Teagasc Gateways 16th November 2017

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

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What are residues?

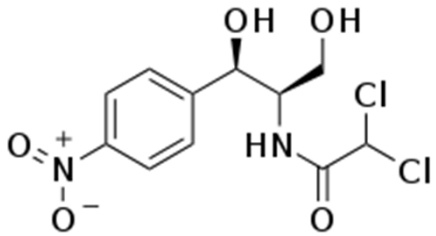
- Residues can arise in milk and milk products from the following sources:
  - Use of licensed veterinary drugs for treatment of dairy cows.
  - Illegal use of banned drugs or growth promoting agents.
  - Pesticides used at farm level to control insect infestations
  - Contaminants from Animal Feed or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

Residue Categories & Sampling

Group	Drug Class	Milk
A6	Banned Drugs	X
B1	Antibiotics	X
B2a	Anthelmintics	X
B2b	Anticoccidials	
B2c	Carbamates / Pyrethroids	
B2d	Sedatives / Tranquilizers	
B2e	NSAIDs	X
B2f	Corticosteroids	

A6. Table 2 Regulation 37/2010

Chloramphenicol



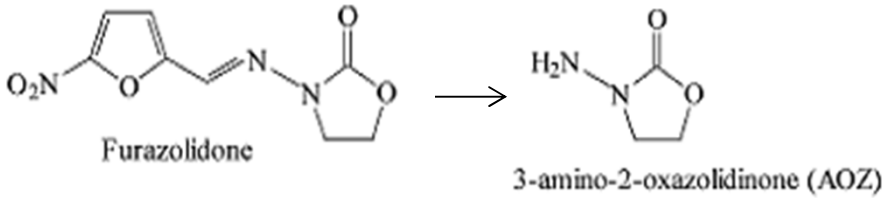
Hazard:
Aplastic anaemia

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

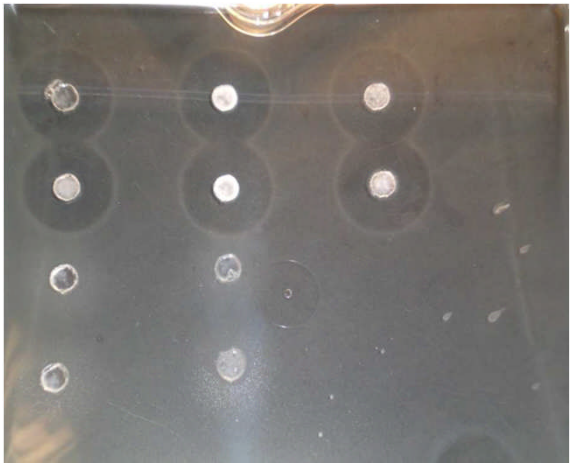
- ✓ Sulphonamides
- ✓ Tetracyclines
- ✓ Macrolides and lincosamides
- ✓ Aminoglycosides
- ✓ Beta-lactams
- ✓ Quinolones
- ✓ Amphenicols
- ✓ Peptide antibiotics

Methods of analysis of antimicrobials can be grouped in

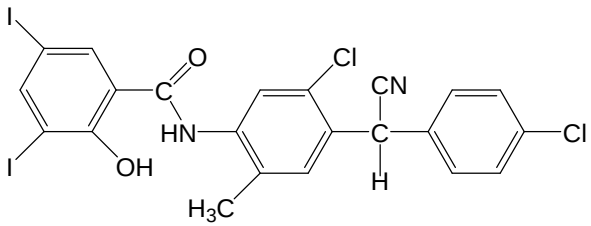
- ❖ Microbiological=> fast screening, limited information
- ❖ Immunochemical=> rapid, selective and sensitive (e.g. ELISA)
- ❖ Physico-chemical=> accurate identification and quantification

Inhibition Assays Overview

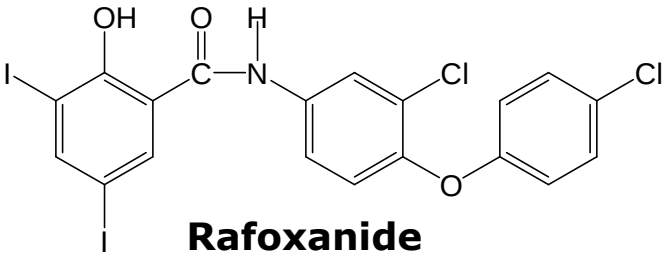
- Low cost, suitable for industry and rapid
- No one method will do all
- Validation can be challenging.
- Results should be confirmed because tests are not quantitative
- Unsuitable for chloramphenicol and nitrofurans etc



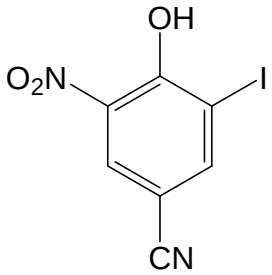
B2a. Anthelmintics



Closantel



Rafoxanide



Nitroxynil

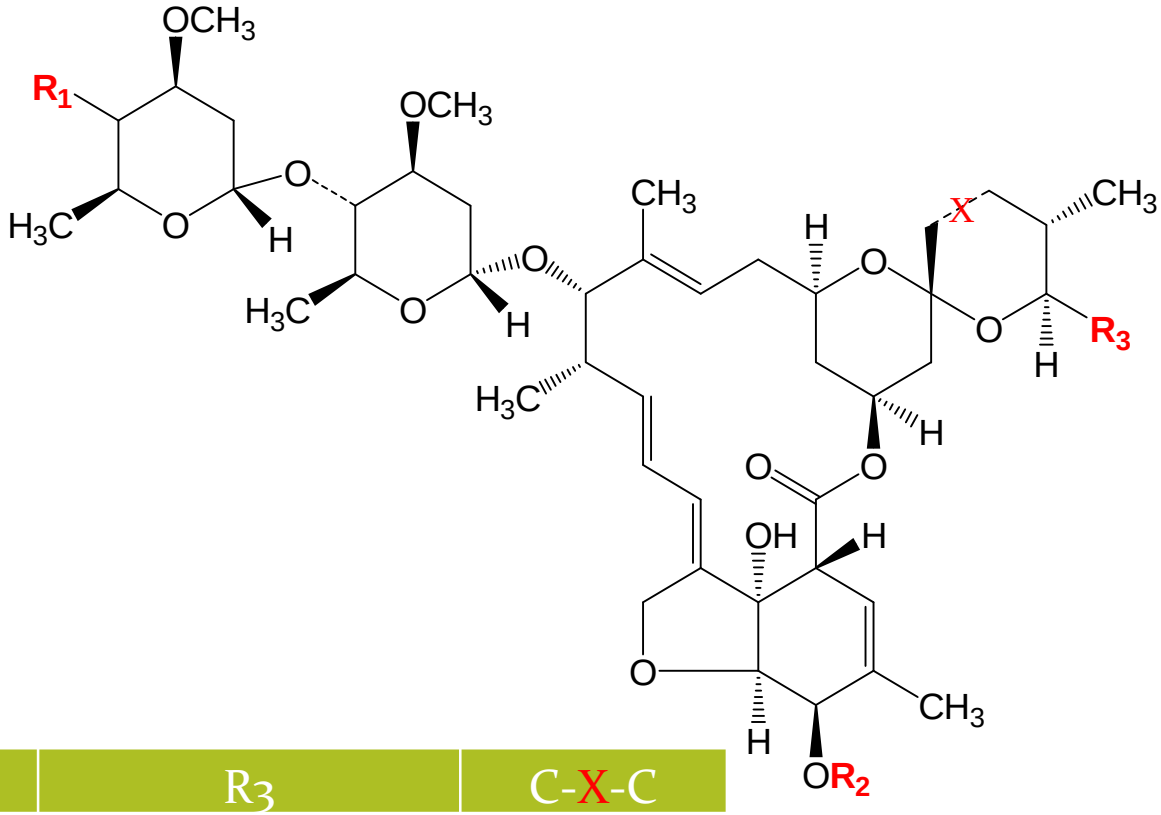
Use and hazard

- Control of
  - Nematodes (roundworms)
  - Cestodes (tapeworms)
  - Trematodes (flukes)
- 3 classes of drugs:
  - Benzimidazoles
  - Macrocyclic Lactones
  - Flukicides
- Some drugs are teratogenic or neurotoxic
- Many products not licensed in lactating animals

Analysis

- Detection: HPLC-UV/FLD and LC-MS/MS

B2a. Anthelmintics - Endectocides



Compound	R1	R2	R3	C-X-C
Abamectin	OH	H	$CHCH_3CH_2CH_3$	-CH=CH-
Doramectin	OH	H	C_6H_{11}	-CH=CH-
Emamectin	$C_6H_5COOHCH_3NH$	H	$CHCH_3CH_2CH_3$	-CH=CH-
Ivermectin	OH	H	$CHCH_3CH_2CH_3$	$-CH_2-CH-$

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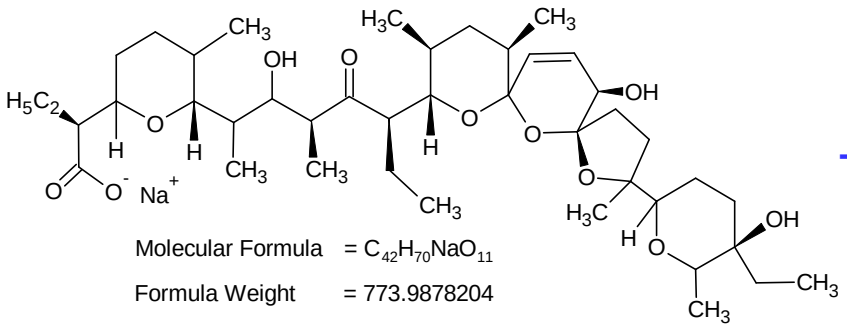
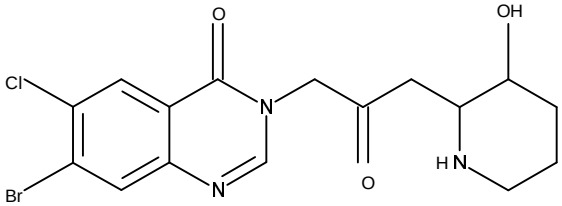
B2b. Anticoccidials



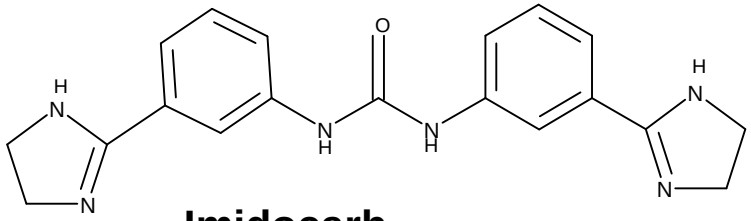
Molecular Formula = $C_{42}H_{70}NaO_{11}$

Formula Weight = 773.9878204

Salinomycin



Halofuginone



Imidocarb

Use and hazard

- Control/Treatment of:
  - Coccidiosis
  - Acute bovine respiratory disease
  - Cryptosporidiosis
  - Babesiosis
  - Isosporiasis
- Two main classes:
  - Ionophores
  - Chemical anticoccidials
- Some anticoccidials are cardiotoxic, neurotoxic

Analysis

- Low LODs required for milk
- Detection: LC-MS/MS

B2b. Regulations

Commision 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

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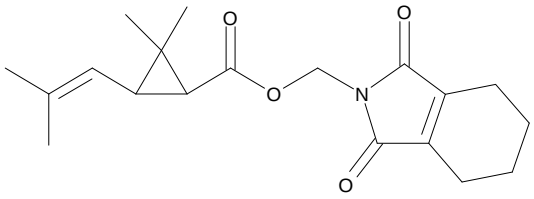
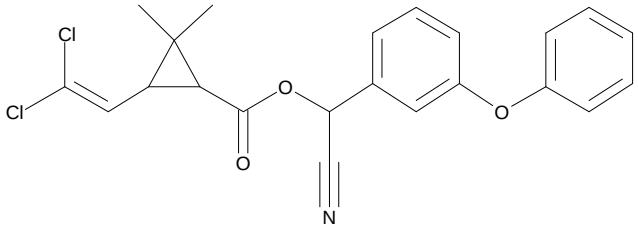
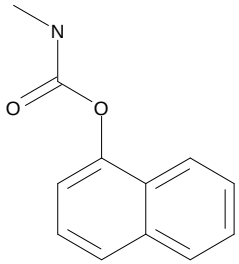
B2c. Carbamate and Pyrethroids



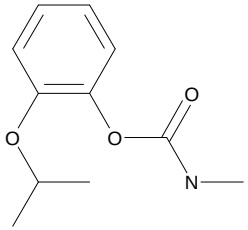
Tetramethrin



Cypermethrin



Carbaryl



Propoxur

Use and hazard

- ➢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

Analysis

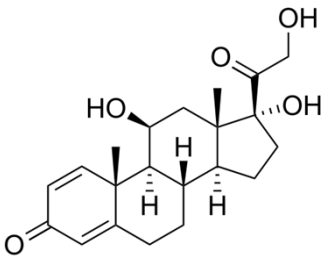
- ➢ Detection: HPLC-FLD, GC-ECD , GC-MS/MS, LC-MS/MS

B2e. NSAID's & B2f. Other

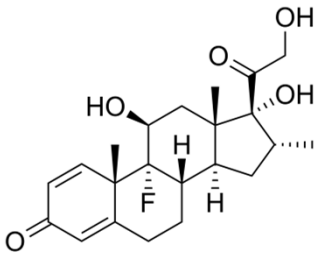
Heterogeneous drug group:

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



Prednisolone



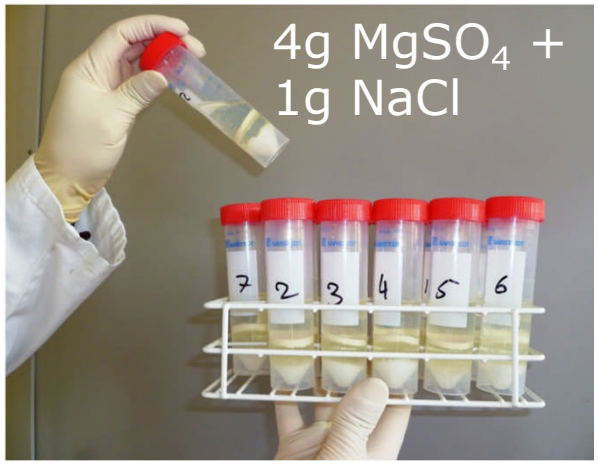
Dexamethasone

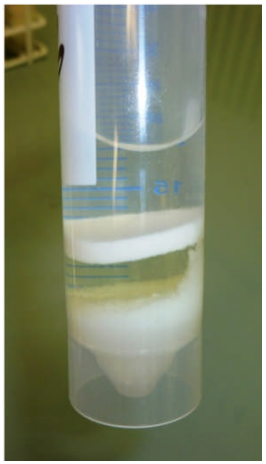
Might be used in cocktails with other ilegal substances in animal feeding (beta-agonists/anabolic steroids)

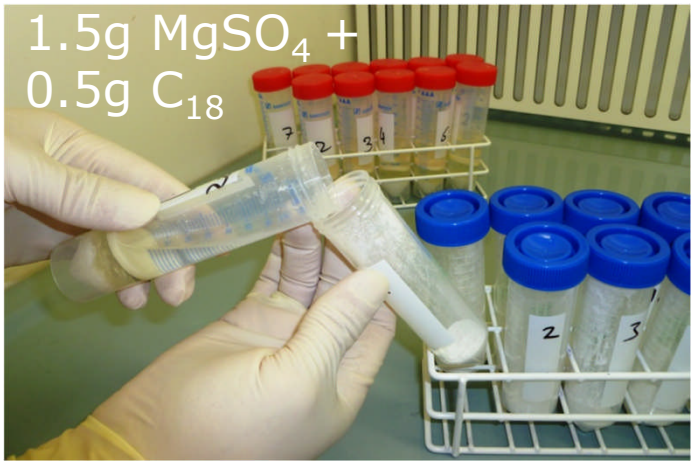
Analytical Developments

Improving the throughput of veterinary drug residue analysis using vibrational shaking technology

QuEChERS Approach



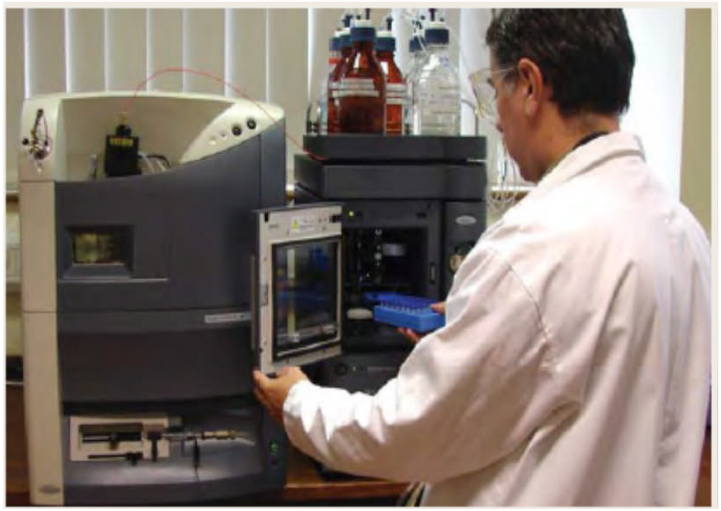




Phase Separation

D-SPE

Concentration

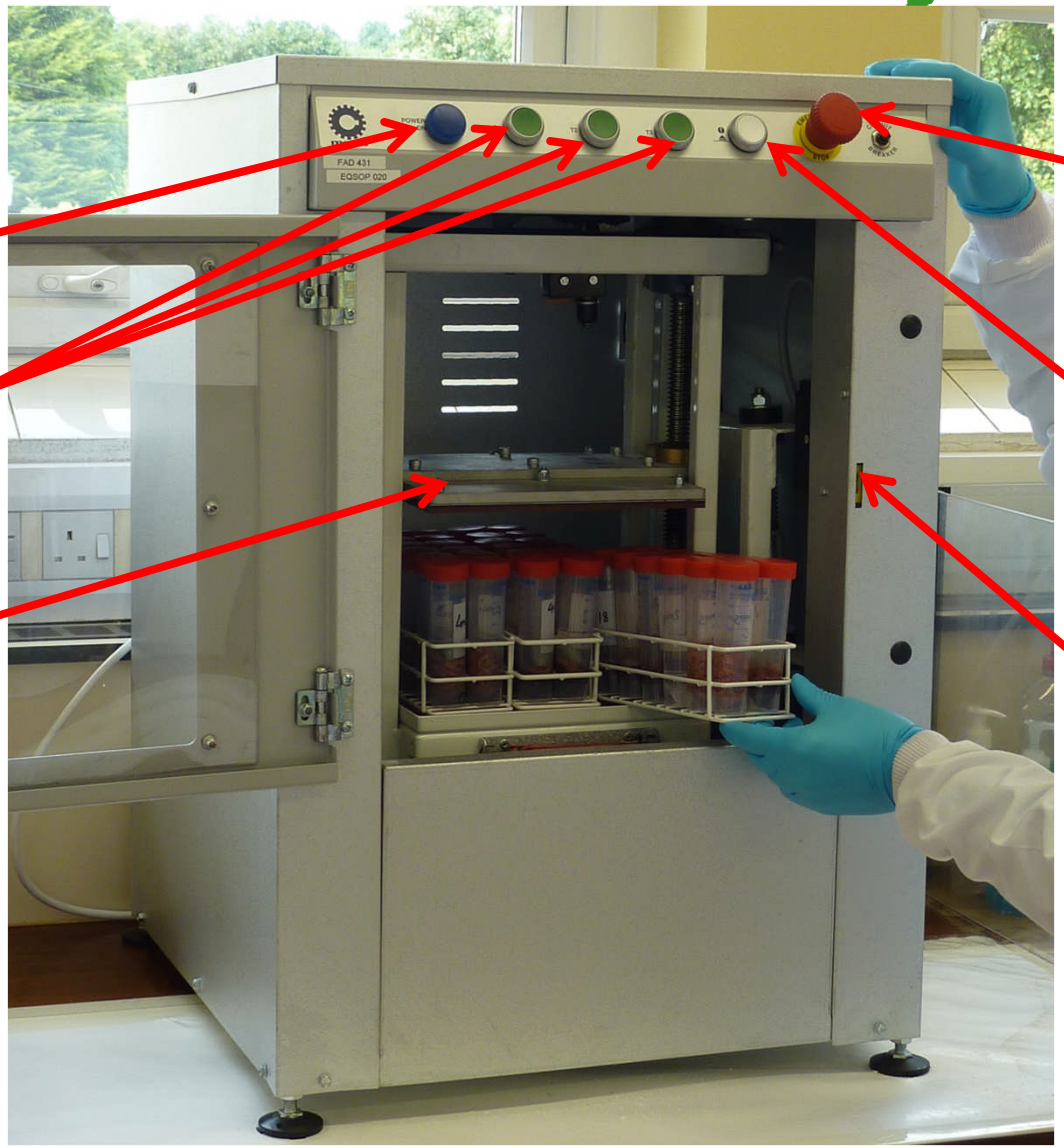


Analysis

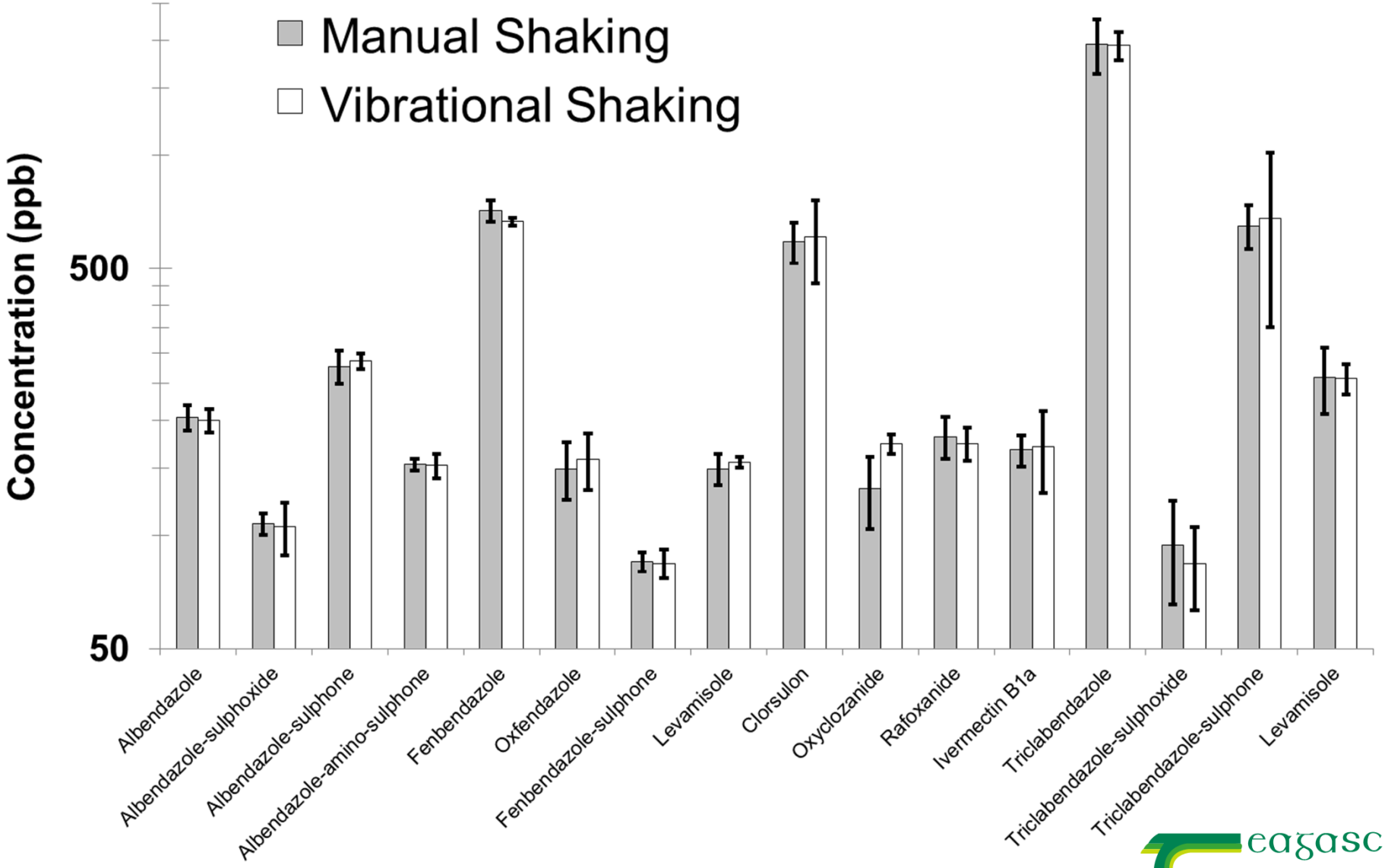
Automated shaking

- 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



Incurred samples study



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

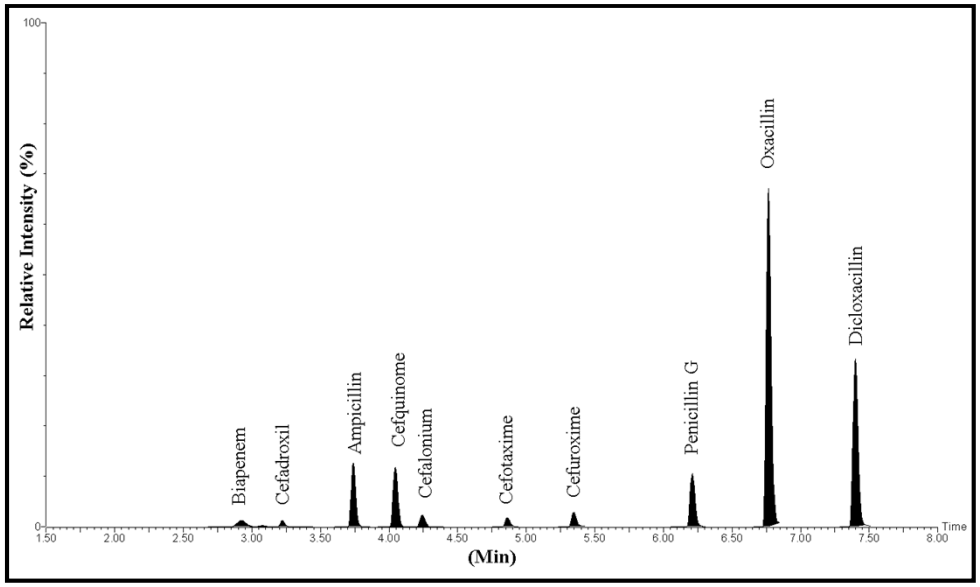
Issues with current approaches

- 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.



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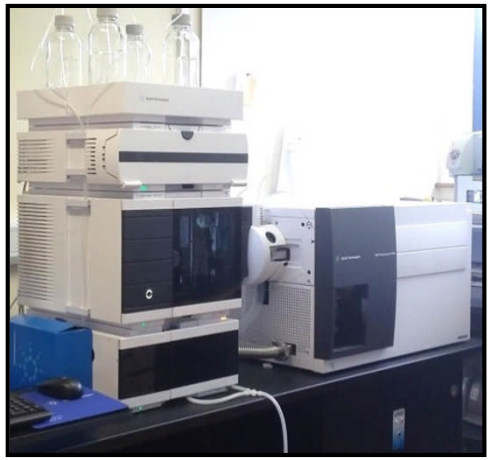
Method overview for milk

Weigh 2 g of milk → Add 100 µL of isotope std → Add 1 mL H_2O & 7 mL MeCN → Vortex 1 min → Centrifuge 2800 x*g* (15 min, 4°C) → d-SPE clean-up (500 mg C_{18}) → Centrifuge 3500 rpm (15 min, 4°C) → N_2-EVAP 5 mL to <1 mL → Reconstitute to 1 mL, filter →



Chromatography conditions

Analytical column: Agilent Phenyl Hexyl column

Binary gradient of:

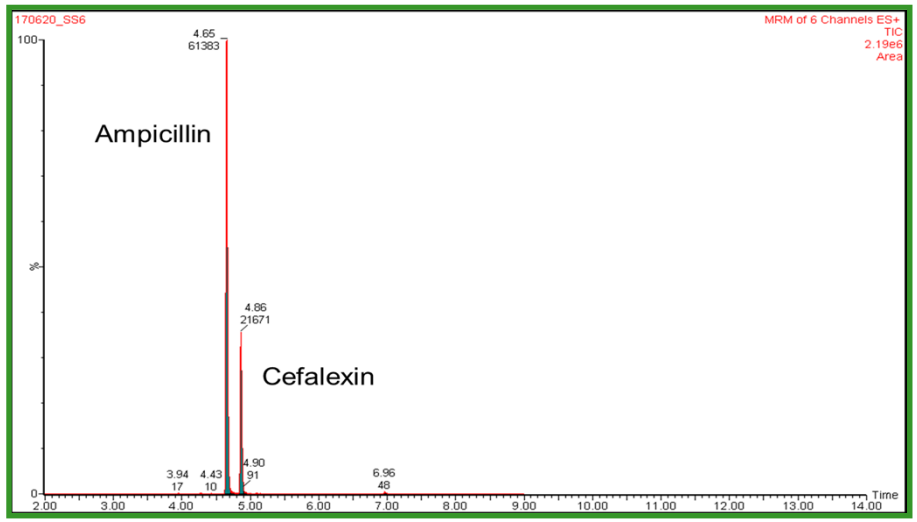
Mobile phase A: HCOOH 0.01% + 0.2 mM ammonium acetate in water

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

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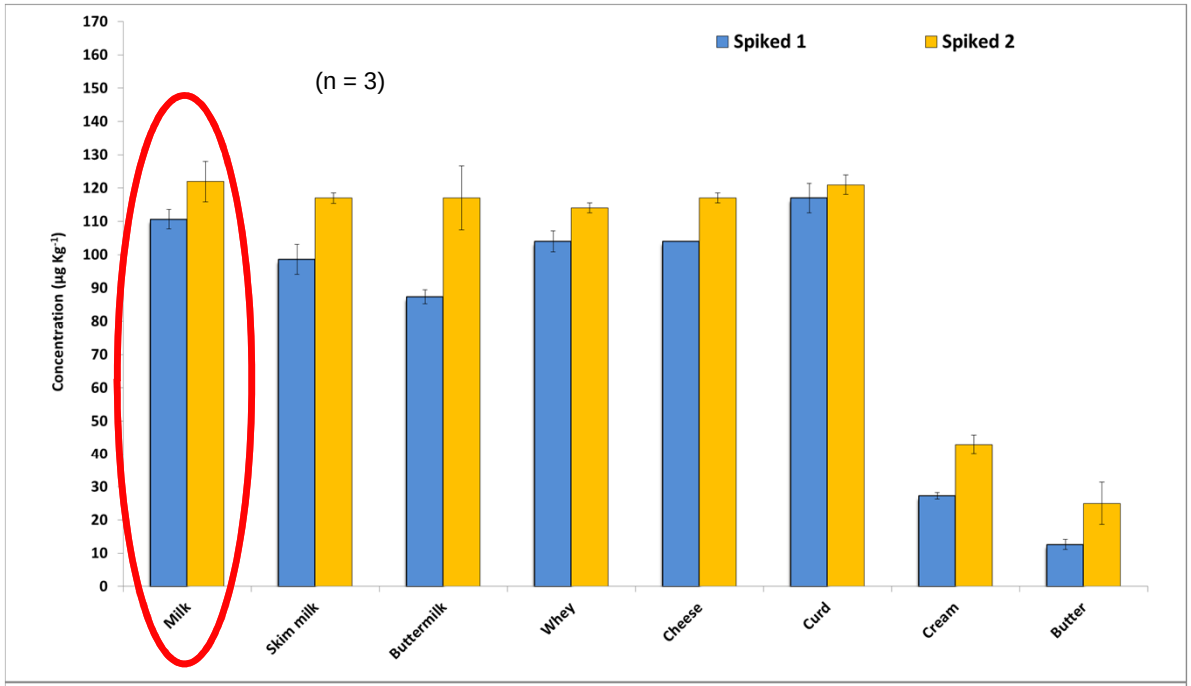
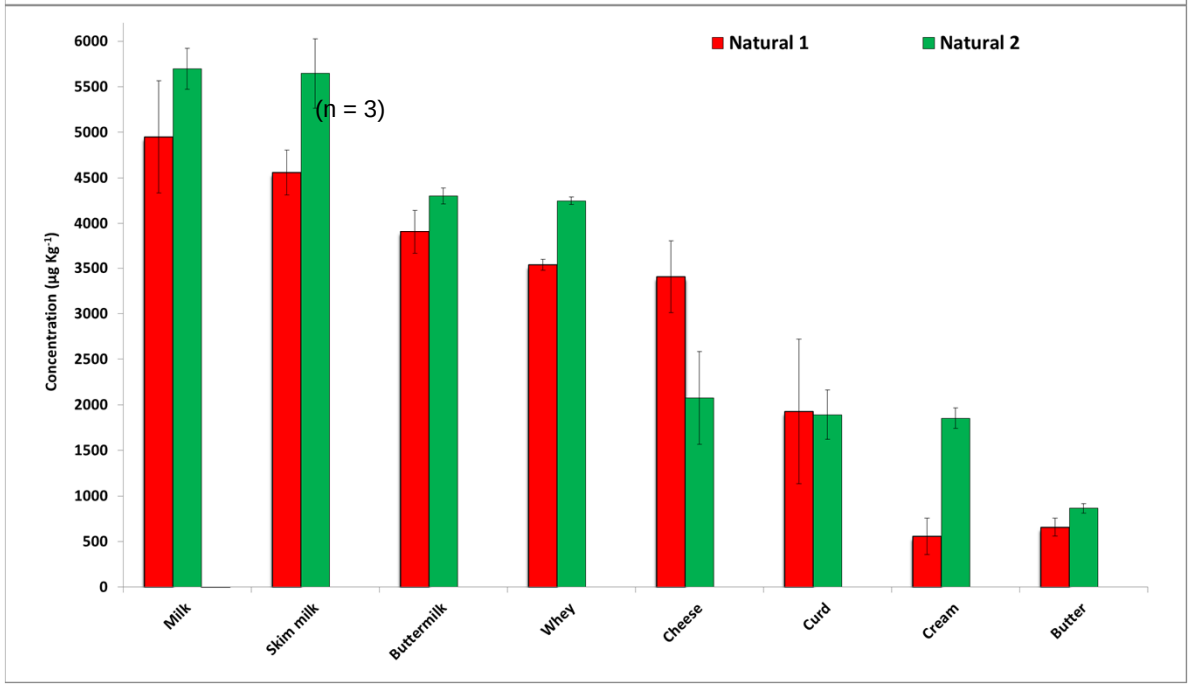
Analysis of cefquinome in dairy products

Matrix	Fortification level ($\mu g\ kg^{-1}$)	Mean ± SD ($\mu g\ kg^{-1}$)	RSD(%)	Trueness(%)
Liquid samples (samples = 11; days = 5)				
Milk	4.0	4.1 ± 0.15	3.8	101
	250	258 ± 9.8	3.8	103
Skimmed milk	4.0	4.0 ± 0.26	6.4	100
	250	251 ± 8.8	3.5	100
Buttermilk	4.0	3.5 ± 0.33	9.3	87
	250	226 ± 7.9	3.5	91
Whey	4.0	3.5 ± 0.20	5.8	86
	250	214 ± 5.6	2.6	85
Cream	4.0	4.0 ± 0.20	5.1	99
	250	256 ± 9.2	3.6	102
Solid samples (samples = 11; days = 3)				
Curd	4.0	3.6 ± 0.25	6.8	90
	250	237 ± 5.5	2.3	95
Cheese	4.0	3.9 ± 0.34	8.6	98
	250	244 ± 7.5	3.1	97
Butter	4.0	3.9 ± 0.16	4.1	99
	250	259 ± 7.2	2.8	104



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

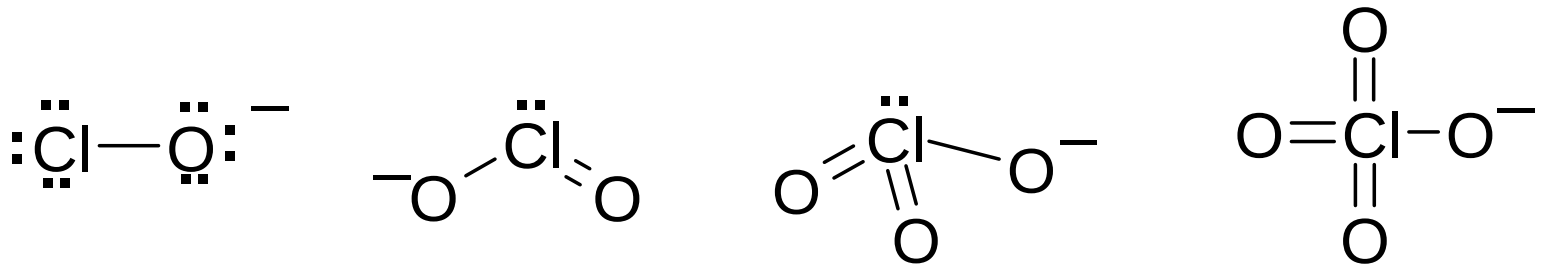
Reconstituted IF = 25.2 g powder + 180 mL H2O

= 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



Hypochlorite Chlorite Chlorate Perchlorate

- 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

Weigh 5g of Milk

Add 100 µL Isotopically labelled internal standard

Centrifuge 10 min @ 3500 rpm

Take 2ml (~2g) skimmed milk carefully avoiding the fat layer at the top

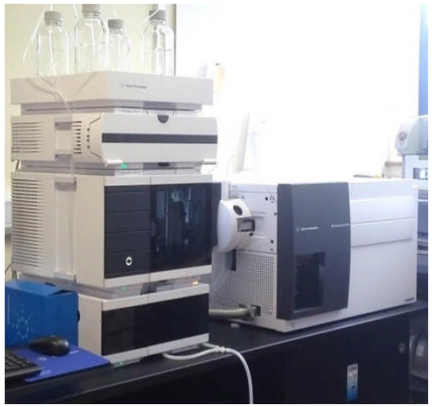
Add 8 ml ACN and 100 µl acetic acid

Shakie at 200 rpm for 20 min

Centrifuge 10 min @ 3500 rpm

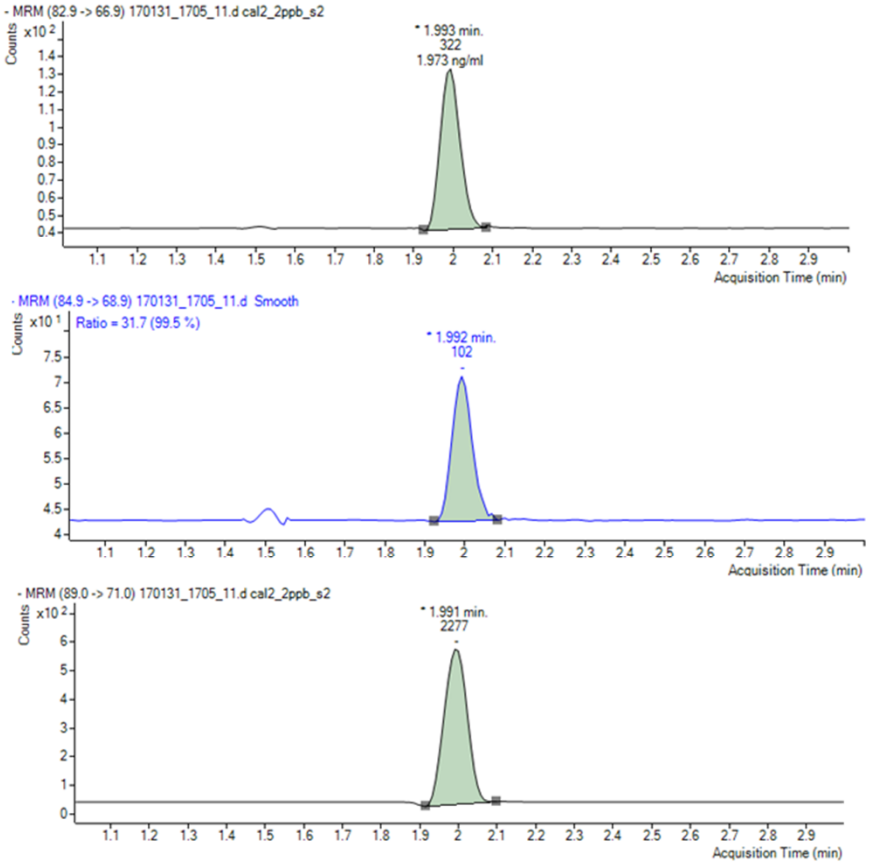
Take 5 ml and Conc. under N_2, @ 30 °C

Filter (0.2 µm PTFE) and inject in UHPLC-MS/MS

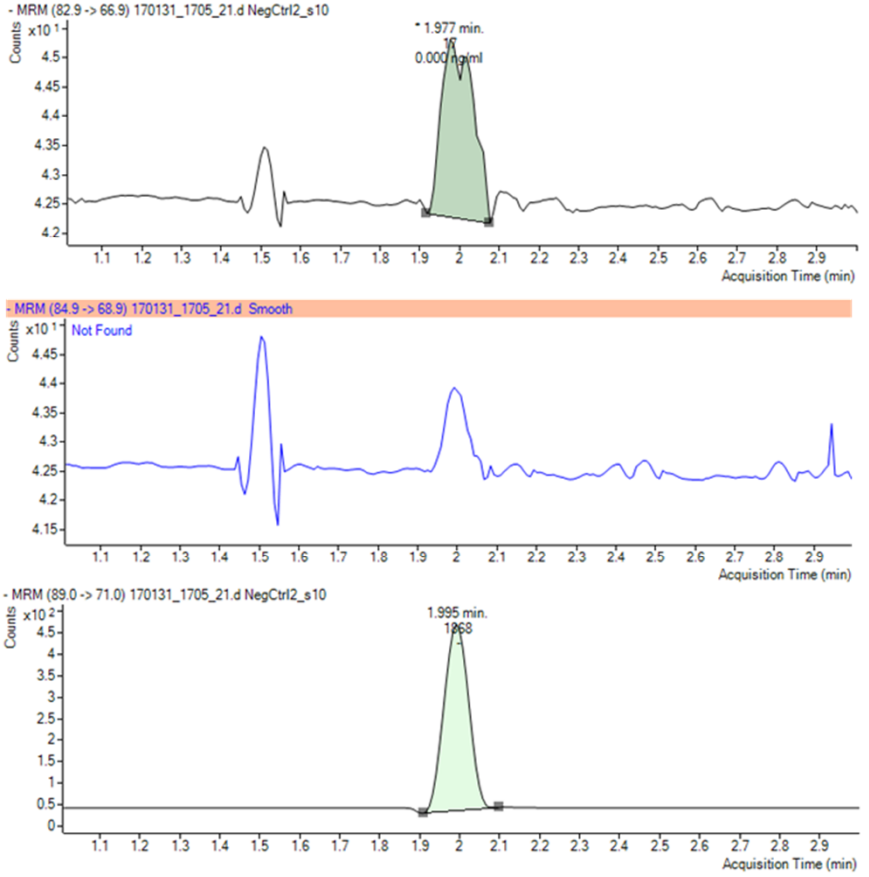


Chlorate Chromatography

2 µg/kg in milk (full fat)



Neg control milk (full fat)



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Matrix Effects study (raw milk)

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

Chlorate
-0.2 to 9.2 (%)
Slight Suppression

Perchlorate
-27.2 to -8.7 (%)
Enhancement

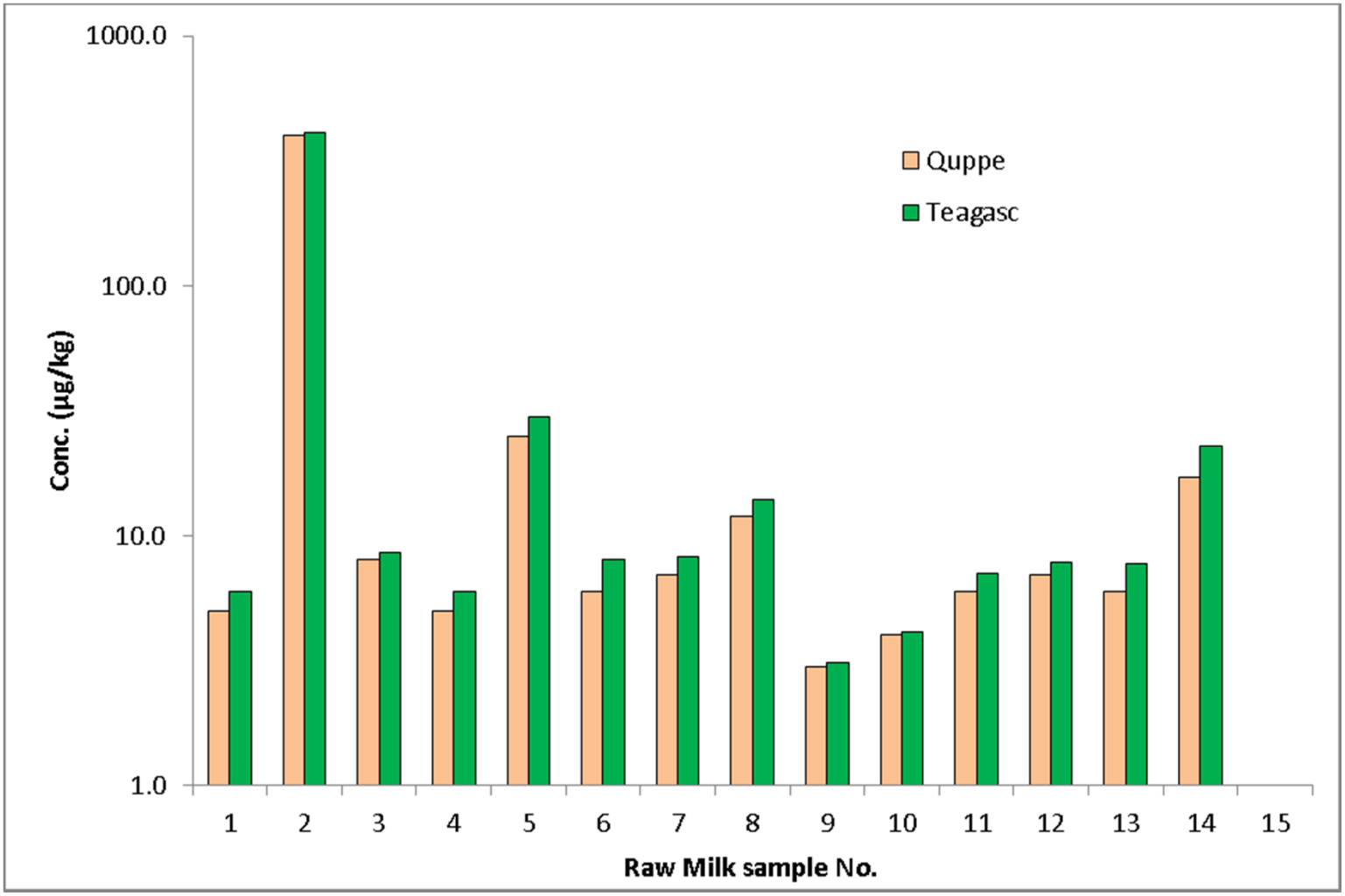
Performance over 10 runs

	Chlorate			Perchlorate		
Run No.	R2	Accuracy	Slope	R2	Accuracy	Slope
1	0.9996	94-109	0.059089	0.9996	95-106	0.169937
2	0.9995	95-105	0.059293	0.9995	91-112	0.169796
3	0.9995	95-107	0.056390	0.9995	95-105	0.163613
4	0.9978	95-107	0.055180	0.9997	96-105	0.159428
5	0.9968	80-109	0.057818	0.9999	96-105	0.166732
6	0.9996	93-110	0.056007	0.9998	91-107	0.166914
7	0.9996	96-106	0.056731	0.9998	96-107	0.164428
8	0.9998	95-105	0.058336	0.9998	97-104	0.162944
9	0.9998	97-105	0.059273	0.9993	87-108	0.164696
10	0.9999	97-103	0.059349	0.9996	94-123	0.165849

Accuracy and Precision

Analyte	Fortification Level (µg/kg)	Between days study (n =2 x 10d) Mean (µg/kg)	S.D. (µg/kg)	CV (%)	Trueness (%)
Chlorate	2	2.04	0.18	8.6	92-112
	100	99.0	2.5	2.5	95-105
Perchlorate	2	2.04	0.13	6.2	95-108
	100	98.8	1.46	1.48	94-101

Milk Comparison (inter-lab)



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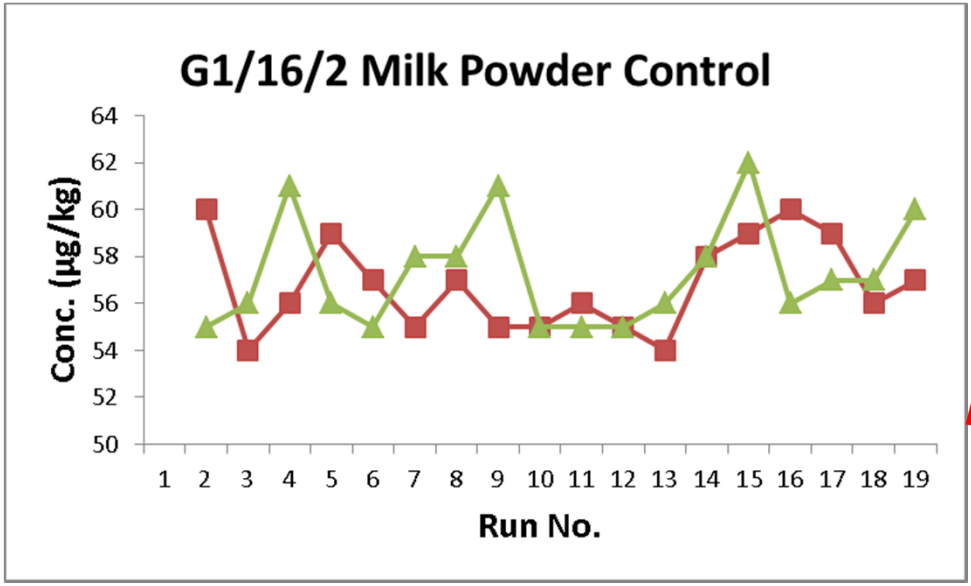
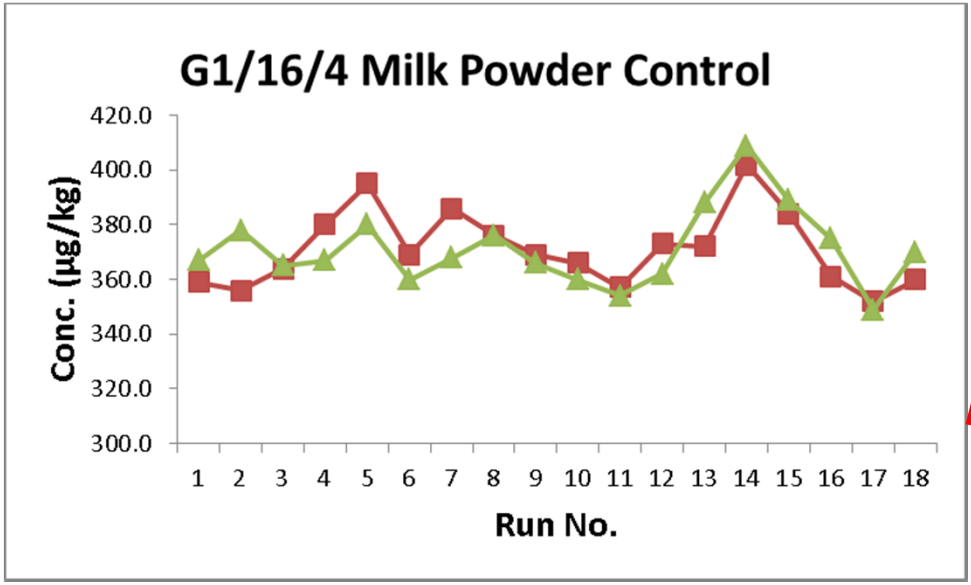
Chlorate Milk Powder QC



Mean:	**57 (µg/kg)**
S.D.	**2.1**
CV	**3.8%**



Mean:	**371 (µg/kg)**
S.D.	**1.4**
CV	**3.7%**

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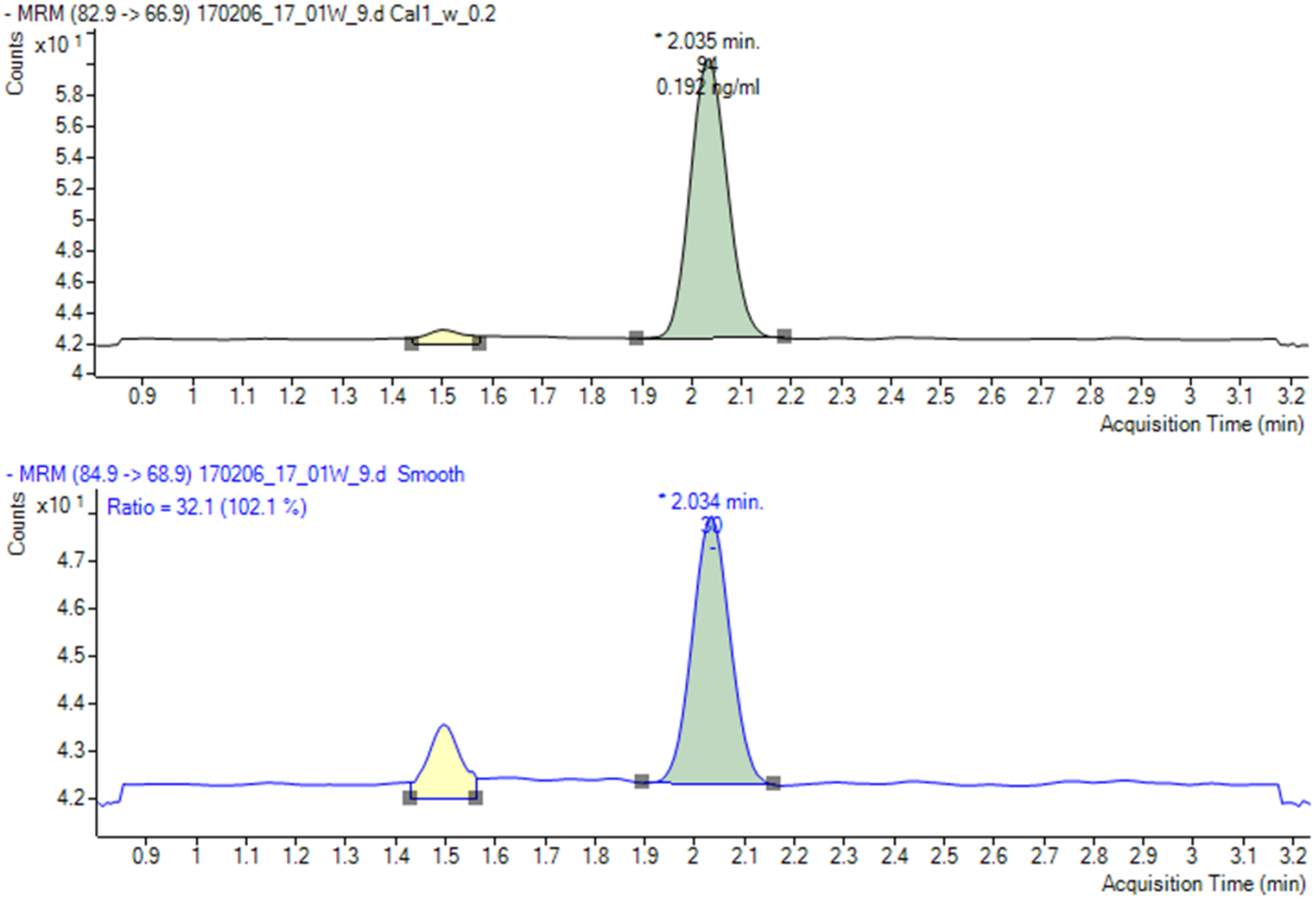
Chlorate in water



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