High-throughput determination of neonicotinoid insecticides in pollen and nectar using liquid chromatography with tandem mass spectrometry detection.

Celebrating 30 Years Serving Ag

Excellence, Passion and Leadership in Agriculture
Overview

• Purpose of Methodology
• Analytes
• Matrices
• Sample Size
• Detection/Quantitation Limits
• Analytical Methodology
Purpose

• Support Pollinator Health Studies
  – Magnitude Of Residue (MOR)
  – Feeding/Dosing Studies
Analytes

- Thiamethoxam (TMX)

- Clothianidin (CLO)
Matrices and Challenges

- Leaves and Flowers
- Nectar
- Pollen
- Reproductive structures (Anthers)
- Greatest analytical challenges
Sample Size

- Nectar: 100-300 mg
- Pollen: 5-100 mg
- “One and done” for most samples
- No sample homogenization
- Good fit with high throughput technology
- No cryomilling!!!
Sample Size Comparison
Initial Conventional Procedure

- Weigh 100 mg sample into a 15 mL centrifuge tube. Add 2 mL of extraction solvent and shake/vortex.
- Centrifuge and transfer 2.0 mL of the supernatant extract to another centrifuge tube. Add 4.0 mL of DI water to the tube.
- Condition Waters HLB SPE columns with methanol, then water.
- Transfer the diluted sample to the SPE and elute under vacuum.
- Rinse the SPE cartridge with 2 mL hexane and apply vacuum to dry.
- Elute compounds of interest with 6 mL of ACN.
- Solvent exchange to 90:10 water:methanol, with a 1 mL target final volume.
Sample Clean Up with SPE

1. Condition Sorbent
2. Apply sample & analyte
3. Interference elution
4. Analyte elution

Eluted Interferences

Analyte
LOQ / LOD

- Nectar: TMX/CLO LOQ = 0.5 ppb LOD = 0.25 ppb
- Pollen/Anthers: TMX/CLO LOQ = 1.0 ppb LOD = 0.5 ppb
- 2.1x100 mm XB-C18 Column, AB Sciex 6500
Calibration Range
0.004 ng/mL to 1.0 ng/mL

Calibration for Thiamethoxam (Q): y = 1.00076 x + 0.01551 (r = 0.99999) (weighting: 1/x)
Recovery

- Sample recovery can be measured using spiking experiments.
- Controls are samples in a study that are not treated with the analyte that is being applied and should be free of the analyte.
- In the lab subsamples of the control have a known amount of the analyte added to them. This sample is now considered a spiked sample. Spike samples are usually prepared in triplicate.
- The spiked sample is prepared using the proposed procedure and analyzed.
- The recovery is calculated by dividing the amount measured by the amount added to the sample. The recovery is usually expressed as a percentage.
## Recovery From Citrus Nectar
### conventional methodology

<table>
<thead>
<tr>
<th>Fort. Level (ppb)</th>
<th>Analyte</th>
<th>Recovery % (n=3)</th>
<th>Std. Dev %</th>
<th>RSD %</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOQ (0.5 ppb)</td>
<td>Thiamethoxam</td>
<td>87</td>
<td>7.7</td>
<td>8.8</td>
</tr>
<tr>
<td>10x LOQ (5 ppb)</td>
<td>Thiamethoxam</td>
<td>89</td>
<td>0.58</td>
<td>0.65</td>
</tr>
<tr>
<td>Overall (n=6)</td>
<td>Thiamethoxam</td>
<td>88</td>
<td>4.9</td>
<td>5.6</td>
</tr>
<tr>
<td>LOQ (0.5 ppb)</td>
<td>Clothianidin</td>
<td>80</td>
<td>7.3</td>
<td>9.1</td>
</tr>
<tr>
<td>10x LOQ (5 ppb)</td>
<td>Clothianidin</td>
<td>92</td>
<td>2.6</td>
<td>2.9</td>
</tr>
<tr>
<td>Overall (n=6)</td>
<td>Clothianidin</td>
<td>86</td>
<td>8.2</td>
<td>9.6</td>
</tr>
</tbody>
</table>
Modified Procedure

- Weigh 100 mg sample into a 50 mL centrifuge tube or cut pipette tip containing pollen into a centrifuge tube. Add 4 mL of extraction solvent and shake/vortex.
- Centrifuge and transfer 2.0 mL of the supernatant extract to another centrifuge tube. Add 8.0 mL of DI water to the tube.
- Condition Waters HLB SPE columns with methanol, then water.
- Transfer the diluted sample to the SPE and elute under vacuum.
- Rinse the SPE cartridge with 2 mL hexane and apply vacuum to dry.
- Elute compounds of interest with 6 mL of ACN
- Solvent exchange to 90:10 water:methanol, with a 1 mL target final volume.
- Final volume is adjusted for pollen samples to match the weight.
Pipette Tip Processing
Processed Pollen Sample
## Recovery From Hive Pollen

### conventional methodology

<table>
<thead>
<tr>
<th>Fort. Level (ppb)</th>
<th>Analyte</th>
<th>Recovery % (n=3)</th>
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</thead>
<tbody>
<tr>
<td>LOQ (1.0 ppb)</td>
<td>Thiamethoxam</td>
<td>23</td>
<td>2.3</td>
<td>10.2</td>
</tr>
<tr>
<td>10x LOQ (10 ppb)</td>
<td>Thiamethoxam</td>
<td>20</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Overall (n=6)</td>
<td>Thiamethoxam</td>
<td>22</td>
<td>2.0</td>
<td>9.5</td>
</tr>
<tr>
<td>LOQ (1.0 ppb)</td>
<td>Clothianidin</td>
<td>52</td>
<td>7.2</td>
<td>13.9</td>
</tr>
<tr>
<td>10x LOQ (10 ppb)</td>
<td>Clothianidin</td>
<td>43</td>
<td>1.9</td>
<td>4.3</td>
</tr>
<tr>
<td>Overall (n=6)</td>
<td>Clothianidin</td>
<td>47</td>
<td>6.9</td>
<td>14.5</td>
</tr>
</tbody>
</table>
Matrix Suppression (e.g. Pollen)

Sample at LOQ
Area: 5461

Standard at LOQ
Area: 24152
Improvement Opportunities

• Stable Isotope Internal Standards
  - Compensate for suppression
  - Commercially available
  - Deuterated forms
TMX and CLO IS commercially available

Pollen Suppression is severe and variable from sample to sample

Matrix match standards not viable, sample amounts too small to produce matrix matched standards

Commercially available pollen is hive pollen and is not representative of flower pollen

Internal standards are added to both standards and samples. Based on the response of the internal standard in the samples compared to the standards a correction is applied to the sample results.
Matrix Variability

- Internal Standard Response
Analytical Methodology
(Internal Standards)

– Weigh 100 mg sample into a centrifuge tube or cut pipette tip containing pollen into a centrifuge tube. Add 4 mL of extraction solvent and shake/vortex.
– Centrifuge and transfer 2.0 mL of the supernatant extract to another centrifuge tube. Add 8.0 mL of DI water to the tube.
– Internal standard added to samples prior to SPE.
– Condition Waters HLB SPE columns with methanol, then water.
– Transfer the diluted sample to the SPE and elute under vacuum.
– Rinse the SPE cartridge with 2 mL hexane and apply vacuum to dry. 
– Elute compounds of interest with 6 mL of ACN
– Solvent exchange to 90:10 water:methanol, with a 1 mL target final volume.
– Final volume is adjusted for pollen samples to match the weight.
## Recovery From Hive Pollen
### Internal Standard Methodology

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<tr>
<th>Fort. Level (ppb)</th>
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<tr>
<td>LOQ (1.0 ppb)</td>
<td>Thiamethoxam</td>
<td>103 (23)</td>
<td>9.9</td>
<td>9.6</td>
</tr>
<tr>
<td>10x LOQ (10 ppb)</td>
<td>Thiamethoxam</td>
<td>84 (20)</td>
<td>5.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Overall (n=6)</td>
<td>Thiamethoxam</td>
<td>93 (22)</td>
<td>12.6</td>
<td>13.5</td>
</tr>
<tr>
<td>LOQ (1.0 ppb)</td>
<td>Clothianidin</td>
<td>99 (52)</td>
<td>14.0</td>
<td>14.1</td>
</tr>
<tr>
<td>10x LOQ (10 ppb)</td>
<td>Clothianidin</td>
<td>90 (43)</td>
<td>2.1</td>
<td>2.4</td>
</tr>
<tr>
<td>Overall (n=6)</td>
<td>Clothianidin</td>
<td>95 (47)</td>
<td>10.3</td>
<td>10.9</td>
</tr>
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</table>
The Path Forward

• High throughput technology
  - Geno Grinder for extraction
  - 96 well plates with SPE
  - 96 well plate automation
96 Well Plate

- High throughput technology
- Space saving in weigh room
- Stainless steel beads added for thorough extraction
- Fast and simple sample closure
Bench space!

- High throughput technology
  - No cumbersome racks.
  - No caps to take on and off.
High Throughput Equipment

- Geno Grinder for extraction
  - Up to 6 well plates (576 samples at a time)
- Centrifugation of samples
  - Up to 4 well plates (384 samples at a time)
96 Well Plate SPE

• High throughput technology
  - Same sorbent beds as 3 or 6 ml tubes
  - Space saving on Lab bench
  - Easier to collect samples eleunts
96 Well plate SPE space requirements

A single 96 well plate takes the place of 4 standard 24 place SPE sample manifold.
High Throughput Procedure

- Weigh 50 mg sample into a 96 well plate. Add 1.0 mL of extraction solvent, a 2 mm stainless steel bead and cap.
- Shake on Geno grinder for 5 minutes.
- Centrifuge and transfer 0.5 mL of the supernatant extract to another 96 well plate. Add 1.0 mL of DI water to the tube.
- Internal standard added to samples prior to SPE.
- Condition Waters HLB SPE columns with methanol, then water.
- Transfer the diluted sample to the SPE and elute under vacuum.
- Rinse the 96 well SPE with 2 mL hexane and apply vacuum to dry.
- Elute compounds of interest with 2 mL of ACN
- Solvent exchange to 90:10 water:methanol, with a 0.5 mL target final volume.
- Final volume can be adjusted for pollen samples to match the weight.
### 96 Well Recovery From Hive Pollen
#### Internal Standard Methodology

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<tr>
<th>Fort. Level (ppb)</th>
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<td>LOQ (1.0 ppb)</td>
<td>Thiamethoxam</td>
<td>107 (103)</td>
<td>14.9</td>
<td>13.8</td>
</tr>
<tr>
<td>10x LOQ (10 ppb)</td>
<td>Thiamethoxam</td>
<td>108 (84)</td>
<td>4.7</td>
<td>4.4</td>
</tr>
<tr>
<td>Overall (n=6)</td>
<td>Thiamethoxam</td>
<td>108 (93)</td>
<td>9.9</td>
<td>9.1</td>
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<tr>
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<td>Clothianidin</td>
<td>96 (99)</td>
<td>2.6</td>
<td>2.7</td>
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<tr>
<td>10x LOQ (10 ppb)</td>
<td>Clothianidin</td>
<td>78 (90)</td>
<td>10.2</td>
<td>13.0</td>
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<td>Overall (n=6)</td>
<td>Clothianidin</td>
<td>87 (95)</td>
<td>11.8</td>
<td>13.6</td>
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</table>
Sample Handling

- Simplified transfer of extracts
  - 12 samples moved each time
  - Dilutions performed in multiples of 12
  - Less handling of samples
Sample Transfer

- Simplified transfer of extracts
  - Now the pipets tips can be transferred to 96 well plate
  - The samples can run through the processes going forward
High Throughput SPE

- Simplified SPE procedure
  - Common well for SPE washes
  - No need to worry about alignment of eluent collection tubes
  - Fast transfers from extraction plates.
High Throughput Solvent evaporation

- Current capability of 96 samples
- Can expand capability to 192 samples or more by adding additional evaporators.
Autosampler High Throughput

- 96 well plates fit into UPLC
- Possibility exists to perform dilution using autosampler
## Pros and Cons

<table>
<thead>
<tr>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Much less space used</td>
<td>Greater difficulty weighing</td>
</tr>
<tr>
<td>Less handling than tubes</td>
<td>More difficult to label</td>
</tr>
<tr>
<td>Much easier to transfer</td>
<td>Necessary to keep track of steps</td>
</tr>
<tr>
<td>3x less solvent use</td>
<td>15% more for 96 well plate vs tube</td>
</tr>
<tr>
<td>No centrifuge tubes or UPLC vials</td>
<td></td>
</tr>
<tr>
<td>Easy transfer to UPLC autosampler</td>
<td></td>
</tr>
<tr>
<td>4x more samples in same time</td>
<td></td>
</tr>
</tbody>
</table>
Questions