A Multi-Class Method for the Determination of Antibiotics and Growth Promoters in Aquaculture Products by LC-MS

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2017 North American Chemical Residue Workshop

July 26, 2017
Outline

1. Overview
2. Current Testing
3. New Multi-Class Method
4. Method Validation
5. Confirmation Approaches
6. Handling Difficult Compounds
7. Conclusion
Outline

1. Overview
   - CFIA Food Safety Laboratory Network
   - Dartmouth Lab
2. Current Testing
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7. Conclusion
CFIA Food Safety Labs

• CFIA created in 1997
  – Mission Statement: “Dedicated to safeguarding food, animals and plants, which enhances the health and well-being of Canada's people, environment and economy”

• 9 food safety laboratories across Canada
  – Other laboratories for plant and animal health

• 7 Chemistry laboratories specialized to deliver specific testing programs
Dartmouth Lab

Dartmouth Laboratory is the national center of expertise for:

- Therapeutants in fish
- Toxic elements in food
- Marine shellfish biotoxins

Accredited by Standards Council of Canada

- CAN-P-4E (ISO 17025)
- ISO 1595
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Current Testing
Therapeutants in Fish

- Constantly expanding scope of analysis
- Antibiotics, pesticides and growth promoters
- Currently 14 single class methods

- 4 Tetracyclines (TC)
- 20 Sulfonamides (SULF)
- 11 Quinolones (FQL)
- 5 Triphenylmethane Dyes (DYES)
- 4 Amphenicols (FEN)
- 1 Macrolide (ERY)
- 7 Nitroimidazoles (NI)
- 3 Stilbenes (SB)
- 5 Steroids (STR)

- 4 Nitrofurans (NF)
- 2 Avermectins (IVR/EMA)
- 1 Benzoylureas (TEF)
- 2 Pyrethroids (CYP)
- 4 Carbapenems (CARB)

Starting Fall 2017
Outline

1. Overview
2. Current Testing
3. New Multi-Class Method
   - Extraction
   - Chromatography
4. Method Validation
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New Multi-Class Method

- Simple acidic organic extraction
- Remove some organic phase with N₂
- Spin to remove particulate
- Analysis by LC-MS
- Matrix matched calibration standards

1. Weigh sample (2g)
2. Add acidic MeCN (80% + 0.1% FA)
3. Homogenize, sonicate, centrifuge (5,250 g’s)
4. Transfer supernatent and make to 20 mL
5. Evaporate 5 mL portion to 3 mL
6. Reconstitute to 5 mL w/ H₂O
7. Centrifuge 1 mL portion (16k g’s)
8. Analyze (LC-MS)
New Multi-Class Method

- 2 injections per sample to optimize peak shape and sensitivity

### UHPLC Conditions

<table>
<thead>
<tr>
<th></th>
<th>Mobile Phase A</th>
<th>Mobile Phase B</th>
<th>Column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile Phase A</td>
<td>5 mM NH$_4$HCO$_2$ + 0.1% Formic Acid</td>
<td>0.1% AcOH in MeCN</td>
<td>50 x 2.1 mm, 1.8 µm Waters HSST3 (w/ guard)</td>
</tr>
<tr>
<td>Column</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow (mL/min)</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Column Temperature (°C)</td>
<td></td>
<td>30 °C</td>
<td></td>
</tr>
<tr>
<td>Injection Volume (µL)</td>
<td></td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

### Gradient Conditions

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>3.5</td>
<td>95</td>
</tr>
<tr>
<td>4.5</td>
<td>95</td>
</tr>
</tbody>
</table>
New Multi-Class Method

Instrumentation

Waters I-Class UPLC coupled to a TQ-S Micro
New Multi-Class Method

Instrumentation

Agilent 1290 UHPLC coupled to a Sciex 5500 QTRAP
New Multi-Class Method
Instrumentation

Waters 1st Gen UPLC coupled to a Thermo Q-Exactive for HRAM work
New Multi-Class Method

Ammonium Formate

Time (Min)

Sulfonamides
TPMDs
Quinolones
Steroids
Erythromycin
Sulfonamides
TPMDs
Quinolones
Steroids
Erythromycin
New Multi-Class Method

Acetic Acid
New Multi-Class Method

Workflow

• Single transition monitored initially (screen)

• Re-inject/Re-extract suspected positives (confirm/quantify)
  – QqQ System with 2 or 3 transitions monitored
  – HRAM system with SIM – targeted MS/MS
    • 70k resolution
New Multi-Class Method

0.3 ng/g CAP In Shrimp
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Method Validation

Overview

1. Multiple goals of this method validation:
   - Ensure method is fit for purpose WRT LOQs, accuracy, precision, uncertainty, analyst/instrument bias, etc.
   - Prove QqQ confirmatory capability
   - Evaluate accurate mass confirmation criteria

2. Method to be used in a Food Safety Survey in September 2017
## Method Validation
### Detection Limits

<table>
<thead>
<tr>
<th>Class</th>
<th>LOD Range (ng/g)</th>
<th>LOQ Range (ng/g)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfonamides</td>
<td>0.2 – 1</td>
<td>0.3 – 3</td>
<td>SNL/SGD LOD @ 17/30</td>
</tr>
<tr>
<td>Quinolones</td>
<td>0.2 – 0.7</td>
<td>0.3 – 2</td>
<td></td>
</tr>
<tr>
<td>Amphenicols</td>
<td>0.1 – 0.7</td>
<td>0.3 – 2</td>
<td>FLRA LOD @ 30</td>
</tr>
<tr>
<td>Nitromidazoles</td>
<td>0.7 – 2</td>
<td>2 – 5</td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>4 – 7</td>
<td>10 – 20</td>
<td></td>
</tr>
<tr>
<td>TPMDs</td>
<td>0.1 – 0.3</td>
<td>0.3 – 1</td>
<td></td>
</tr>
<tr>
<td>Steroids</td>
<td>0.2 – 0.4</td>
<td>0.5 – 1</td>
<td></td>
</tr>
<tr>
<td>Stilbenes</td>
<td>0.5 – 1</td>
<td>2 – 3</td>
<td></td>
</tr>
</tbody>
</table>

Used USEPA 40 CFR, Part 136, Appendix B procedure for LODs.
Method Validation

Plan

• Salmon, Shrimp & Tilapia used as representative matrices

• For each matrix, mixed standard was spiked on tissue
  – 3 x levels (LOQ, 3LOQ, 25LOQ)
  – 5 x replicates/day
  – 3 days
  – Repeated by second analyst on another instrument with independently prepared solutions
Method Validation

Accuracy

Deviation from 100% Recovery

- 56 of 60 compounds between 80-110%

Outliers
- Erythromycin (~65%)
  - Formic acid in extraction
- TC & CTC (~75%)
  - Epimerization
- Sulfamoxole (~115%)
  - LOQ level = 144%
  - 3LOQ and 25 LOQ are fine
Method Validation

Precision @ LOQ

Single Analyst Repeatability
• 57 of 60 compounds ≤22%

Multi-Analyst Precision
• 56 of 60 compounds ≤22%
Method Validation

Precision @ 3 x LOQ

Single Analyst Repeatability
- 57 of 60 compounds ≤15%

Multi-Analyst Precision
- 53 of 60 compounds ≤15%
Method Validation
Precision @ 25 x LOQ

Single Analyst Repeatability
• 58 of 60 compounds ≤15%

Multi-Analyst Precision
• 57 of 60 compounds ≤15%

Outliers
• ERY
  • Acid Stability
  • Leuco Forms of TPMDs
  • Conversion?

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   - Unit Resolution QqQ
   - HRAM
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Confirmation Approaches

QqQ

- Same chromatographic method
- Monitor 2 or 3 structurally significant fragments
  - Maximize dwell time
- Criteria
  - Retention time ± 2.5%
  - Peak-to-Peak S/N of 3x for at least 2 transitions
  - Ion ratios within 2002/657/EC limits
    - (Typically ±20%)
Confirmation Approaches

HRAM

• Same Chromatographic Method

• Alternating SIM / Targeted MS/MS scans
  – 70k SIM resolution, 35k MS/MS resolution

• Criteria
  – Retention time ± 2.5%
  – ±5 ppm mass accuracy on SIM precursor and at least 2 fragments
Confirmation Approaches

HRAM

1.0 ng/g ENRO spiked in Salmon
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Handling Difficult Compounds

- Method is fit for purpose for the majority of compounds

- A few problematic compounds exist
  - Tetracyclines (epimerization)
  - Erythromycin (Accuracy & Precision)
  - TPMDs (Precision)
  - Poor chromatography for some of the polar early eluting compounds
Handling Difficult Compounds

• Tetracyclines:
  Recovery for TC and CTC is low due to conversion
  - ~95% recovery for total tetracycline
Handling Difficult Compounds

- Erythromycin recovery and precision poor
  - Acid in extraction solution causes breakdown
  - Even with low recovery, method is fit for purpose to screen for ERY
  - Targeted single class method for follow up quantitation
  - Evaluating ERY in ongoing carbapenem method validation
Handling Difficult Compounds

• Poor TPMD precision
  – Potentially caused by conversion between chromic and leuco forms
  – Radiolabelled internal standards available
  – Consider IS for future quantitative work
Handling Difficult Compounds

• Early Eluters
  – Small polar molecules partially un-retained when injected in ~40% MeCN
    • SNL, SGD, FLRA, MNZ-OH
  – Compromise on injection volume
  – “Peak” gives acceptable results
  – Fit for screening purposes
    • Confirm with single class method
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Conclusion

- Multi-Class method in aquaculture products validated for 60 compounds
  - Fully quantitative/confirmatory method for 52 compounds
  - Acceptable screening method for FLRA, SNL, SGD, LMG, LCV and ERY
  - Summed peaks are acceptable for “total” tetracyclines
  - QqQ and HRAM confirmatory options
Conclusion

• Replacing multiple single class methods with a multi-class method provides significant efficiency gains
  – Analyst time
  – Consumable costs
  – Instrument Capacity/Maintenance
  – Quality system maintenance requirements

• Increased laboratory testing capacity