Determination of antibiotic residues in feedingstuffs.

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Plan

- Introduction
  - Problematic
  - Feed in the food chain
  - Laws and regulations
  - Feed, feed additives & Veterinary medicinal products
- Objective
- Screening method
- Confirmatory method
- Conclusion
Contamination of animal feeds

- Can cause deleterious health effects in animals
- And in the consumers by “secondary exposure”.
- Examples:
  - BSE in cattle,
  - Use of growth promoting antibiotics and bacterial resistance,
  - Contamination with toxic contaminants with deleterious health effects on the animals,
  - Contamination with veterinary medicinal products with potentially harmful effects.

“WE ARE WHAT WE EAT”
Feed in the total food chain...

- Pesticides
- Dioxins
- Mycotoxins
- Veterinary drugs
- Heavy metals...

Feed → Farm → Food processor → Consumer

• Feed is the starting point of the food chain.
Animal nutrition sector

- Production: around 120 million tonnes of animal feedingstuffs annually in EU.

- Good quality animal nutrition is essential.

- Affect animal health, food safety & human health.
Laws, rules and regulation

- EU Legislation regulates food safety and animal nutrition
  - Labelling, marketing and circulation of feedingstuffs
  - Requirements for feed hygiene
  - Official feed and food controls
  - Use of additives,
  - Genetically modified feedingstuffs,
  - Preparation and marketing of medicated foodstuffs
  - ...
The General Food Law EC 178/2002

I

(Acts whose publication is obligatory)

of 28 January 2002
laying down the general principles and requirements of food law, establishing the European Food
Safety Authority and laying down procedures in matters of food safety

Applies to all stages of the production, processing and distribution of food and also of feed produced for, or fed to, food producing animals “farm to fork” approach
Definition of Feed

Materials for feedingstuffs is defined by directive 96/25/EC

Feed materials definition:
- various products of vegetable or animal origin,
- in their natural state, fresh or preserved,
- and products derived from the industrial processing thereof,
- and organic or inorganic substances,
- whether or not containing additives,
- which are intended for use in oral animal feeding either directly as such, or after processing, in the preparation of compound feedingstuffs or as carriers of premixtures.
Main feed materials
Annex B directive 96/25/EC

- 1 Cereal grains, their products and by-products
- 2 Oil seeds, oil fruits, their products and by-products
- 3 Legume seeds, their products and by-products
- 4 Tubers, roots, their products and by-products
- 5 Others seed and fruits, their products and by-products
- 6 Forages and roughage
- 7 Others plants, their products and by-products
- 8 Milk products
- 9 Land animal products
- 10 Fish, other marine animals, their products and by-products
- 11 Minerals
- 12 Miscellaneous
Feed additives - Definition

  – “Feed additives” means substances, micro-organisms or preparations, other than feed material and premixtures which are internationally added to feed or water in order to perform, in particular one or more of the functions mentioned in Article 5 (3).

• Regulation 1831/2003 amending Directive 70/524/EEC, Additives may be classified into the following categories:
  – Technological additives (e.g. preservatives, antioxidants, emulsifiers, stabilising agents, acidity regulators, silage additives)
  – Sensory additives (e.g. flavours, colorants)
  – Nutritional additives (e.g. vitamins, minerals, aminoacids, trace elements)
  – Zootechnical additives (e.g. digestibility enhancers, gut flora stabilizers)
  – Coccidiostats and histomonostats
Veterinary medicinal products

- Veterinary medicines in animal feedingstuffs:
  “Any mixture of a veterinary medicinal product or products and feed or feeds which is ready prepared for marketing and intended to be fed to animals without further processing, because of its curatives or preventive properties or other properties as a medicinal product”.

- Directive 2001/82/EC governs:
  - Production,
  - Marketing,
  - Distribution and
  - Use of veterinary medicinal products.
Substances  Council directive 96/23/EC, annex 1

• **Group A:** anabolic effect and unauthorised substances
  - Stilbenes,
  - Antithyroid agents
  - Steroids
  - Resorcylic acid lactones
  - Beta-agonists
  - Compounds included in annex IV No 2377/90 & 470/2009 (Lists of pharmacologically active substances for which no maximum levels can be fixed)

• **Group B:** veterinary drugs & contaminants
  - Antibacterial substances
  - Others veterinary drugs
    - Anthelmintics
    - Anticoccidials
    - Carbamates and Pyrethroids
    - Sedatives
    - NSAIDS
    - Other pharmacologically active substances
  - Other substances and environmental contaminants
    - Organochlorine compounds
    - Organophosphorus compounds
    - Chemical elements
    - Mycotoxins
    - Dyes
    - Others

Banned substances, lower limits

**Substances with MRL**
Safety = Controls !!!

- Official feed and food controls Regulation 882/2004

To assure chemical safety and quality in the European food supply; support of EC policies and competitiveness of food and feed industries......
Objective of this project:

- Development of methods to detect
  - **What**: veterinary drugs (antibiotics, coccidiostats)
  - **Where**: in feedstuffs
  - **To**: Luxembourgish agriculture sector ASTA
  - **Why**: To control the fraudulence (illegal or unauthorized drugs) and cross contamination.
    Lack of control methods in these matrix.

- Made “Animal Feeds” feel secure…..
# High number of molecules

<table>
<thead>
<tr>
<th>Antimicrobial family</th>
<th>Substances</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polypeptides</td>
<td>Bacitracin Zn <em>(Banned 99)</em>, Colistin</td>
</tr>
<tr>
<td>Pleuromutilins</td>
<td>Valnemulin</td>
</tr>
<tr>
<td>Ionophores</td>
<td>Lasalocid, Maduramycin, Narasin, Monensin <em>(Banned 06)</em>, Salinomycine <em>(Banned 06)</em>,</td>
</tr>
<tr>
<td>Glycolipide</td>
<td>Flavophospholipol <em>(Banned 06)</em></td>
</tr>
<tr>
<td>Macrolides</td>
<td>Spiramycin <em>(Banned 99)</em>, Tilmicosin, Tylosin <em>(Banned 99)</em></td>
</tr>
<tr>
<td>Lincosamides</td>
<td>Lincomycin</td>
</tr>
<tr>
<td>Streptogramines</td>
<td>Virginiamycin <em>(Banned 99)</em></td>
</tr>
<tr>
<td>Penicillines</td>
<td>Penicillin V &amp; G, Amoxycillin</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Tetracycline, Chlortetracycline, Oxytetracycline, Doxycycline</td>
</tr>
<tr>
<td>Diaminopyrimidines</td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>Sulfadiazin</td>
</tr>
<tr>
<td>Not define</td>
<td>Robenidine</td>
</tr>
<tr>
<td>Quinoxalines</td>
<td>Carbadox, Olaquindox <em>(Banned 99, carcinogenic genotoxic</em>)</td>
</tr>
<tr>
<td>Phenicol</td>
<td>Florfenicol, Chloramphenicol <em>(Annexe 4 LMR)</em></td>
</tr>
<tr>
<td>Quinolones</td>
<td>Ac nalidixic, Ac oxolinic, Flumequin, Ciprofloxacin, Enrofloxacin, Marbofloxacin, Danofloxacin</td>
</tr>
</tbody>
</table>

**35 compounds**  
**15 families**
Structure & properties very different

Oxytetracycline (Tetracyclines)

Salinomycin (ionophores coccidiostats)

Enrofloxacin (Quinolones)

Tilmicosin (macrolides)

Amoxycillin (Beta-lactams)
Classes of antibiotics introduced

1940
- Sulfa drugs
  - β-Lactams

1950
- Aminoglycosides
- Chloramphenicol, Tetracyclines

1960
- Macrolides
- Glycopeptides
- Quinolones, Streptogramins

1970
- ß-Lactams

1980
- Mutilins
- Lipopeptides
- Oxazolidones

1990

2000

2010

How antibiotics work

- Mechanism of action

  - Glycopeptides, Beta-lactams.
  - Bacterial cell wall synthesis
  - Plasmic membrane
  - Polymyxines
  - Protein synthesis
  - DNA or RNA synthesis
  - Cell metabolism, biosynthesis of folic acid
  - Diaminopyrimidines & Sulfonamides
  - Quinolones, Rifamycins
  - Aminosides & Tetracyclines (30S), Macrolides, Lincosamides & Streptogramines, Phenicols (50S).
How bacteria reacts

- **Antibiotic resistance:**
  - 2 types:
    - Natural (intrinsic) resistance
    - Acquired resistance
  - Mutational resistance / Acquisition or transfer of resistance
  - Expression of resistance
    - Production of enzyme
    - Cell permeability modified
    - Efflux mechanisms
    - Alteration of target
    - Bypass of metabolic pathway
Coccidiostats

- Used to treat the protozoan parasite causing coccidiosis (parasitic diseases)
- Infectious site: gastrointestinal tract
- Used as additives to prevent (narasin, monensin, lasolacid, robenidine, flavomycin, …)
- Also an antimicrobial activity,
- Complex effect on the intestinal flora.
Animal feed is a complex matrix

- Different species targeted (bovine, porcine, avian…)
- Different age and production groups (cattle, dairy cow, beef)
- Different forms (liquid or solid form, powder, ….)
Animal Feed Composition

- **Composition:**
  - Carbohydrates: 70-80% (starch)
  - Proteins: 15%
  - Lipids: < 5%
  - Mineral compounds
  - ....
Selected procedure for the analysis

Sample

First step

Screening method

Non-compliant

Second step

Confirmatory method

Non-compliant

Compliant

Screening method

• Main requirements
  – Large number of samples
  – Multiple residues analysed
  – Easy to use
  – Low costs
  – Reduced time for results
  – Sensitivity (no positives are lost)
  – Specificity (minimum of false positives)
  – Repeatability

• Techniques available
  – Microbiological inhibition tests
  – Thin-Layer Chromatography
  – ELISA
  – High Performance Liquid Chromatography (HPLC)
  – …
Screening method: bacterial inhibition

**Principle:**
- Based on antimicrobial activity of the compound against bacterial strain
- Bactericide or Bacteriostatic effect

**Observation:** inhibition of bacterial growth

**Factors:**
- Bacteria (reference strain!) / Molecule
- pH acid / neutral / basic
- Ions (Mg2+)
- Enzymes (Penases)
- Synergic and antagonist factors (TMP + Sulfamides)
Protocol

1. Bacterial suspension
2. Microbiological culture media
3. Reagents

Inoculation

Screening plates

Diffusion and incubation

Read the inhibition bacterial growth area
List of the bacterial strains tested

- **Bacilli Gram +**
  - *Bacillus stearothermophilus* (spores) Merck & ATCC 10149
  - *Bacillus subtilis* (spores) BGA & ATCC 6633
  - *Bacillus cereus* (spores) ATCC 11778
  - *Bacillus pumilis* (spores) CN 607 & ATCC 14884
  - *Bacillus megaterium* (spores) ATCC 10778
  - *Corynebacterium xerosis* NCTC 9755

- **Bacilli Gram –**
  - *Escherichia coli* ATCC 11303, 29998 & 27166
  - *Yersinia ruckeri* NCIMB 13282
  - *Bordetella bronchiseptica* ATCC 4617

- **Cocci Gram +**
  - *Micrococcus luteus* ATCC 9341, 9341a, 10240 & 15957
  - *Staphylococcus aureus* ATCC 29213 & 6538P
  - *Staphylococcus epidermidis* ATCC 12228
  - *Staphylococcus warneri / spp* ATCC 12715

- **Cocci Gram -**
## Results

<table>
<thead>
<tr>
<th>Category</th>
<th>Optimal conditions</th>
<th>Limit of detection (Standard solution) (µg/L)</th>
<th>Feed* (mg/kg)</th>
</tr>
</thead>
</table>
| Beta-lactams, Penicillins (3)   | *B. stearothermophilus* Merck & pH acid to neutral  
*B. subtilis* BGA & pH acid                                                                                                                                                                                                 | 1 à 10  
10 à 50                                                                                            | 1 mg/kg       |
| Tetracyclines (4)               | *B. cereus* ATCC 11778 & pH acid                                                                                                                                                                                                                                           | 10 - 100                                                                             | 0.2 to 1      |
| Quinolones (2) & FluoroQ (5)    | *E. coli* ATCC 11303 & pH acid to neutral                                                                                                                                                                                                                                       | 500 - 1000 NA / 50 - 100 OA  
50 - 500 Flu / 1 - 10 FQ                                                                                                          | 5             |
| Macrolides (3)                  | *M. luteus* ATCC 9341 ou 9341A ou 10240  
& K₂HPO₄ & 7.5 µg/L Tylosin & pH basic                                                                                                                                                                                                                                           | 50 à 100                                                                             | 1             |
| Lincosamines (1)                | *M. luteus* ATCC 9341 ou 9341A & pH basic                                                                                                                                                                                                                                       | 500                                                                                   | 1             |
| Streptogramines (1)             | *M. luteus* ATCC 9341A ou 10240 & pH basic  
*M. luteus* ATCC 9341 & pH neutral                                                                                                                                                                                                                                           | 500                                                                                   | 2             |
| Pleuromutilins (1)              | *B. Stearo* ou *M. luteus* ATCC 9341 ou 9341A ou 10240 & pH basic                                                                                                                                                                                                              | 50 - 100                                                                             | ND            |

* Limit of detection (mg/kg) with the SIMBAG FEED method
## Results

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<tbody>
<tr>
<td>Polypeptides (2)</td>
<td>M. luteus ATCC 10240 ou Corynebacterium xerosis &amp; pH acid Bordetella bronchiseptica &amp; pH acid</td>
<td>&gt; 1000 (no association)</td>
<td>2 mg/kg (Bac) &amp; 50 (col)</td>
</tr>
<tr>
<td>Diaminopyrimidines (1)</td>
<td>B. stearothermophilus Merck &amp; pH neutral</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Sulfonamides (1)</td>
<td>All strains tested &amp; pH acid to neutral</td>
<td>&gt; 1000</td>
<td>75</td>
</tr>
<tr>
<td>Quinoxalines (2)</td>
<td>All strains tested &amp; pH acid to neutral</td>
<td>&gt; 1000</td>
<td>ND</td>
</tr>
<tr>
<td>Phenicols (2)</td>
<td>Yersinia ruckeri &amp; pH neutral</td>
<td>500</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>Coccidiostats (Ionophores 5)</td>
<td>B. stearothermophilus Merck &amp; pH acid &amp; basic</td>
<td>acid: 100 – 500 basic: 100</td>
<td>&gt; 20 to 100</td>
</tr>
<tr>
<td>Coccidiostats (Synthetic Ionophore 1)</td>
<td>E. coli ATCC 27166 &amp; pH neutral</td>
<td>500</td>
<td>&gt; 60</td>
</tr>
</tbody>
</table>

* Limit of detection (mg/kg) with the SIMBAG FEED method
Conclusion

• Microbiological screening can produce:
  – Good level of detection
    • 1 to 10 µg/L: 7 compounds
    • 10 to 100 µg/L: 12 compounds
    • 100 to 500 µg/L: 11 compounds
    • 1000 µg/L & more: 4 compounds
  – Different bacteria
  – Pre identification of family
  – Exception with the phenicol family (ELISA)

• Next step:
  – Application in feed with the extraction protocol.
  – Validation in accordance with the to the European Decision 2002/657/EC
Confirmatory method

• Main requirements
  – Unambiguous identification
  – Quantification above the Maximal Residue Limits (MRL)
  – Specificity with 4 identification points according to the European Directive 2002/657/EC

• Techniques available
  – HPLC – Electrospray ionisation (ESI) tandem Mass Spectrometry (MS-MS)
  – HPLC – Atmospheric Pressure Chemical Ionisation (APCI) tandem Mass Spectrometry (MS-MS)
  – ...
HPLC-ESI-MS-MS Principle

HPLC - Separation system → Electrospray source ESI → Tandem Mass Spectrometer

Signal analysis
HPLC-MS/MS optimization

• 1) Determination of the ESI-MS/MS parameters:
  – Optimization of conditions, by direct injection of each compound individually, for the determination of molecular peak and the different fragments in positive and negative modes.
  – Optimization of the source parameters for the nebulization (potential, temperature, and nebulizer pressure)

• 2) Determination of the chromatographic conditions
  – Chromatographic column
  – Eluent & gradient
MS analysis: positive mode

26 antibiotics

- Conditions: standards 100 ng/mL, column: Zorbax Eclipse plus, elution gradient: eluent A = H₂O + 0.1% FA, eluent B = CH₃CN 70%/MeOH 30% + 0.1% FA, LOQ: 1–20 ng/mL, ion spray voltage = 5500 V, source temperature = 550°C
MS analysis: negative mode

- Conditions: standards 100 ng/mL column: Zorbax Eclipse plus, elution gradient: eluent A = H2O+5mM AcONH4, eluent B= CH3CN 70%/MeOH 30%, ion spray voltage= -4500 V, source temperature= 550°C
Extraction and clean-up optimization

• 1) Optimization of the extraction procedures:
  – the solvent mixture for an efficient extraction
  – between ultrasonic extraction and pressurized liquid extraction (ASE)

• 2) Optimization of the clean-up procedures
  – SPE by using Oasis® HLB and C18-ec cartridges
  – LLE (hexane), TCA 10%
  – dispersive SPE (d-SPE) by using silica, florisil and PSA (primary secondary amine) as sorbent
Optimization of extraction

- Influence of the solvent extraction on the recoveries of the different drug families
  - **M1**: CH$_3$OH/ H$_2$O (75/25, v/v), **M2**: CH$_3$OH/ H$_2$O (75/25, v/v) with formic acid 1%, **M3**: CH$_3$OH/ McIlvaine buffer, pH = 4.6 (75/25, v/v), **M4**: CH$_3$OH/ McIlvaine buffer, pH = 4.6 (75/25, v/v) with 40 µL of EDTA-Na$_2$ (0.5 M), **M5**: CH$_3$CN/ McIlvaine buffer, pH = 4.6 (75/25, v/v) with 40 µL of EDTA-Na$_2$ (0.5 M), **M6**: CH$_3$OH/CH$_3$CN/McIlvaine buffer, pH = 4.6 (37.35/37.5/25, v/v/v) with 40 µL of EDTA-Na$_2$ (0.5 M).
Optimization of extraction

- Ultrasonic-assisted extraction (UAE) and pressurized liquid extraction (PLE) recoveries using different temperatures (50, 60, 70°C) for each antibiotic and coccidiostat families (number of analytes by family; n=3 for each individual analyte).
Optimization of clean-up conditions

- Influence of the amount of primary secondary amine (PSA) on the peaks area of coccidiostats from extracted blanks of piglet feed
Final protocol optimized

- Piglet feed: 4 ± 0.1 g
- Ultrasonic extraction (15 min)
- Centrifugation (6 mL of supernatant)
- d-SPE clean-up (250 mg of PSA)
- Centrifugation (5 mL + PSA)
- Evaporation of 3 mL and reconstitution in water, Vf = 1.5 mL
- Centrifugation and dilution by 4
- LC-MS/MS analysis

15 mL of solvent extraction:
- 37.5% MeOH : 37.5% CH₃CN : 25% buffer
- pH=4.6
- with 0.3% of EDTA
Validation of the confirmatory method

Spiking at 40 ng/g (n=6)

Veterinary drugs

Recovery yields (%)
Conclusion

• 32 analytes from 12 classes were quantifiable

• Recoveries
  – 52% (robenidine) < R < 109% (tilmicosine)
  – Mean for 32 analytes = 88%
  – Repeatability (within-day, n=6) & reproducibility (between-day, n=3) were ACCEPTABLE (RSD means < 16%)

• Specificity & sensitivity are GOOD
  – (4<LOQ<65 ng/g, LOQ mean = 13 ng/g)
Overall conclusion

• Combination of
  – Microbiological methods (screening)
  – And Physico-chemical method (confirmatory)

• Useful tools to control presence of veterinary drugs in feedingstuffs.
Thank for your attention