Targeted Multiresidue Analysis of Veterinary Drugs In Dairy Products Using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

James Wittenberg, Kelli Simon, and Jon Wong

U.S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Regulatory Science
Monitoring Vet Drugs

**USDA**
- Meat
- Poultry
- Egg Products

**FDA**
- Animal drugs
- Animal feed / pet food
- Veterinary devices

**Center for Veterinary Medicine (CVM)**
- Animal drugs
- Animal feed / pet food
- Veterinary devices

**Center for Food Safety and Applied Nutrition (CFSAN)**
- All other food

**Office of Regulatory Affairs (ORA)**
- Field testing
## Vet Drugs In Milk and Dairy Products

### Development and Validation of a Multiclass Method for Analysis of Veterinary Drug Residues in Milk Using Ultrahigh Performance Liquid Chromatography Electrospray Ionization Quadrupole Orbitrap Mass Spectrometry

Jian Wang*, Daniel Leung, Willis Chow, James Chang, and Jon W. Wong

- SPE clean-up utilizing both aqueous and organic phases

### Multi-residue Quantification of Veterinary Drugs in Milk with a Novel Extraction and Cleanup Technique: Salting Out Supported Liquid Extraction (SOSLE)


- SLE clean-up
- 91% average recovery

### Challenges in Implementing a Screening Method for Veterinary Drugs in Milk Using Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry

Sherri B. Turnipseed*, Jack J. Lohne, Joseph M. Storey, Wendy C. Andersen, Susan L. Young, Justin R. Carr, and Mark R. Madson

- Screening LODs determined for 156 veterinary drugs using LC-QToF

### Multi-residue Determination of 115 Veterinary Drugs and Pharmaceutical Residues in Milk Powder, Butter, Fish Tissue and Eggs Using Liquid Chromatography-Tandem Mass Spectrometry

Marilena E. Dasenaki and Nikolaos S. Thomaidis*

- Precipitation of proteins and fats @ -23 °C for 12 h

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### Table: LODs for Veterinary Drugs

<table>
<thead>
<tr>
<th>Compound</th>
<th>LOD (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butter</td>
<td>≤10, 10-100, ≥100</td>
</tr>
<tr>
<td>Fish</td>
<td>≤10, 10-100, ≥100</td>
</tr>
<tr>
<td>Egg</td>
<td>≤10, 10-100, ≥100</td>
</tr>
<tr>
<td>Milk Powder</td>
<td>≤10, 10-100, ≥100</td>
</tr>
</tbody>
</table>

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**Analytica Chimica Acta, 2015, 880, 103-121**

**Analytica Chimica Acta, 2014, 820, 56-68**

**J. Agric. Food Chem., 2015, 63, 9175-9187**


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Project Goals

• Very few methods developed for veterinary drug determination in milk powders
  — 4.6 million metric tons of non-fat milk powder traded globally in 2015

• Create an analytical method capable of determining 60 veterinary drug residues in milk powders and other dairy products
  1) Develop and optimize an LC-MS/MS method for the target compounds
  2) Collect a number of milk powders (non-fat / fat-containing) for fortification studies
  3) Develop a sample preparation method and apply to milk powders
  4) Complete single-laboratory validation
  5) Complete multi-laboratory validation
  6) Extend method to other priority dairy products (cheese, butter, yogurt)
• 60 priority veterinary drugs of interest
  – 13 different classes of compounds with log P values ranging from −7.8 to +5.8
• 8 “Polar” compounds:

  Aminoglycosides (7)

  ![Aminoglycosides diagram]

  Coccidiostat (1)

  ![Coccidiostat diagram]

• Aminoglycosides are very polar compounds
  – Ion-pairing for RP chromatographic compatibility
  – Hydrophilic interaction chromatography (HILIC)
• No column / mobile phase combination resulted in retention of all compounds
  – HSS, Amide, HILIC, ZIC-HILIC
  – MeOH, ACN, H₂O with FA, AA, NH₄⁺ salts
• Addition of ion-pair reagent to final extract for analysis of >150 drug residues, including aminoglycosides, in food animal tissue (Lehotay, et al.)
• 52 “Nonpolar” compounds:

- Benzimidazoles (3)
- Nitroimidazoles (6)
- Sulfonamides (6)
- Quinolones (6)
- Amphenicols (2)
- Lincosamides (2)
- NSAIDs (7)
• Waters Acquity UPLC® coupled to Sciex Qtrap 6500
• Kinetex Biphenyl 2.6 µm, 2.1 × 100 mm @ 40 °C
• MP A = 5 mM NH₄HCO₂ + 0.1% FA in H₂O / MP B = 5 mM NH₄HCO₂ + 0.1% FA in MeOH
• Gradient = 5% B @ 0.5 min to 100% B @ 7 min, flow rate @ 0.5 mL/min, 10 min run
• 5 µL injection in 20% ACN in 0.1 M NH₄OAc
• Scheduled SRM with positive/negative polarity switching
• 2 transitions (quantitation/confirmation) monitored for each analyte

MS Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IonSpray Voltage</td>
<td>5500</td>
</tr>
<tr>
<td>Temperature</td>
<td>400</td>
</tr>
<tr>
<td>Curtain Gas</td>
<td>30</td>
</tr>
<tr>
<td>Ion Source Gas 1</td>
<td>50</td>
</tr>
<tr>
<td>Ion Source Gas 2</td>
<td>70</td>
</tr>
<tr>
<td>Collision Gas</td>
<td>“Medium”</td>
</tr>
<tr>
<td>Entrance Potential</td>
<td>10</td>
</tr>
<tr>
<td>Exit Potential</td>
<td>10</td>
</tr>
</tbody>
</table>
We are interested in analyzing tolerance levels in the powder (not reconstituted)
  – Advantageous if reconstitution not necessary

Extraction solution = high % organic solvent in H₂O / proteins and carbs will not dissolve

Lipid removal with dSPE or SPE
  – Agilent EMR dSPE = “polish” step required / poor recovery of polar compounds
  – Waters HLB PRiME SPE = optimal recovery for fat-containing samples
    ➢ Pass-through SPE
    ➢ Limited loading capacity
Sample Prep for Non-Fat Milk Powder

Weigh 1.0 g powder and place in 50 mL tube

Fortify, shake, and let sit for 15 min

Add 9 mL ACN and shake for 10 s, add 1 mL H₂O and SS ball bearing and shake for 30 min

Remove SS ball bearing and centrifuge for 10 min

Concentrate 5 mL supernatant to near-dryness with N₂ blow-down

Post-spike for MMCS / Reconstitute to 1 mL of 20% ACN in 0.1 M NH₄OAc

Filter through 0.2 µm PVDF, inject, and analyze by LC-MS/MS
Weigh 1.0 g powder and place in 50 mL tube

Fortify, shake, and let sit for 15 min

Add 9 mL ACN and shake for 10 s, add 1 mL H₂O and SS ball bearing and shake for 30 min

Remove SS ball bearing and centrifuge for 10 min

Pass 5 mL supernatant through Waters HLB PRiME SPE

Concentrate to near-dryness with N₂ blow-down

Post-spike for MMCS / Reconstitute to 1 mL of 20% ACN in 0.1 M NH₄OAc

Filter through 0.2 µm PVDF, inject, and analyze by LC-MS/MS

- Additional clean-up step needed for lipid removal from fat-containing powders
• Method detection limits (MDL) determined in non-fat milk powder
  – 40 CFR Pt. 136 Appendix B (8 replicates)
• LOQ = 3 × MDL
• Matrix-matched calibration curves established from 2-5 × LOQ and up
• Samples measured in triplicate
  – % RSD of < 20% required for acceptable repeatability
• Fortified at 3 levels
  – Below, at, and above tolerance level in liquid milk
  – Tolerances set for 18 out of 52 compounds ranging from 0 to 400 ppb
  – If no tolerance set, low level = 2-5 × LOQ
• Acceptable recovery range set to 60 – 120% for majority of compounds
  – FDA Foods Program Guidelines for Chemical Methods
• Recoveries measured without internal standard correction
  – No major benefit with ISTD correction
  – Simplifies method / no need for expensive isotopically-labeled standards
Recovery in Non-Fat Milk Powder

80% reach acceptable recovery at all levels

- **β-Lactams**
  - Amoxicillin
  - Ampicillin
  - Desacetyl Cephapirin
  - Cephapirin
  - Ceftiofur

- **Nitroimidazoles**
  - Dimetridazole
  - Ipronidazole

- **Tetracyclines**
  - Chlortetracycline
  - Tetracycline

- **Lincosamides**
  - Pirlimycin

- **Macrolides**
  - Tylosin

- **NSAIDs**
  - Phenylbutazone
Problematic Vet Drug Classes

**β-Lactams and Nitroimidazoles**

- Tend to degrade
- Relatively polar / poor extraction

**Tetracyclines**

- Metal chelation
- Poor chromatography

**Lincosamides**

- Relatively polar / poor extraction

**Macrolides**

- Form adducts / poor ionization

**NSAIDs**

- Bind to proteins
- Poor ionization
## Recovery in Dairy Powders Overview

<table>
<thead>
<tr>
<th>Commodity</th>
<th>Acceptable Recoveries (Out of 52)</th>
<th>Average % Recovery</th>
<th>% Protein</th>
<th>% Fat</th>
<th>% Carbs</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 non-fat milk powders</td>
<td>40</td>
<td>83</td>
<td>35</td>
<td>0</td>
<td>52</td>
</tr>
<tr>
<td>1 whole milk powder</td>
<td>43</td>
<td>80</td>
<td>26</td>
<td>22</td>
<td>52</td>
</tr>
<tr>
<td>1 milk protein concentrate (MPC)</td>
<td>36*</td>
<td>87</td>
<td>80</td>
<td>0</td>
<td>6.7</td>
</tr>
<tr>
<td>1 whey protein concentrate (WPC)</td>
<td>39</td>
<td>74</td>
<td>80</td>
<td></td>
<td>6.7</td>
</tr>
<tr>
<td>1 whey protein isolate (WPI)</td>
<td>39</td>
<td>78</td>
<td>90</td>
<td>0</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* Ion enhancement observed for imidazoles (6 of 9, >120% recovery)
• Apply (or adapt) method for other finished dairy product matrices
  – Butter
  – Cheese
  – Yogurt
• Optimize sample preparation for lipid removal in high-fat samples
  – Waters HLB PRiME SPE cartridge (limit amount loaded)
  – Agilent EMR dSPE sorbent (adaptation of current protocol)
  – Hexane rinse
  – Other?
• Obtain industry samples for testing
• Attempt to chromatograph all analytes in a single run
  – Ion-pairing agent in extraction solution
• Developed a validated analytical method for the determination of 40 of 52 veterinary drugs in milk powders (both non-fat and fat-containing)

• Recoveries are dependent on contents of matrix
  – Whole milk powder requires additional sample preparation step
  – MPCs, WPCs, WPIs suffer from increase in % protein

• Currently developing a method for aminoglycosides (and amprolium) in milk powders
  – Adapt internal FDA method for aminoglycosides in raw milk

• Method will be applied (adapted) for the determination of vet drugs in other finished dairy products (cheese, butter, yogurt)
  – May require optimized lipid removal step
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