Recent developments in the analysis of residues in milk and dairy products.

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What are residues?

- Residues can arise in milk and milk products from the following sources:
  - Use of licensed veterinary drugs for treatment of dairy cows.
  - Illegal use of banned drugs or growth promoting agents.
  - Pesticides used at farm level to control insect infestations.
  - Contaminants from Animal Feed or the environment e.g. mycotoxins or PCBS.
  - Chemicals used to ensure good hygiene at farm and processing plants.
## Residue Categories & Sampling

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug Class</th>
<th>Milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>A6</td>
<td>Banned Drugs</td>
<td>X</td>
</tr>
<tr>
<td>B1</td>
<td>Antibiotics</td>
<td>X</td>
</tr>
<tr>
<td>B2a</td>
<td>Anthelmintics</td>
<td>X</td>
</tr>
<tr>
<td>B2b</td>
<td>Anticoccidials</td>
<td></td>
</tr>
<tr>
<td>B2c</td>
<td>Carbamates / Pyrethroids</td>
<td></td>
</tr>
<tr>
<td>B2d</td>
<td>Sedatives / Tranquilizers</td>
<td></td>
</tr>
<tr>
<td>B2e</td>
<td>NSAIDs</td>
<td>X</td>
</tr>
<tr>
<td>B2f</td>
<td>Corticosteroids</td>
<td></td>
</tr>
</tbody>
</table>
A6. Table 2 Regulation 37/2010

Chloramphenicol

Hazard: Aplastic anaemia

Potential carcinogenicity and genotoxicity (IARC=>group 2A)

Promotes the formation of the multi-resistance of pathogens

RPA: 0.3 µg/kg

Nitrofurans

Hazard

carcinogenic and mutagenic properties

RPA: 1.0 µg/kg
B 1. Antibacterial substances

- Sulphonamides
- Tetracyclines
- Macrolides and lincosamides
- Aminoglycosides
- Beta-lactams
- Quinolones
- Amphenicols
- Peptide antibiotics

Methods of analysis of antimicrobials can be grouped in:

- Microbiological => fast screening, limited information
- Immunochemical => rapid, selective and sensitive (e.g. ELISA)
- Physico-chemical => accurate identification and quantification
Inhibition Assays Overview

- Low cost, suitable for industry and rapid
- No one method will do all
- Validation can be challenging.
- Results should be confirmed because tests are not quantitative
- Unsuitable for chloramphenicol and nitrofurans etc
B2a. Anthelmintics

- Control of
  - Nematodes (roundworms)
  - Cestodes (tapeworms)
  - Trematodes (flukes)

- 3 classes of drugs:
  - Benzimidazoles
  - Macro cyclic Lactones
  - Flukic sides

- Some drugs are teratogenic or neurotoxic
- Many products not licensed in lactating animals

Use and hazard

Analysis

- Detection: HPLC-UV/FLD and LC-MS/MS
B2a. Anthelmintics - Endectocides

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>$R_3$</th>
<th>C-X-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abamectin</td>
<td>OH</td>
<td>H</td>
<td>CHCH$_3$CH$_2$CH$_3$</td>
<td>-CH=CH-</td>
</tr>
<tr>
<td>Doramectin</td>
<td>OH</td>
<td>H</td>
<td>C$<em>6$H$</em>{11}$</td>
<td>-CH=CH-</td>
</tr>
<tr>
<td>Emamectin</td>
<td>C$_6$H$_5$COOHCH$_3$ $\text{NH}_3$</td>
<td>H</td>
<td>CHCH$_3$CH$_2$CH$_3$</td>
<td>-CH=CH-</td>
</tr>
<tr>
<td>Ivermectin</td>
<td>OH</td>
<td>H</td>
<td>CHCH$_3$CH$_2$CH$_3$</td>
<td>-CH$_2$-CH-</td>
</tr>
</tbody>
</table>
B2b. Anticoccidials

- Control/Treatment of:
  - Coccidiosis
  - Acute bovine respiratory disease
  - Cryptosporidiosis
  - Babesiosis
  - Isosporiasis

- Two main classes:
  - Ionophores
  - Chemical anticoccidials

- Some anticoccidials are cardiotoxic, neurotoxic

Use and hazard

- Low LODs required for milk
- Detection: LC-MS/MS
B2b. Regulations

Commission Regulations and Directives

- No 1831/2003
- No 37/2010 – pharmacologically active substances
- No 124/2009 – MLs for anticoccidials in food resulting from unavoidable carryover in non-target feed

Licensed Feed Additives

Lasalocid Na
Narasin
Salinomycin Na
Monensin Na
Semduramycin
Maduramycin
Robenidine
Decoquinate
Halofuginone
Nicarbazin
Diclazuril
B2c. Carbamate and Pyrethroids

- Control/Treatment of external parasites
  - Flies,
  - Lice,
  - Keds
  - and mites
- The pyrethroids are more widely used.
- Type II pyrethroids have a higher mammalian toxicity and are toxic to the environment.
- Carbaryl may be carcinogenic

Use and hazard

- Detection: HPLC-FLD, GC-ECD, GC-MS/MS, LC-MS/MS

Analysis

Tetramethrin

Cypermethrin

Carbaryl

Propoxur
B2e. NSAID’s & B2f. Other

Heterogeneous drug group:
1. Salicylic acid derivatives (aspirin)
2. Propionic acid derivatives (ibuprofen, ketoprofen)
3. Pyrazoles derivatives (phenylbutazone)
4. Aniline derivatives, including anthracilic and nicotinic acid derivatives (flunixin)

- Corticosteroids
- Quinoxalines
- Amitraz

Might be used in cocktails with other illegal substances in animal feeding (beta-agonists/anabolic steroids)
Analytical Developments
Improving the throughput of veterinary drug residue analysis using vibrational shaking technology
QuEChERS Approach

Phase Separation

4g MgSO$_4$ + 1g NaCl

1.5g MgSO$_4$ + 0.5g C$_{18}$

D-SPE

6 mL Supernatant + 0.5 mL DMSO

Analysis

Concentration
Automated shaking

- Ceramic homogeniser pellets are added to samples.
- Salts are added to samples at same time.
- Samples (n = 36) are placed in the shaker in the test tube racks (n = 3).
- Rack is clamped.
- Samples are shaken at 700 rpm.
- Instrument shaking time is adjusted to give the desired extraction efficiency.
Vibrational Extraction System

Operating light

Shaking speeds

Clamp

Emergency stop

Start/Stop button

Electronic door locking
Incurred samples study

- Manual Shaking
- Vibrational Shaking

Concentration (ppb)
Gaps in analysis: Improving chemical analysis of Beta-lactam antibiotics
β-Lactams usage in animal products

Penicillins and Cephalosporins:
- Oral, parenteral and intramammary administration
- Therapeutic use in ruminants, monogastrics and poultry
- Prophylactically at sub-therapeutic doses

Carbapenems:
- Not licensed in food-producing animals
Issues with current approaches

• No multi-residue LC-MS/MS methods incorporating cephalosporins currently available in Ireland

• Outsource of samples to other countries for confirmatory analysis
  • Long turnaround time
  • Degradation of samples during transport
    • Impact on integrity of results
  • Cost implications
Why LC-MS/MS?

- Required to identify and quantify the residues in non-compliant samples.
- Very sensitive, selective and specific.
- Gives very accurate and precise results.

![Image of LC-MS/MS instrument and chromatogram](image.png)
Method overview for milk

1. Weigh 2 g of milk
2. Add 100 µL of isotope std
3. Add 1 mL H₂O & 7 mL MeCN
4. Vortex 1 min
5. Centrifuge 3500 rpm (15 min, 4°C)
6. N₂-EVAP 5 mL to <1 mL
7. d-SPE clean-up (500 mg C₁₈)
8. Reconstitute to 1 mL, filter
9. Centrifuge 2800 xg (15 min, 4°C)
Chromatography conditions

Analytical column: Agilent Phenyl Hexyl column

Binary gradient of:

*Mobile phase A:* HCOOH 0.01% + 0.2 mM ammonium acetate in water

*Mobile phase B:* HCOOH 0.01% in acetonitrile

**Column temperature:** 30°C

**Flow rate:** 0.4 mL min⁻¹

**Injection volume:** 10 µL

**Run time** = 12 min
# Analysis of cefquinome in dairy products

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Fortification level (μg kg(^{-1}))</th>
<th>Mean ± SD (μg kg(^{-1}))</th>
<th>RSD(%)</th>
<th>Trueness(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liquid samples (samples = 11; days = 5)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk</td>
<td>4.0  250</td>
<td>4.1 ± 0.15  258 ± 9.8</td>
<td>3.8</td>
<td>101</td>
</tr>
<tr>
<td>Skimmed milk</td>
<td>4.0  250</td>
<td>4.0 ± 0.26  251 ± 8.8</td>
<td>3.5</td>
<td>100</td>
</tr>
<tr>
<td>Buttermilk</td>
<td>4.0  250</td>
<td>3.5 ± 0.33  226 ± 7.9</td>
<td>3.5</td>
<td>91</td>
</tr>
<tr>
<td>Whey</td>
<td>4.0  250</td>
<td>3.5 ± 0.20  214 ± 5.6</td>
<td>2.6</td>
<td>85</td>
</tr>
<tr>
<td>Cream</td>
<td>4.0  250</td>
<td>4.0 ± 0.20  256 ± 9.2</td>
<td>3.6</td>
<td>102</td>
</tr>
<tr>
<td><strong>Solid samples (samples = 11; days = 3)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Curd</td>
<td>4.0  250</td>
<td>3.6 ± 0.25  237 ± 5.5</td>
<td>6.8</td>
<td>90</td>
</tr>
<tr>
<td>Cheese</td>
<td>4.0  250</td>
<td>3.9 ± 0.34  244 ± 7.5</td>
<td>8.6</td>
<td>98</td>
</tr>
<tr>
<td>Butter</td>
<td>4.0  250</td>
<td>3.9 ± 0.16  259 ± 7.2</td>
<td>4.1</td>
<td>99</td>
</tr>
</tbody>
</table>
Spiked studies

Animal treatment studies
Emerging residues: Analysis of Chlorate & Perchlorate Residues
Monitoring data infant formula

- Concern because chlorates are a competitive inhibitor of iodine uptake in the thyroid, making its presence in food a potential health concern for vulnerable groups, particularly infants.
Proposed Temporary MRL

- 0.200 mg/kg for chlorate in milk (includes sodium, potassium and magnesium chlorate expressed as chlorate).

- The default MRL of 10 µg/kg applies to infant formula “as consumed” (Article 10 (1) of CD 2006/141).

- Chlorate residues are present at levels that frequently exceed the default MRL of 0.01 mg/kg (10 µg/kg) and that the levels vary depending on the source and the product.
Interpretation for IF

MRL for Reconstituted IF = 0.01 mg/kg

Reconstituted IF = 25.2 g powder + 180 mL H2O

= 25.2 g powder + 180 g H2O

Dilution factor (w/w) = (25.2g + 180 g)/25.2 g = 8.14

0.01 mg/kg Recon. IF ~ 0.0814 mg/kg IF
Analytical challenge

- Very small polar molecules, which make it difficult to achieve selective analysis.
- Need selective detection i.e. MS or MS/MS to achieve low levels of detection.
- Due to high water solubility speciality chromatographic columns or ion chromatography is required.
Sample preparation for milk

1. Weigh 5g of Milk
2. Add 100 μL isotopically labelled internal standard
3. Centrifuge 10 min @ 3500 rpm
4. Take 2ml (~2g) skimmed milk carefully avoiding the fat layer at the top

5. Take 5 ml and Conc. under N₂, @ 30 °C
6. Centrifuge 10 min @ 3500 rpm
7. Shakie at 200 rpm for 20 min
8. Add 8 ml ACN and 100 μl acetic acid

9. Filter (0.2 μm PTFE) and inject in UHPLC-MS/MS
Chlorate Chromatography

2 µg/kg in milk (full fat)  Neg control milk (full fat)
## Matrix Effects study (raw milk)

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>ME% Chlorate</th>
<th>ME% Perchlorate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.4</td>
<td>-11.3</td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
<td>-15.2</td>
</tr>
<tr>
<td>3</td>
<td>4.7</td>
<td>-19.8</td>
</tr>
<tr>
<td>4</td>
<td>3.4</td>
<td>-16.5</td>
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<tr>
<td>5</td>
<td>3.4</td>
<td>-24.9</td>
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<tr>
<td>6</td>
<td>0.8</td>
<td>-17.0</td>
</tr>
<tr>
<td>7</td>
<td>9.2</td>
<td>-27.2</td>
</tr>
<tr>
<td>8</td>
<td>5.1</td>
<td>-15.6</td>
</tr>
<tr>
<td>9</td>
<td>3.3</td>
<td>-16.1</td>
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<tr>
<td>10</td>
<td>2.2</td>
<td>-8.7</td>
</tr>
<tr>
<td>11</td>
<td>5.5</td>
<td>-17.7</td>
</tr>
<tr>
<td>12</td>
<td>0.9</td>
<td>-18.7</td>
</tr>
<tr>
<td>13</td>
<td>3.3</td>
<td>-16.2</td>
</tr>
<tr>
<td>14</td>
<td>2.4</td>
<td>-26.4</td>
</tr>
<tr>
<td>15</td>
<td>0.3</td>
<td>-25.0</td>
</tr>
<tr>
<td>16</td>
<td>2.0</td>
<td>-19.9</td>
</tr>
<tr>
<td>17</td>
<td>-0.2</td>
<td>-12.2</td>
</tr>
<tr>
<td>18</td>
<td>3.5</td>
<td>-21.4</td>
</tr>
<tr>
<td>19</td>
<td>5.9</td>
<td>-18.1</td>
</tr>
<tr>
<td>20</td>
<td>7.2</td>
<td>-11.4</td>
</tr>
<tr>
<td>21</td>
<td>2.1</td>
<td>-14.0</td>
</tr>
</tbody>
</table>

**Chlorate**
-0.2 to 9.2 (%)
Slight Suppression

**Perchlorate**
-27.2 to -8.7 (%)
Enhancement
# Performance over 10 runs

<table>
<thead>
<tr>
<th>Run No.</th>
<th>Chlorate R2</th>
<th>Chlorate Accuracy</th>
<th>Chlorate Slope</th>
<th>Perchlorate R2</th>
<th>Perchlorate Accuracy</th>
<th>Perchlorate Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.9996</td>
<td>94-109</td>
<td>0.059089</td>
<td>0.9996</td>
<td>95-106</td>
<td>0.169937</td>
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<tr>
<td>2</td>
<td>0.9995</td>
<td>95-105</td>
<td>0.059293</td>
<td>0.9995</td>
<td>91-112</td>
<td>0.169796</td>
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<tr>
<td>3</td>
<td>0.9995</td>
<td>95-107</td>
<td>0.056390</td>
<td>0.9995</td>
<td>95-105</td>
<td>0.163613</td>
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<tr>
<td>4</td>
<td>0.9978</td>
<td>95-107</td>
<td>0.055180</td>
<td>0.9997</td>
<td>96-105</td>
<td>0.159428</td>
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<td>5</td>
<td>0.9968</td>
<td>80-109</td>
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<td>0.9999</td>
<td>96-105</td>
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<td>6</td>
<td>0.9996</td>
<td>93-110</td>
<td>0.056007</td>
<td>0.9998</td>
<td>91-107</td>
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<tr>
<td>7</td>
<td>0.9996</td>
<td>96-106</td>
<td>0.056731</td>
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<tr>
<td>8</td>
<td>0.9998</td>
<td>95-105</td>
<td>0.058336</td>
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<td>97-104</td>
<td>0.162944</td>
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<tr>
<td>9</td>
<td>0.9998</td>
<td>97-105</td>
<td>0.059273</td>
<td>0.9993</td>
<td>87-108</td>
<td>0.164696</td>
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<tr>
<td>10</td>
<td>0.9999</td>
<td>97-103</td>
<td>0.059349</td>
<td>0.9996</td>
<td>94-123</td>
<td>0.165849</td>
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</table>
# Accuracy and Precision

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Fortification Level (µg/kg)</th>
<th>Mean (µg/kg)</th>
<th>S.D. (µg/kg)</th>
<th>CV (%)</th>
<th>Trueness (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorate</td>
<td>2</td>
<td>2.04</td>
<td>0.18</td>
<td>8.6</td>
<td>92-112</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>99.0</td>
<td>2.5</td>
<td>2.5</td>
<td>95-105</td>
</tr>
<tr>
<td>Perchlorate</td>
<td>2</td>
<td>2.04</td>
<td>0.13</td>
<td>6.2</td>
<td>95-108</td>
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<tr>
<td></td>
<td>100</td>
<td>98.8</td>
<td>1.46</td>
<td>1.48</td>
<td>94-101</td>
</tr>
</tbody>
</table>
Milk Comparison (inter-lab)
Chlorate Milk Powder QC

**G1/16/2 Milk Powder Control**
- Mean: 57 (µg/kg)
- S.D.: 2.1
- CV: 3.8%

**G1/16/4 Milk Powder Control**
- Mean: 371 (µg/kg)
- S.D.: 1.4
- CV: 3.7%
Chlorate in water

- MRM (82.9 -> 66.9) 170206_17_01w_9.d Cal1_w_0.2
  * 2.035 min.
  0.192 mg/ml

- MRM (84.9 -> 68.9) 170206_17_01w_9.d Smooth
  Ratio = 32.1 (102.1 %)
  * 2.034 min.
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