
<td>2.7</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>Epinephrine-$^{13}\text{C}_2\text{ }^{15}\text{N}$</td>
<td>11.6</td>
<td>0.3</td>
<td>4.4</td>
<td>20–2500</td>
<td>0.986</td>
<td>5.56</td>
<td>17</td>
<td>87</td>
<td>3.3</td>
</tr>
<tr>
<td>GABA</td>
<td>GABA-d$_6$</td>
<td>12.1</td>
<td>0.3</td>
<td>3.5</td>
<td>40–3000</td>
<td>0.979</td>
<td>19.3</td>
<td>58</td>
<td>81</td>
<td>4.2</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Glutamate-d$_5$</td>
<td>13.6</td>
<td>0.8</td>
<td>2.2</td>
<td>20–3000</td>
<td>0.960</td>
<td>2.47</td>
<td>7.4</td>
<td>63</td>
<td>18</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Glutamine-$^{13}\text{C}$-$^{15}\text{N}$</td>
<td>13.3</td>
<td>0.3</td>
<td>2.5</td>
<td>5–3000</td>
<td>0.948</td>
<td>1.51</td>
<td>4.5</td>
<td>79</td>
<td>1.7</td>
</tr>
<tr>
<td>Histamine</td>
<td>L-Tryptophan-d$_3$</td>
<td>18.3</td>
<td>0.5</td>
<td>1.5</td>
<td>100–3000</td>
<td>0.973</td>
<td>25.2</td>
<td>76</td>
<td>89</td>
<td>1.7</td>
</tr>
<tr>
<td>Histidine</td>
<td>L-Tryptophan-d$_3$</td>
<td>15.8</td>
<td>0.4</td>
<td>2.5</td>
<td>20–4000</td>
<td>0.930</td>
<td>0.31</td>
<td>0.9</td>
<td>89</td>
<td>1.7</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>L-Tryptophan-d$_3$</td>
<td>10.7</td>
<td>0.4</td>
<td>3.2</td>
<td>5–2500</td>
<td>0.993</td>
<td>0.54</td>
<td>1.6</td>
<td>89</td>
<td>1.7</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>L-Tyrosine-d$_4$</td>
<td>12.1</td>
<td>0.5</td>
<td>3.2</td>
<td>40–3000</td>
<td>0.948</td>
<td>8.22</td>
<td>25</td>
<td>72</td>
<td>3.0</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>Norepinephrine-d$_6$</td>
<td>12.7</td>
<td>0.5</td>
<td>4.2</td>
<td>40–2500</td>
<td>0.942</td>
<td>13.2</td>
<td>40</td>
<td>89</td>
<td>4.2</td>
</tr>
<tr>
<td>Nornetanephrine</td>
<td>Nornetanephrine-d$_6$</td>
<td>10.7</td>
<td>0.2</td>
<td>3.6</td>
<td>1–1000</td>
<td>0.995</td>
<td>0.28</td>
<td>0.9</td>
<td>89</td>
<td>4.2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Epinephrine-$^{13}\text{C}_2\text{ }^{15}\text{N}$</td>
<td>10.3</td>
<td>1.0</td>
<td>3.2</td>
<td>0.1–1500</td>
<td>0.988</td>
<td>0.03</td>
<td>0.1</td>
<td>87</td>
<td>3.3</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Serotonin-d$_4$</td>
<td>10.5</td>
<td>0.8</td>
<td>3.9</td>
<td>0.5–2500</td>
<td>0.994</td>
<td>0.10</td>
<td>0.3</td>
<td>89</td>
<td>1.6</td>
</tr>
<tr>
<td>Tyramine</td>
<td>3-MT-d$_4$</td>
<td>9.7</td>
<td>0.5</td>
<td>4.8</td>
<td>1–1000</td>
<td>0.997</td>
<td>0.35</td>
<td>1.0</td>
<td>95</td>
<td>2.2</td>
</tr>
</tbody>
</table>
**Exposure to imidacloprid**

**Conditioning period of 2 days for adaptation**  
Refreshing and egg counting every second day

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Day 8</th>
<th>Day 9</th>
<th>Day 10</th>
<th>Day 11</th>
<th>Day 12</th>
<th>Day 13</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selection</td>
<td>Condition Condition</td>
<td>Exposure</td>
<td>Exposure</td>
<td>Exposure</td>
<td>Exposure</td>
<td>Exposure</td>
<td>Exposure</td>
<td>Exposure</td>
<td>Exposure</td>
<td>Exposure</td>
<td>Exposure</td>
<td>Exposure</td>
<td>Dissection</td>
</tr>
<tr>
<td>Food</td>
<td>Refreshing</td>
<td>Egg counting</td>
<td>Food</td>
<td>Egg counting</td>
<td>Food</td>
<td>Egg counting</td>
<td>Food</td>
<td>Egg counting</td>
<td>Food</td>
<td>Egg counting</td>
<td>Food</td>
<td>Egg counting</td>
<td>Egg counting</td>
</tr>
</tbody>
</table>

**10 DAYS**

**CTRL GROUP**
- 0.1 µg/L
- 1 µg/L
- 10 µg/L
- 100 µg/L

*Tufi et al., under review* Env Sci Technol 2015
Data analysis workflow

- Metabolic changes visible at low, environmentally relevant concentrations
- Indication of inflammation and neuron cell injury
- Involvement of the cholinergic system, possibly through an increase in cholinergic gene expression
- Indication of GABA receptor antagonist activity
- Insight in the involvement of different or unexpected metabolic pathways
Acknowledgment

• Victoria Osorio Torrens and the EDA-EMERGE network
• Pim Leonards
• Jacob de Boer

THANK YOU

More info: sara.tufi@vu.nl
marja.lamoree@vu.nl