Technologies for detection of chemical and biological contaminants in foods

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Agricultural Research Service
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Outline

1) Project Overview
2) Steven Lehotay and Yelena Sapozhnikova
   a) veterinary drug residue analysis in meats and eggs
   c) pesticides and environmental contaminants analysis
   d) sample processing and test portions study
   b) automated sample prep and analysis, including data handling and identification
3) Guoying Chen
   mercury analysis
4) Johnny Perez
   mass spectrometry in antimicrobial resistance research
Mission Statement and Goal

Develop and transfer to stakeholders effective, efficient, and useful analytical approaches for the screening, quantification, and/or identification of chemicals of concern in food and food-related matrices, including aspects related to antimicrobial resistance.

The goal of our work is to better protect the food supply for the benefit of human health, the environment, and agriculture, and conduct outstanding research, disseminate the findings, transfer the technologies, and have rewarding interactions in the process.
Sample Throughput to analyze Chemical Residues in foods → QuEChERS + LC- & GC-MS (/MS)
Some Recent Publications of Note

• Lehotay et al. (2016) "Automated mini-column solid-phase extraction cleanup for high-throughput analysis of chemical contaminants in foods by low-pressure gas chromatography – tandem mass spectrometry" Chromatographia, 79, 1113-1130

• Han et al. (2016) “Method validation for 243 pesticides and environmental contaminants in meats and poultry by tandem mass spectrometry coupled to low-pressure gas chromatography and ultrahigh performance liquid chromatography” Food Control 66, 270-282


Currently, 219 vet. drugs (including >100 antibiotics) are on our list, but have targeted and evaluated ≈180 so far in (UHP)LC-MS/MS.
UHPLC-MS/MS of AMGs w/o Ion-Pairing Agent

- Spectinomycin
- Spectinomycin hydrate
- Hygromycin
- Streptomyacin
- Dihydrostreptomyacin
- Kanamycin
- Amikacin
- Apramycin
- Gentamicin(C2+C2a)
- Neomycin
50 mM sodium 1-heptanesulfate in final extract
## Updated Vet. Drug Residue Method for FSIS

### Aminoglycosides Multiclass, Multiresidues

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>2 g tissue + 20 mL of 10 mM NH₄OAc, 0.4 mM EDTA, 2% trichloroacetic acid, and 0.5% NaCl in water + IS</td>
</tr>
<tr>
<td>2.</td>
<td>Shake 5 min on pulsed vortex platform shaker (80% setting, max pulsation)</td>
</tr>
<tr>
<td>3.</td>
<td>Centrifuge 3 min at 3700 rcf</td>
</tr>
<tr>
<td>4.</td>
<td>Transfer 10.75 mL (1 g equiv. sample) to 15 mL tube</td>
</tr>
<tr>
<td>5.</td>
<td>Adjust pH to 6.5 ± 0.1 using a pH meter</td>
</tr>
<tr>
<td>6.</td>
<td>Condition 50 mg WCX* DPX† tips with 3 mL each of methanol and water</td>
</tr>
<tr>
<td>7.</td>
<td>Load extract in 3 portions onto 50 mg WCX DPX tips</td>
</tr>
<tr>
<td>8.</td>
<td>Wash DPX tips with 5 mL water</td>
</tr>
<tr>
<td>9.</td>
<td>Elute DPX tips with 1 mL 10% formic acid in water</td>
</tr>
</tbody>
</table>

*WCX = weak cation exchange sorbent  †DPX = dispersive pipette extraction

Yields 95 mg/mL final extract for each method in 34/66 (v/v) acetonitrile/water containing 50 mM IP reagent and 0.85% HO₂CH → 4 µL injection = 0.38 mg equiv. sample on column

### Multiclass, Multiresidues

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>2 g tissue + 10 mL 4/1 (v/v) acetonitrile/water + IS</td>
</tr>
<tr>
<td>2.</td>
<td>Centrifuge 3 min at 3700 rcf</td>
</tr>
<tr>
<td>3.</td>
<td>Tissue equivalence 0.174 g/mL</td>
</tr>
<tr>
<td>4.</td>
<td>407 µL extract (71 mg sample equiv.)</td>
</tr>
<tr>
<td>5.</td>
<td>+ 272 µL 138 mM sodium 1-heptanesulfate ion-pairing (IP) reagent in water/acetonitrile</td>
</tr>
</tbody>
</table>

4 µL injection = 0.38 mg equiv. sample on column
Table 1: Results for the veterinary drugs spiked at 0.5X, 1X, and 2X levels, n=10 each, in the bovine tissues; (tR = retention time), aminoglycosides in blue text.

<table>
<thead>
<tr>
<th>Drug</th>
<th>tR (min)</th>
<th>0.5X</th>
<th>1X</th>
<th>2X</th>
<th>Kidney</th>
<th>Liver</th>
<th>Muscle</th>
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</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>4.88</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>1.68</td>
<td>1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primaquine</td>
<td>5.54</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Quinupristin</td>
<td>4.53</td>
<td>10</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zwittertetracycline</td>
<td>1.38</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Matrix Effects in Different Bovine Tissue Extracts

- Kidney
- Liver
- Muscle

Recoveries and RSDs in Bovine Liver

- abamectin
- doramectin
- eprinomectin
- ivermectin
- rafoxanide
- closantel

Validation Results

- Gold = 80-110% Recovery, 15% RSD
- Silver = 70-120% Recovery, 25% RSD
- Bronze = 50-150% Recovery, 40% RSD

84% of analytes within the box

Recovery

- off-scale
- %Recovery (%RSD)
  - desacetyl cephalirin 238 (11)
  - fenbendazole 170 (46)
  - moxidectin 28 (167)
  - 6-methyl-2-thiouracil ND
  - ivermectin ND
  - clencyclohexerol ND
  - closantel ND
Multi-Application, Multiresidue Analysis

**Goal:** Develop a multi-class, multi-residue method for analysis of pesticides as well as legacy and emerging environmental contaminants in food:

- Pesticides
- Polychlorinated biphenyls (PCBs), including dioxin-like PCB congeners
- Polycyclic aromatic hydrocarbons (PAHs)
- Polybrominated diphenyl ethers (PBDEs)
- Novel alternate flame retardants (FRs)
High Throughput Efficiency Start-to-Finish

1) Sample processing (Blixer → 2-5 g test portions)

2) QuEChERS batch extraction by platform pulsed vortexing followed by centrifugation

3a) UHPLC-MS/MS analysis of LC-amenable analytes

3b) Automated cleanup + fast, low-pressure (LP) GC-MS/MS

4a+b) Trustworthy automatic peak integrations and analyte identifications without human review
>240 Analytes in Parallel 10 min Analyses

- **Low pressure (LP)GC-MS/MS**: 150 pesticides, > 50 environmental contaminants & internal/QC standards
- **UHPLC-MS/MS**: 55 overlapping pesticides, 99 pesticides & internal/QC standards
Comminuted Broccoli

Blixer

Blixer + Cryomill

Robot Coupe Blixer has a spatula in the lid to ease and improve comminution

**Conclusion**: Cryomill = Overkill
**Instrument Top Sample Preparation (ITSP)**

Determined performance results in the use of automated mini-SPE cleanup in the LPGC-MS/MS analysis of pesticides and other contaminants in QuEChERS extracts of 10 different matrices.

Robotic liquid handler:
- 3 min cleanup step at 2 µL/s
- + 5 min for addition of APs and switching/washing syringes

Used mini-cartridges showing removal of chlorophyll and other matrix components.

Final extract volumes $= 278 \pm 5 \, \mu L \, (n = 255)$ after 50 µL addition of APs (and/MeCN) solution.
ITSP+LPGC-MS/MS takes 13 min per injection cycle

Agilent 7010 enabled 1:9 split injection (0.1 mg sample equivalent) rather than 10-fold higher amount to still achieve <10 ng/g LOQs and LOIs (quantification and identification) for nearly all analytes in LPGC-MS/MS, entailing hundreds of injections over many days before maintenance is needed.
Summation Integration in Chromatography

SIMPLIFY, don’t COMPLIFY!

• Draw a straight line at the baseline just before the start of the expected peak to just after its expected end → EASY PEASY!

• *e.g.* Elkin *et al.* “Computer-controlled mass fragmentography with digital signal processing” *J. Chromatogr.* **81** (**1973**) 47-55

• **Advanced ≠ Better**

• **Function ≠ Beauty**

<table>
<thead>
<tr>
<th>LOQ/LOI (ng/g)</th>
<th>Qualitative Result</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Height</strong></td>
<td><strong>0.9/0.9</strong></td>
</tr>
<tr>
<td><strong>Area</strong></td>
<td><strong>1.4/1.8</strong></td>
</tr>
</tbody>
</table>

2 ng/g Pyriproxyfen in Orange

Quant. Ion

\[ m/z 198 \rightarrow 129 \]

\[ t_R = 5.6 \text{ min} \]

Qual. Ion

\[ m/z 198 \rightarrow 102 \]
Summation integration is consistent and reliable.
p,p’-DDD and o,p’-DDT partially co-elute but can be consistently integrated individually.
Continued:

Orange

1 ng/g Spikes

10 ng/g Spikes

100 ng/g Spikes

Tilapia

after ≈140 injections

after ≈170 injections

after ≈200 injections

Original QuEChERS  Acetate-Buffered  Original QuEChERS  Acetate-Buffered
Rules in Automatic Post-Run Identification 
(e.g. in Excel or Instrument Software)

1) Ret. time ($t_R$) for each ion (Quant. and Qual.) must be $\leq |0.1|$ min from the contemporaneous $t_R(\text{ref.})$

2) Ion Ratio (IR) = (peak area ion 2)/(peak area ion 1), 3/1, 4/1, etc. (in %); IR(\text{ref.}) and $t_R(\text{ref.})$ = avg. of contemporaneous high conc. calibration stds in solvent [note: IR(\text{ref.}) $\leq 110\%$

IR must be $|\pm 10|$ for $\geq 1$ ion or $|\pm 20|$ for $\geq 2$ ions vs. IR(\text{ref.})

3) Conc. must be $> \text{reporting level}$
Conclusions

• Smaller test portions are possible using the Blixer for many food samples.

• High quality, rugged results can be achieved for hundreds of ultratrace analytes in diverse foods using automated high-throughput analysis by QuEChERS + ITSP + LPGC-MS/MS and UHPLC-MS/MS without matrix-matched calibration followed by summation function chromatographic peak integrations + defined post-run processing to yield accurate determinations and identifications with minimal need for human review.
Other Current Work

• Identification and monitoring of food packaging components in processed foods
• Evaluation of EMR-Lipid, Chlorofiltr, and other sorbents for cleanup
• Analysis of seafoods for veterinary drugs and other chemical contaminants
• Analyses to establish Certified Reference Material for veterinary drugs in bovine muscle
• Interlaboratory study report on rapid method to monitor inorganic arsenic in rice
• Flow-injection analysis for mass spectrometric detection
Speciation of trace mercury (Hg) impurities in fish oil by differential photochemical vapor generation-atomic fluorescence spectrometry (PVG-AFS)

Guoying Chen and Bunhong Lai

**Summary**

1. Differential photochemical vapor generation using UV-B and UV-C
2. Math-based approach avoided chemical or chromatographic separation
3. 0.4% anthranilic acid in 20% formic acid was an effective PVG solution
4. Cost-effective instrumentation, operation, and chemical reagents
5. Issue: cleanup of cysteine in fish muscle that interfered in the analysis
Methylmercury (MeHg⁺) toxicity: Minamata disease

- Chisso Factory discharged 70-150 ton MeHg⁺ to Minamata Bay, Japan (1932-68)
- MeHg⁺, lipophilic and hydrophilic, easily passes blood-brain and placental barriers
- MeHg⁺ is especially damaging to brain development for fetus and children
- 11,540 fell victim by consuming local fish/shellfish, total damage: 12.6B Yen

Symptoms:
- uncontrollable tremors
- loss of motor control
- loss of auditory and visual senses
- ataxia: loss of muscle control during voluntary movements
- numbness in the extremities like hands and feet
- speech impairment
- children with congenital disease
- paralysis, coma, even death

Mother bathing a 16-year-old daughter

Minamata, Japan
Human exposure to Hg

Background

• Ubiquitous presence
• Highly toxic Class 1 metals
• Human Hg exposure: seafood consumption
• 70-95% of Hg in fish is MeHg⁺
• Species-dependent toxicity: MeHg⁺ > Hg++

Regulations

MeHg⁺:

• FAO/WHO Joint Expert Committee on Food Additives (JECFA) provisional tolerable weekly intakes (PTWI): **1.6 µg/kgbw**
• JECFA warned a greater risk for pregnant/breastfeeding women.
• FAO/WHO Codex Alimentarius guideline: **1 mg/kg in predatory fish; 0.5 mg/kg in other fish**
• Most countries: **0.5 mg/kg**

Hg++: **PTWI: 4 µg iHg/kgbw**
HPLC–ICPMS vs. non-HPLC-MS methods

1. **HPLC-MS methods**: expensive instrumentation, operation, and personnel
   - **Separation by HPLC**: slow and expensive instrumentation
   - **Quantification by ICPMS**: $200/sample

   Regulations only target iAs or MeHg⁺; complete speciation is not needed

2. **Non-HPLC-MS methods**: low-cost, sensitive, rapid, green chemistry
   - **Separation**: 1. stepwise chemical reduction
     2. mathematical approach by UV vapor generation (UVG) (green chemistry)
     3. cryogenic trapping (CT) using sorbent (green chemistry)
   - **Sample Introduction**: cold vapor (CVG) or hydride generation (HG)
   - **Quantification**: atomic absorption or fluorescence spectrometry (AAS or AFS)
**Hg\(^{++}\)/MeHg\(^{+}\) speciation by PVG under UV-B and UV-C**

- **Prerequisites:**
  1. Hg\(^{++}\) and MeHg\(^{+}\) are the only detectable species in fish (observed globally)
  2. AFS responses are linear (under adequate conditions)
  3. AFS responses are additive (theoretically valid)
  4. Prior elimination of interfering cysteine

- **Linear equations:**

  \[ I_B = m_B[Hg^{++}] + n_B[MeHg^{+}] \]  \( (1) \)

  \[ I_C = m_C[Hg^{++}] + n_C[MeHg^{+}] \]  \( (2) \)

Solved using junior high algebra.
PVG–AFS instrumentation setup

- **Photoreactor coil** is illuminated by UV-C or UV-B lamps
- **Gas-liquid separator** separates Hg vapor from matrix components
- **Dryer** eliminates moisture from Hg vapor
- **AFS** registers atomic fluorescence signal
# Design of a dual-source photochemical reactor

<table>
<thead>
<tr>
<th>Light source</th>
<th>UV-B</th>
<th>Philips fluorescent lamp PL-S 9W/01 (311 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-C</td>
<td>UVP low-pressure mercury lamp 3SC-9 (254 nm)</td>
<td></td>
</tr>
<tr>
<td>Reductant</td>
<td>20% formic acid – 0.4% anthranilic acid</td>
<td></td>
</tr>
<tr>
<td>Photoreactor</td>
<td>Quartz coil of 16.2 mL, 110 s exposure time</td>
<td></td>
</tr>
</tbody>
</table>

![Diagram of the photochemical reactor](image-url)
Significance of Hg speciation in fish oil supplement

- **Health benefits of fish oil supplement**
  - Rich omega-3 fatty acids especially EPA and DHA
  - FDA approval to lower triglycerides levels
  - Benefits for >60 conditions especially cardiovascular system
  - Global fish oil production: 1-1.25 million tons (2010)

- **Raw material of fish oil**
  - Mackerel, herring, tuna, anchovy, salmon, sardine, cod liver, krill, etc.

- **Widespread concerns on impurities**
  - MeHg$^+$ and Hg$^{++}$ or other environmental contaminants

- **Challenges in Hg speciation in fish oil**
  - Low-level presence at ppb to sub-ppb level
  - So far only total Hg is measured in fish oil; speciation is not carried out.
  - Speciation data will shed light on purification practice
Procedure

1. **Liquid-liquid extraction (LLE)**
   - Mix 2 mL of fish oil with 40 mL of water in a 50 mL flask
   - Shake for 10 min on a platform vortexer
   - Centrifuge at 4000 rpm for 10 min
   - Separate aqueous extract

2. **Photochemical vapor generation (PVG)**
   - Mix aqueous extract with 20% FA–0.4% AA in a quartz coil
   - Expose to 254 nm (UV-C) or 311 nm (UV-B)
   - Sweep the resulting Hg$^0$ vapor with high-purity Ar to gas/liquid separator (G/L)
   - Remove moisture from Hg$^0$ using a Nafion membrane dryer

3. **Atomic fluorescence spectrometry (AFS)**
   - Illuminate Hg$^0$ with a Hg hollow cathode lamp at 254 nm
   - Detect resulting resonance fluorescence with a photomultiplier tube

4. **Calculation**
   - Obtain 4 slopes from 4 calibration curves: $m_B$, $n_B$, $m_C$, and $n_C$
   - Solve the following equation set:
     \[ I_B = m_B[Hg^{++}] + n_B[MeHg^+] \] (1)
     \[ I_C = m_C[Hg^{++}] + n_C[MeHg^+] \] (2)
**Performance and results**

1. **Ultra-High sensitivity**
   - LOD: $\text{Hg}^{++}: 0.3 \text{ ng/mL}$; $\text{MeHg}^{+}: 1.0 \text{ ng/mL}$
   - LOQ: $\text{Hg}^{++}: 1.7 \text{ ng/mL}$; $\text{MeHg}^{+}: 5.6 \text{ ng/mL}$

2. **Rapid LLE with reasonable recoveries**
   - $\text{MeHg}^{+}$: $\approx 73\%$
   - $\text{Hg}^{++}$: should be higher because $K_{ow} < 1$

3. **Green chemistry**
   - No strong or unstable reductant
   - No strong acid or base for digestion

4. **Ultralow Hg impurities**
   - Average total Hg = 2.54 ng/mL
   - $\text{MeHg}^{+}/\text{Hg}^{++}$ ratio is 3.5

5. **Conclusion:**
   - Hg binds to fish meal rather than fish oil
   - Effective purification by: (a) water washing, (b) bleaching, and (c) molecular distillation.

### Hg impurities in fish oil

<table>
<thead>
<tr>
<th>#</th>
<th>iHg</th>
<th>MeHg</th>
<th>#</th>
<th>iHg</th>
<th>MeHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.70</td>
<td>&lt;LOD</td>
<td>21</td>
<td>0.30</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>2</td>
<td>0.33</td>
<td>&lt;LOD</td>
<td>22</td>
<td>0.45</td>
<td>&lt;LOD</td>
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<tr>
<td>3</td>
<td>3.18</td>
<td>&lt;LOD</td>
<td>23</td>
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<td>&lt;LOD</td>
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<tr>
<td>4</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>24</td>
<td>0.99</td>
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<tr>
<td>5</td>
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<tr>
<td>6</td>
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<td>&lt;LOD</td>
<td>26</td>
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<td>7</td>
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<td>&lt;LOD</td>
<td>27</td>
<td>0.98</td>
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<tr>
<td>8</td>
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<td>16</td>
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<tr>
<td>19</td>
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<td>Average</td>
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<tr>
<td>20</td>
<td>0.38</td>
<td>&lt;LOD</td>
<td>LOD</td>
<td>0.30</td>
<td>1.68</td>
</tr>
</tbody>
</table>
Here’s Johnny........
Rapid Detection for Aminoglycoside Resistance using UHPLC-MS/MS

• Last couple years, HPLC-MS was employed for rapid detection of β-lactam resistance by the appearance of the modified antibiotic

• Due to the surge in resistance to aminoglycosides, another highly essential antibacterial treatment agent, we wanted to develop a rapid detection UHPLC method for modified aminoglycosides

• Currently, no reporting of LC-MS methods for modified aminoglycosides
Aminoglycoside Modification

- Acetyltransferases (AAC)
- Phosphotransferases (APH)
- Nucleotidyltransferases (AAD)

Kanamycin + Acetyl-CoA $\xrightarrow{\text{Acetyltransferase}}$ Acetyl-Kanamycin + CoA

1. Acquired the MP443 (aac) plasmid from Dr. Martin Pavelka (University of Rochester Medical School)
2. Transformation to a competent E-Coli strain
3. Grew bacteria overnight in LB broth and diluted to an OD = 1
4. Pelleted the bacteria and inoculated 10 ug/mL Kanamycin (aq)
5. Incubated for 5 hours prior to injection of the supernatant
Measurements of E. Coli

Currently, acetylated kanamycin observed from 100 pg/mL - 100 µg/mL

**E. Coli MP443 (aac gene)**

Acetylated Kanamycin

\[ [M + COCH + 2H]^+ \text{ m/z } 264.2 \]

**E. Coli NEB-5α (Susceptible)**

Acetylated Kanamycin

\[ [M + COCH + H]^+ \text{ m/z } 527.2 \]

RT = 3.609 min

RT = 3.609 min
Conclusions (2)

• Further streamlined sample preparation to “dilute and shoot”

• Feasibility of FI-MS/MS demonstrated for veterinary drug residue monitoring at tolerance levels in bovine muscle and kidney

• Refinements needed to improve results for some drugs at 10 ng/g (can we inject more equivalent sample?)

• Simple screening/identification approach devised needs further evaluation to assess rates of false positives/negatives

.... to be continued...
Sample Size and Extract Volume

- **Croaker**
  - 2 g + 2 mL: [Bar Chart Value]
  - 4 g + 4 mL: [Bar Chart Value]
  - 4 g + 8 mL: [Bar Chart Value]
  - 10 g + 10 mL: [Bar Chart Value]

- **Salmon**
  - 2 g + 2 mL: [Bar Chart Value]
  - 4 g + 4 mL: [Bar Chart Value]
  - 4 g + 8 mL: [Bar Chart Value]
  - 10 g + 10 mL: [Bar Chart Value]

- **SRM 1947**
  - 2 g + 2 mL: [Bar Chart Value]
  - 4 g + 4 mL: [Bar Chart Value]
  - 4 g + 8 mL: [Bar Chart Value]
  - 10 g + 10 mL: [Bar Chart Value]
**Additional Future Work (2015)**

Evaluate flow-injection tandem mass spectrometry (FI-MS/MS) to provide 3 min screening analysis of 130+ drugs (and other electrospray-amenable contaminants, such as many pesticides) with automatic software identifications of positives.

Investigate new automated sample cleanup tool (ITSP) for use in FI-MS/MS and QuEChERS applications.

Assess new chromatographic column stationary phases to include aminoglycosides in the same analysis as other veterinary drugs.
Possible Future Plans (2015-2020)

• Develop better methods for speciation analysis of arsenic, and mercury
• Develop rapid analytical methods for emerging contaminants of concern
• Validated criteria for identification purposes in FI-MS/MS and other types of analyses
• Collaborate in studies involving antibiotic resistance
• Investigate chemical/MS-based methods for the monitoring of biological analytes and processes (e.g. metabolomics, exposomics)
Updated LC-MS/MS Method Logistics

10 min sample prep for a few samples, or 1 chemist was able to process 60 pre-homogenized samples in 3 hours (for overnight LC-MS/MS run)

(longest steps involved labeling tubes/vials, weighing, and preparing calibration standards)

No glassware to be cleaned afterwards

Waste = 10 mL MeCN, pipet tips, and a 50 mL tube

Review of results for 135 drugs x 3 transitions x 67 injections (>27,000 data points) took 8 hours
Sample Preparation

We are currently evaluating the approach for analysis of beef, pork, and chicken muscle for possible implementation by FSIS.

Sample preparation (final method):

- 10 g homogenized fish + internal standards
- Add 10 mL MeCN and shake 10 min on vortex shaker at 80% setting with max. pulsing
- Add 5 g HCO₂NH₄, shake 1 min, centrifuge 2 min at 3700 rcf

**Filter-vial dispersive-SPE:**

- Add 0.5 mL extract to the PVDF (0.2 μm) filter-vial shell containing 75 mg each anh. MgSO₄ + 1/1/1 PSA/C18/Z-Sep
- Partially depress the filter-vial plunger and shake for 30 s in an autosampler vial tray
- Fully depress the plunger into the filter-vial shell
Conclusions of Extraction Study

• 1 min extraction with the pulsed vortex shaker is sufficient for extraction of many incurred contaminants in the homogenized fish tissues, but 10 min extraction time is better as a precaution – **batch analysis of 50 samples at a time**

• Extraction with a probe blender was rapid and complete, but it limited sample throughput and was inconvenient

• 1:1 sample:MeCN (g:mL) ratio was sufficient to achieve full extraction, and 2 g homogenized sample gave equivalent results as 4 and 10 g samples

• Spiking with an int. std. does not compensate for lower extraction efficiency in incurred samples vs. spikes
**Updated LC-MS/MS Method for Veterinary Drugs**

<table>
<thead>
<tr>
<th>Extraction</th>
<th>Clean-up</th>
</tr>
</thead>
</table>
| 2 g tissue in a 50 mL tube  
add IS mix (SMZ-IS; flunixin-d3) | 0.4 mL supernatant + 25 mg C18 in filter-vial d-SPE;  
vibrate AS tray for 30 s and filter through 0.2 μm  
PVDF by pressing plungers to seal the vials |
| add 10 mL of 4/1 (v/v) MeCN/water  
vortex briefly, shake for 5 min  
centrifuge for 5 min >3500 rcf | |

The sensitive MS/MS instrument allows >100-fold less injected sample equivalent (0.17 mg)!
3-Day Validation Experiment

Day 1:
- Analyst 1, Reagents Lot A, 10 matrix blanks from different sources, 6 spikes at 3 levels each in 6 matrices + 4 spikes each at same levels in mixed matrices (using filter-vial d-SPE); 6-point calibration in mixed-matrix and reagent-only stds

Days 2 & 3:
- Analysts 2 & 3 repeat using Reagents Lot B with different sources of matrices
Status of LC-MS/MS Study

• Validated the method using core-shell Kinetex column for 134 vet. drugs in bovine muscle (submitted paper).

• Validated method using unique “Select DA” column for 134 vet. drugs in bovine kidney, liver, and real samples (experiments completed, data review pending).

• UHPLC-MS/MS instrument was purchased, and method will be optimized for implementation by FSIS.
Oh, and we may have eliminated matrix effects in GC-MS ... 

... via use of appropriate int. stds + analyte protectants in split inj’n
Sampling and Sample Processing

- For particulate materials
- Finite Elements
- Infinite Elements & Increments
- Compositional Heterogeneity and Fundamental Error
- Distributional Heterogeneity
- Sample Correctness and Tools

Slide adapted from Jo Marie Cook
Fast Low-Pressure (LP)GC-MS/MS

Review of dozens of publications using LPGC-MS(/MS):

Injector

Restriction Capillary

5 m × 0.18 mm

Mega-Bore Column

15 m × 0.53 mm × 1 μm x̅-5ms

GC Oven

No special adaptations needed; can be implemented in any GC-MS(/MS).
LPGC-MS is Much Faster

Traditional GC-MS

25 min

LPGC-MS

6 min

and more sensitive
Analyte Protectants

Strongly interact with active sites in GC system (inlet, column and ion source) to decrease degradation and adsorption of co-injected analytes.

Sharper peaks, less tailing, more ruggedness, lower LOD

Mastovska et al., Anal. Chem. 77 (2005) 8129-8137
Effect of Analyte Protectants

![Graph showing the effect of analyte protectants on Dimethoate retention time. The solid line represents 'w/ analyte protectants' and the dotted line represents 'w/o analyte protectants.' The x-axis represents retention time (min) from 2.95 to 3.35, and the y-axis represents abundance from 0 to 7000.]
Injection liner and septum after 325 injections in 5 days including 230 matrix extracts (1 mg equiv.) of 10 diverse food commodities

A little “dirt” here and there, but the analyte protectants did their job and results still looked great from start to finish.
Acknowledgments

CTC Analytics          Jessie Matarrita          Alan Lightfield
ITSP Solutions         Robyn Moten              Limei Yun
Gerstel                Tawana Simons            Lijun Han
Restek                 Yelena Sapozhnikova

Disclaimer

Mention of brand or firm name does not constitute an endorsement by the USDA above others of a similar nature not mentioned.

Thank You!

Contact: Steven. Lehotay@ars.usda.gov
**FDA Sampling for Pesticides**

<table>
<thead>
<tr>
<th>Item</th>
<th>Sampling Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 25 g units (berries)</td>
<td>1 kg (2.2 lbs)</td>
</tr>
<tr>
<td>25 – 250 g (apples)</td>
<td>1 kg (≥ 10 units)</td>
</tr>
<tr>
<td>&gt; 250 g (cabbage)</td>
<td>2 kg (≥ 5 units)</td>
</tr>
<tr>
<td>Grains, Tree Nuts</td>
<td>1 kg</td>
</tr>
<tr>
<td>Herbs</td>
<td>0.5 kg</td>
</tr>
<tr>
<td>Spices</td>
<td>0.1 kg</td>
</tr>
</tbody>
</table>

**CODEX:** 1 kg (2.2 lbs)

**Pesticide Data Program:** 3–5 lbs fresh, 2 lbs processed

**USDA-FSIS:** 1 lb meat, poultry, fish
Cryogenic Sample Processing

Spex FreezerMill (Cryomill)
Instrument Top Sample Preparation (ITSP) (2)

Morris and Schriner (2015) “Development of an automated column solid-phase extraction cleanup of QuEChERS extracts, using a zirconia-based sorbent, for pesticide residue analyses by LC-MS/MS” J. Agric. Food Chem. 63, 5107-5119
5-year project plan

Sample processing
- Bulk Comm- inution
- Cryogenic milling

Sample preparation
- Automated high-throughput
- Better cleanup

Chemical analysis
- Fast GC-MS/MS
- Fast LC-MS/MS
- FI-MS/MS

Data processing
- Fast and accurate
- No human review
- Identification
Sample processing
Cryogenic milling

Dried bacon

Salmon
Automated high-throughput sample preparation and data processing to monitor veterinary drugs, pesticides, and environmental contaminants.

**Automated Sample Cleanup – Instrument Top Sample Preparation (ITSP)**

- Robotic liquid handler:
  - 3 min cleanup + 5 min for addition of analyte protectants and switching/washing syringes

**Automated Data Processing**

- Summation Integration Function
- 1 min to integrate a batch of >60 samples of ≈660 data points per sample
- WITHOUT REVIEW!

Used mini-cartridges showing removal of chlorophyll and other matrix components.
Multiclass, multiresidue analysis of pesticides, and environmental and emerging contaminants in foods

Simultaneous analysis method for diverse pesticides, legacy and emerging environmental contaminants in meats:

- Pesticides & environmental contaminants: PAHs, PCBs, PBDEs, flame retardants (≈300 total)
- High throughput, fast, simple sample preparation based on QuEChERS extraction and streamlined clean-up
- Cost of materials ≈$3/per sample
- Fast Gas & Liquid chromatography tandem mass spectrometry analysis, 10 min each in parallel
Simultaneous analysis method for diverse pesticides, legacy and emerging environmental contaminants in meats

- Pesticides (EPA list) & environmental contaminants (≈300 total)
- QuEChERS extraction & d-SPE clean-up
- Fast GC & LC-MS/MS analysis, 10 min each
Automated SPE cleanup

- ITSP = Instrument Top Sample Preparation
- Mini-SPE cleanup
- 45 mg anh. MgSO$_4$/PSA/C$_{18}$/Z-Sep/CarbonX

Efficient cleanup

Co-extractive

Recoveries

(70-120%)

RSDs<20%
Multi-class, multiresidue method

- **Pesticides**
- Polychlorinated biphenyls (PCBs)
- Polycyclic aromatic hydrocarbons (PAHs)
- Polybrominated diphenyl ethers (PBDEs)
- Novel alternate flame retardants (FRs)
Novel analytical methods for inorganic and organometallic toxic metals: mercury (Hg) and arsenic (As)

- Speciation of As and Hg
- Solid phase extraction (SPE) for cleanup and enrichment of inorganic As
- Hg$^{++}$ and MeHg$^+$ speciation in fish oil supplement by photochemical vapor generation (PVG)
- Atomic fluorescence spectrometric quantification – sensitive, rapid, low cost
- Patent filing on a cryogenic trap system for As speciation
Bioanalytical methods to monitor for antibiotic resistant organisms and/or their biomarkers

- Developing bioanalytical methods (including mass spectrometry) to monitor for antibiotic resistant organisms and/or their biomarkers in conjunction with antibiotic residues in seafood and meats.
- Developing rapid antimicrobial resistance (AMR) assays based on high resolution mass spectrometry (HRMS)
QuEChERS extraction with acetonitrile

Batch of 12 pre-homogenized samples can be prepared in 1 hr

Automated SPE cleanup

Waste = 1-2 mL acetonitrile & disposable pp tube

Cost of materials ≈ $3-4/sample

LPGC & UHPLC-MS/MS: 10 min run in parallel
Multiresidue method for food packaging contaminants in packaged foods

**Phase 1.** Identification of food packaging contaminants leaching from stretch plastic films used as food packaging:
- In food simulants
- In packaged food (e.g. ground beef, pork, chicken)

Non-targeted analysis by GCxGC-TOF-MS

**Phase 2.** Method development

**Phase 3.** Market survey & data for risk assessment
Sample preparation - QuEChERS

Quick, Easy, Cheap, Effective, Rugged, Safe
93 organic chemicals identified in food simulants 
(with >80% match similarity to the standard NIST mass spectral library)

<table>
<thead>
<tr>
<th>Chemical Class: Uses/Sources</th>
<th>Hexafluorobisphenol A: polymer additive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkylated naphthalene: lubricant additive</td>
<td></td>
</tr>
<tr>
<td>Polycyclic aromatic hydrocarbon (PAH): combustion, biogenic, petroleum</td>
<td>2,4,7,9-Tetramethyl-5-decyn-4,7-diol: adhesive, surfactant, plastic additive</td>
</tr>
<tr>
<td>Linear alkylbenzene (LAB): precursor of biodegradable detergents</td>
<td></td>
</tr>
<tr>
<td>Adipates (DEHA, DOA, and five other adipic acids): plasticizer</td>
<td>13-Isopropylpodocarpa-8,11,13-trien-19-al, 10,18-Bisnorabieta-8,11,13-triene, and Methyl dehydroabietate: thermal degradation</td>
</tr>
<tr>
<td>Phthalates and salicylates: plasticizer</td>
<td>1H-Indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl-, and Galaxolide (musk): fragrance</td>
</tr>
<tr>
<td>Homosalate: UV filter</td>
<td>Phenyl/Biphenyl/diphenyl compounds (miscellaneous)</td>
</tr>
</tbody>
</table>
Some examples of identified chemicals

<table>
<thead>
<tr>
<th>Identified compound</th>
<th>Use</th>
<th>Concern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzyl chloride</td>
<td>Manufacturing of plasticizers</td>
<td>Probable human carcinogen</td>
</tr>
<tr>
<td>Benzyl benzoate</td>
<td>Flavor and fragrance agent</td>
<td>Endocrine disruptor</td>
</tr>
<tr>
<td>Furan, 2-pentyl</td>
<td>Flavoring agent</td>
<td>Suspected genotoxicity</td>
</tr>
<tr>
<td>Benzophenone</td>
<td>UV blocker</td>
<td>Endocrine disruptor</td>
</tr>
<tr>
<td>2,4-trimethyl-1,3-pentanediol diisobutyrate (TXIB)</td>
<td>Low-viscosity plasticizer</td>
<td>Reproductive/developmental toxicity</td>
</tr>
<tr>
<td>2-ethylhexyl methyl isophthalate</td>
<td>Commonly used plasticizer</td>
<td>Genetic mutation, reproduction toxicity</td>
</tr>
</tbody>
</table>
Current work

- Currently identifying chemicals leaching into meats

Scan chromatograms of plastic film extract, control beef extract and beef exposed to plastic film packaging for 24 hours
Retention Times and Peak Widths are Rock Solid in UHPLC-MS/MS

3-Day Validation Experiment of 101 Pesticides analyzed by UHPLC-MS/MS
40 matrix (muscle) spks and blks + QC = 65 injections per day
Avg $t_R$ (min) of reagent stds and matrices throughout the run (SD <0.020)

<table>
<thead>
<tr>
<th>#</th>
<th>Analyte</th>
<th>Day 1 = 7/17/15</th>
<th>Day 2 = 7/22/15</th>
<th>Day 3 = 7/28/15</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Methamidophos</td>
<td>0.965 0.963</td>
<td>0.950 0.955</td>
<td>0.962 0.963</td>
</tr>
<tr>
<td>8</td>
<td>Oxamyl</td>
<td>1.977 1.970</td>
<td>1.950 1.950</td>
<td>1.962 1.963</td>
</tr>
<tr>
<td>28</td>
<td>Oxadixyl</td>
<td>4.030 4.030</td>
<td>4.012 4.010</td>
<td>4.020 4.022</td>
</tr>
<tr>
<td>42</td>
<td>Metalaxyl</td>
<td>5.023 5.017</td>
<td>5.000 5.000</td>
<td>5.008 5.010</td>
</tr>
<tr>
<td>90</td>
<td>Profenophos</td>
<td>7.023 7.018</td>
<td>7.003 7.007</td>
<td>7.017 7.018</td>
</tr>
<tr>
<td>100</td>
<td>Methoprene</td>
<td>8.013 8.013</td>
<td>8.000 8.005</td>
<td>8.010 8.010</td>
</tr>
</tbody>
</table>

New mobile phase added for each sequence
And Fast, Low-Pressure GC-MS/MS, Too

3-Day Validation Experiment of 202 Pesticides analyzed by LPGC-MS/MS

40 matrix (muscle) spks and blks + QC = 70 injections per day

Avg $t_R$ (min) of reagent stds and matrices throughout the run (SD <0.040)

Analyte Protectants Added to all Final Extracts

- Phenanthrene
- Azinphos
- Dibenz(ah)-anthracene

Retention Time (min)
What the Heck?

Integrations of replicate injections were correct 2 out of 3 times

In analytical chemistry, 2 out of 3 ain’t good

Analytical chemists are hard-working lazy people who look for short-cuts rather than make manual re-integrations. Real samples can’t be dropped as outliers.
In chromatography, the primary parameters are ret. time \((t_R)\) and peak shape (width, height/area).

If \(t_R\) and peak widths are so important and consistent in good methods, why do most (all?) sophisticated (and expensive) chromatographic peak integration software programs so often choose peaks at the wrong \(t_R\) with quite variable peak shapes?
Don’t Trust the “Advanced” Software

And don’t trust the analyst, either. This mistake was caught after preparing the previous slide for this presentation.
Summation Integration Function

- ≈1 min to integrate a batch of >60 samples of ≈660 MRMs per sample **WITHOUT REVIEW!**

- This is a >40 year-old integration function, but **LACKING IN SOME DATA PROCESSING SYSTEMS!**
Chrysene 1 µL 9:1 split injection after ITSP

Partial co-elution with benz(a)anthracene – summation integration at mid-point

1 ng/g Stds

Second sequence of 107 injections
(214 total with same liner)
184 of matrix extracts

R² = 0.9996
RESOLVED: Garbage In = Garbage Out
Correct and consistent chromatographic peak integration is essential to achieving high quality final results.

RESOLVED: Despite technology and software advancements, no set of peak integration parameters works consistently for all analytes, concentrations, and matrices in the real-world (at least not yet in my experience).

RESOLVED: Good analysts are able to conduct peak integrations better than current advanced software tools (but good analysts are hard to find, earn wages, get bored reviewing data, and still make mistakes).
RESOLVED: Human review takes too long!

High-throughput (or even low-throughput) multi-analyte monitoring applications:

G.F. Pang et al. (Beijing, China) include 1,138 pesticides in their GC- and LC- MS/MS monitoring approach. **Large team of chemists conduct analyses and review results.**

**USDA:** 240 analytes × 2-4 ion transitions × 50 samples/batch = 36,000 peaks! Analyst review and re-integration at 1 s per peak = **10 hours w/o breaks on each instrument!**
5 ng/mL endosulfan sulfate in reagent-only and matrix-matched calibration standards

LOQ ≈2 ng/mL in all matrices; even after 325 injections, including 230 food extracts
## Mathematical approaches to speciate As or Hg

### Comparison

<table>
<thead>
<tr>
<th>As hydrides</th>
<th>4</th>
<th>4</th>
<th>4</th>
<th>n/a</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Volatile species</strong></td>
<td><strong>Analytes</strong></td>
<td><strong>Equations</strong></td>
<td><strong>Reductants</strong></td>
<td><strong>Wavelengths</strong></td>
<td><strong>Calibration curves</strong></td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><strong>Hg$^0$ vapor</strong></td>
<td>$I_B = m_B[Hg^{++}] + n_B[MeHg^+]$</td>
<td>$I_C = m_C[Hg^{++}] + n_C[MeHg^+]$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Hg speciation by PVG-AFS at 2 UV wavelengths, this work)

As speciation by HG-AFS under 4 sets of HG conditions (Cava-Montesinos, et al., Talanta, 2005, 66, 895-901)

$I(A) = m_a[As(III)] + n_a[As(V)] + p_a[MMA] + q_a[DMA]$,  
$I(B) = m_b[As(III)] + n_b[As(V)] + p_b[MMA] + q_b[DMA]$,  
$I(C) = m_c[As(III)] + n_c[As(V)] + p_c[MMA] + q_c[DMA]$,  
$I(D) = m_d[As(III)] + n_d[As(V)] + p_d[MMA] + q_d[DMA]$,  

Volatile species

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Equations</th>
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<th>Wavelengths</th>
<th>Calibration curves</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>