CONTROL OF RESIDUES: EXAMPLE OF ANTIMICROBIALS IN FOOD

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Antimicrobials (antibiotics and synthetic compounds) are used in ruminants, swine, poultry, and aquaculture to:

- therapy and prophylaxis,
- increase the productivity of the food producing animals.

The presence of antimicrobial residues

- constitutes a potential human health hazard,
- has significant impact on international food trade.
- has implications on technological process in dairy industry.
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Definition 1 residues of veterinary drugs

« All pharmacologically active substances, whether active principles, excipients or degradation products, and their metabolites which remain in foodstuffs obtained from animals to which the veterinary medicinal product in question has been administered »

Council Regulation EEC 2377/90
Definition 2: Maximum residue limit (MRL) (Tolerance levels)

«The maximum concentration of residue resulting from the use of a veterinary medicinal product expressed in part per billion (µg/kg) on a fresh weight basis which may be accepted by the Community to be legally permitted or recognized as acceptable in or on a food»

_Council Regulation EEC 2377/90_

MRL is specific for:

• Each antimicrobial compound.

Exception: the combined residues of all substances in the sulfonamide group should not exceed 100 µg/kg in tissues and milk.

• Each animal species in which residues may be present,

• Each of the tissues (muscle, liver, kidney, fat) or product (milk, eggs, honey) obtained from the treated animal.

If no MRL for egg or milk has been established, the substance is not to be used in animals from which eggs or milk are produced for human consumption.
**Classification**

_Council Regulation 2377/90_

- Establishes MRLs
- Classifies pharmacologically active substances in 4 annexes:
  - Annex I: List of substances with definitive MRLs.
    Antimicrobials: category B1 substances
  - Annex II: List of substances not subject to MRLs.
  - Annex III: List of substances for which temporary MRLs are fixed for a defined period of time (< 5 years) because scientific data are incomplete.
  - Annex IV: List of substances for which no MRLs can be established (risk to human health).
  - Banned antimicrobials in food-producing animals: chloramphenicol, nitrofurans, nitroimidazols, dapson
  - Dyes: malachite green.
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   2.4. Interpretation of results

Conclusion
1. Methods of detection of antimicrobial residues

> **Analysis of antimicrobial residues in foodstuffs is highly complex:**

- 12 families
- More than 60 substances,
- Analysis of parent compound and/or metabolite (e.g. 3-amino-oxazolidinone for furazolidone),
- Marked difference in the physicochemical properties between the different classes of antibiotic families, and between individual compounds belonging to the same family,
- Wide variety of matrices: muscle, liver, kidney, fat, milk, eggs and honey
  (animal matrix may affect the detection capability)
- Requires sample preparation: deproteinisation, extraction, cartridge cleanup.
- 6 species
1. Methods of detection of antimicrobial residues

> Many regulatory authorities worldwide: EU, JECFA, EMEA, CAC ...

> Different concepts:
  - No consensus protocol for analysis of antimicrobial residues.
  - Lack of a unified and transparent approach: e.g. MRL Neomycin in milk EU: 1500 µg/kg; Mercosur: 500 µg/kg; USA: 150 µg/kg

> Validation of analytical methods of antimicrobial residues is very elaborate, time-consuming.

> Expensive materials: liquid chromatography (LC) tandem mass spectrometry (LC/MS-MS)

Residue analysis involves:
  - Screening methods
  - Confirmatory methods.
1. 1. Screening Methods

> Should be available for a wide range of antimicrobial
> Are used to detect the presence of an analyte or class of analyte at the level of interest (MRL).
> Have a false positive (non-compliant) result of less than 5 % at the level of interest (Directive 96/23/EC).
> Specifically designed to avoid false negative (compliant) results.
> Screening methods are:
  - Microbial inhibition assay
  - Enzymatic assay
  - Receptor assay
  - Immuno assay
  - Radio immuno assay
  - Biosensor assay
  - Thin layer chromatography
1.1.1 : Microbial inhibition assay

Two microbial inhibition assays can be used: agar diffusion plate and tube test

1) AGAR DIFFUSION PLATE

- Various sensitive strains of microorganisms are inoculated into 4, 5, 6 or 7 agar plates with specific mediums and different pH values that maximise the response from the antibiotics.
- Discs of filter paper moistened with milk or meat juice or thin slices of muscle are placed on the surface of the medium.
- After incubation, any zone of inhibition on the agar greater than 2 mm in diameter is a positive result.
## 1.1.1: Microbial inhibition assay

### Microbial screening methods

<table>
<thead>
<tr>
<th>AGAR DIFFUSION PLATE</th>
<th>GERM -TEST</th>
<th>MATRIX</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 - plate test</td>
<td>3 plates with <em>B. subtilis</em> at pH 6; 7.2 + trimethoprim; pH 8; 1 plate with <em>Kocuria rhizophila</em> (<em>Micrococcus luteus</em>)</td>
<td>Muscle</td>
</tr>
<tr>
<td>5 - plate test STAR</td>
<td><em>B. subtilis BGA</em>; <em>B. stearothermophilus ATCC 7953</em>; <em>B. cereus var. mycoides</em>; <em>K. rhizophila ATCC 15957</em>; <em>E. coli</em></td>
<td>Milk</td>
</tr>
<tr>
<td>6 - plate test</td>
<td><em>B. subtilis</em> at pH 6 and 7.2; <em>K. rhizophila</em> at pH 6 and 8; <em>B. cereus</em>; <em>E. coli</em></td>
<td>Muscle, Kidney</td>
</tr>
</tbody>
</table>
1.1. Screening methods

1.1.1: Microbial inhibition assay

2) TUBE Test

A tube test consists of an agar medium inoculated with spores of B. stearothermophilus var. calidolactis and incorporated with a pH-indicator (bromocresol purple) or redox-indicator (brilliant black).

After incubation at the appropriate temperature, the germ-test grows, produces acid resulting in a change in pH or in redox and also a change in colour from purple or blue to yellow.

The presence of antimicrobial residues will prevent bacterial growth, and this is indicated by the absence of color change.
### 1.1. Screening methods

#### 1.1.1: Microbial inhibition assay

<table>
<thead>
<tr>
<th>Features</th>
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<tbody>
<tr>
<td>- Easy to perform</td>
<td></td>
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<tr>
<td>- High capacity</td>
<td></td>
</tr>
<tr>
<td>- Inexpensive</td>
<td></td>
</tr>
<tr>
<td>- Multi-residue screening tests</td>
<td></td>
</tr>
<tr>
<td>- Lack of specificity: false positive results</td>
<td></td>
</tr>
<tr>
<td>- Insufficient sensitivity to certain substances (&gt; MRL): colistine, sulfamides, quinolones, nitrofurans.</td>
<td></td>
</tr>
<tr>
<td>- Incubation time: 3 - 18 hours</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Examples</th>
<th></th>
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<tbody>
<tr>
<td><strong>MILK</strong> : - Delvotest® P - Copan® Milk Test - Delvotest® SP</td>
<td>- Valio T 101® Test (S. thermophilus) - Eclipse Farm® Test - BR Test® AS Brilliant</td>
</tr>
<tr>
<td><strong>MUSCLE</strong> : Premi® Test, Explorer®</td>
<td></td>
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<tr>
<td><strong>KIDNEY</strong> : Kidney Inhibition Swab (KIS™) Test</td>
<td></td>
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</tbody>
</table>
1.1. Screening methods

1.1.2: Enzymatic assay

> This test is based on the principle that β-lactams prevent bacterial multiplication by inhibiting the activity of the enzyme DD-carboxypeptidase.

> During the test, DD-carboxypeptidase activity liberates D-alanine from an enzyme substrate (R-D-Ala-D-Ala) which is visualised by a colour change.

> In the presence of a β lactams containing sample, the activity of the enzyme is inhibited, no D-alanine can be liberated and no colour change occurs.

Milk: Penzym® test; Penzym S® test
1.1. Screening methods

1.1.3: Receptor assay

> Milk or muscle is incubated with a receptor. \( \beta \) lactams eventually present in the milk, will bind with the receptor.

> After migration over filtration paper in a dipstick, the remaining quantity of free receptor is usually controlled by comparing the colour intensity of the sample spot and the control spot.

> The colour intensity at the captation zone is inversely related to the concentration of \( \beta \) lactam antibiotics in the sample.
1.1. Screening methods

1.1.3: Receptor assay

- Detection a class of antimicrobials
- Rapid (5 -15 mn)
- Easy to perform
- Clear visual interpretation
- High sensitivity: β-lactams: 3-5 ppb; Tetracyclines: 20-30 ppb

- **MILK:** Beta Start®, Snap Beta®
  Snap Tetra®, Charm MRL®
  Twinsensor BT® (detection of β-lactam & tetracyclines)
- **Muscle:** Tetrasensor®
1.1. Screening methods

1.1.4: Immuno assay

**Competitive ELISA (Enzyme Linked Immuno Sorbent Assay)**

- A target antibiotic group is captured by immobilised specific antibodies. Antibiotics in the sample compete with an internal antibiotic standard for the immune receptor.
- The antibody-antibiotic complex is linked to an enzyme that catalyses a colour reaction.
1.1. Screening methods

1.1.4: Immuno assay

- a **high intensity** is considered as 'negative',
- a **low intensity** is considered as 'positive'.
1.1. Screening methods

1.1.4: Immuno assay

**Features**
- Narrow spectrum detection
- Specific results
- High sensitivity
- High capacity
- Long time of analysis: several incubation and wash steps
- Instrumental interpretation
- Difficult to automate

**Examples**
Milk, Meat, Eggs, Shrimp: Ridascreen® Chloramphenicol
Fluorophos Betascreen®
1.1. Screening methods

1.1.5: Radio Immuno assay

> The sample is incubated with a binding agent (bacteria with specific antibodies attached) and a tracer (the radio-labelled version of the antibiotic to be detect). The amount of tracer on the binding agent is measured using a scintillation counter.

> If contaminating antibiotic is present, it will prevent the binding of the tracer by occupying the receptors on the binding agent.

Samples with low count are considered positive.

Samples with high count are considered negative.

This method involves radioactive substances and requires the use of expensive equipment.

Milk and Meat: Charm II Kit tests: β lactams, aminoglycosides, amphenicols, tetracyclines, sulfonamides.
### 1.1. Screening methods

#### 1.1.6: Biosensor assay

- A biosensor consists of a biological sensing element (ligand) that interacts with the analyte and is in close contact with a physical transducer that senses the physico-chemical change that follows the interaction.
- Many different types of biosensors, classified according to the transducing system applied are used. The biosensor assay is performed on a surface plasmon resonance (SPR).

<table>
<thead>
<tr>
<th>Features</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>- No or low sample preparation requirements</td>
<td>- <strong>Milk, Muscle</strong>: Qflex® Kits - Sulfonamides, - Streptomycin, Chloramphenicol</td>
</tr>
<tr>
<td>- Fast response time</td>
<td>- <strong>Honey</strong>: Qflex® Kits - Streptomycin - Chloramphenicol</td>
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<tr>
<td>- High sensitivity</td>
<td></td>
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<td>- Low rate of false positives</td>
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<tr>
<td>- Multi-flow channel systems</td>
<td></td>
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<td>- Fully automated samples handling and analysis system</td>
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<tr>
<td>- Limited availability of test kits</td>
<td></td>
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<tr>
<td>- Expensive</td>
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</tbody>
</table>
1.1. Screening methods

1.1.7: Thin Layer Chromatography

- Inexpensive
- Quick
- Sufficient precision
- Relatively unspecific

Muscle: Sulfonamides
1.1. Methods of analysis of antimicrobial residues

1.2. Confirmatory methods

Definition

«Methods that provide full or complementary information enabling the analyte to be identified unequivocally and if necessary quantified at the level of interest» Directive 96/23/EC

In the case of a suspect-non compliant result, screening result shall be confirmed by a confirmatory method.

Chromatographic methods essentially **LC** (various stationary phases and mobile phases) equipped with **various detectors** (UV, fluorescence, DAD, mass spectrometry).

> The false positive rate is less than 5 % for MRLs- authorized substances.
> The false positive rate is less than 1 % for banned substances.
## 1.2. Confirmatory methods

The following methods or method combinations are considered suitable for the identification of antimicrobial residues.

<table>
<thead>
<tr>
<th>Identification methods</th>
<th>Criterion</th>
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<td>2 D TLC - full scan UV/vis</td>
<td>- 2-dimensional HPTLC (High Performance Thin Layer Chromatography) and co-chromatography are mandatory</td>
</tr>
<tr>
<td>LC-UV/Vis (single wavelength) (e.g. tetracyclines, β lactams, sulfonamides)</td>
<td>- Only if at least 2 different chromatographic systems or second, independent detection method are used.</td>
</tr>
<tr>
<td>LC- full scan DAD (e.g. tetracyclines, β-lactams, sulfonamides)</td>
<td>- Specific requirements for absorption in UV spectrometry have to be met.</td>
</tr>
<tr>
<td>LC-immunogram</td>
<td>- Only if at least 2 different chromatographic systems or a second, independent detection method are used.</td>
</tr>
<tr>
<td>LC - fluorescence (e.g. fluoroquinolones, tetracyclines)</td>
<td>- Only for molecules that exhibit native fluorescence and to molecules that exhibit fluorescence after either transformation or derivatisation.</td>
</tr>
<tr>
<td>GC-elektron capture detection</td>
<td>- Only if 2 capillary columns of different polarity is used.</td>
</tr>
<tr>
<td>LC or GC with mass- spectrometric detection</td>
<td>- Only if following either an on-line or an off-line chromatographic separation</td>
</tr>
<tr>
<td></td>
<td>- Only if full scan techniques are used using at least 3 identification points (IP) for allowed substances and 4 IP for banned substances.</td>
</tr>
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  - 2.4. Interpretation of results
- Conclusion
Objective

To demonstrate through systematic evaluation that an analytical procedure is suitable for its intended use.

⇒ Validation is needed for each matrix and each species separately
⇒ Analytical methods should be validated prior to their use for screening methods and for confirmatory methods. (*Commission Decision 882/2004 /EC*)

> **QUALITATIVE METHOD** « means an analytical method which identifies a substance on the basis of its chemical, biological or physical properties ».

> **QUANTITATIVE METHOD** « means an analytical method which determines the amount or mass fraction of a substance so that it may be expressed as a numerical value of appropriate units ».
2. Validation of analytical methods for residue controls

2.1 Validation for qualitative methods.

> QUALITATIVE SCREENING METHODS are used for:

- **Banned antimicrobials**
  
  Minimum required performance levels (MRPLs)
  
  « Minimum content of an analyte in a sample, which at least has to be detect and confirmed» (2004/2726/CE)
  
  Chloramphenicol: < 0.3 ppb; Nitrofuran metabolites: < 1.0 ppb

- **Illicit use of antibacterial compounds**

  → Screening method: Detection capability (CC$\beta$:µg/kg)
  
  « The lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1-\(\beta\) (\(\beta = 5\%)\).»

  The value for CC$\beta$ must be less than or equal to the MRPL.
2. Validation of analytical methods for residue controls

2.1 Validation for qualitative methods.

> QUALITATIVE CONFIRMATORY METHODS

→ Confirmatory method: Decision limit (CCα: µg/kg)

« The limit at and above which it can be concluded with an error probability of α that a sample is non-compliant »

Consequently, the value of CCα must always be less than the MRPL.

| Performance characteristics determined in validation of qualitative methods (2002/657/EC) | S: screening; C: confirmatory |
| CCβ | CCα | Trueness/Recovery | Precision | Selectivity/Specificity | Applicability/Ruggedness/Stability of analyte |
| Qualitative methods | S | + | - | - | - | + | + |
| C | + | + | - | - | + | + |
2. Validation of analytical methods for residue controls

2.2. Validation for quantitative methods.

> QUANTITATIVE SCREENING METHODS

For banned antimicrobials and substances with fixed MRLs

\[ CC_\beta = CC_\alpha + 1.64 \times \text{the standard deviation at } CC_\alpha \]

> QUANTITATIVE CONFIRMATORY METHODS

- Banned antimicrobials

\[ CC_\alpha = \text{Concentration at the } y\text{-intercept} + 2.33 \times \text{the standard deviation of the intercept} \]

- Substances with fixed MRLs

\[ CC_\alpha = \text{MRL value} + 1.64 \times \text{the standard deviation at the MRL} \]

### Performance characteristics determined in validation for quantitative methods

<table>
<thead>
<tr>
<th></th>
<th>CCβ</th>
<th>CCα</th>
<th>Trueness/Recovery</th>
<th>Precision</th>
<th>Selectivity/Specificity</th>
<th>Applicability/Ruggedness/Stability of analyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantitative methods</td>
<td>S</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

S: screening; C: confirmatory
2. Validation of analytical methods for residue controls

2.3. Validation for quantification methods.

The validation of quantification method is based on the accuracy expressed by «the closeness of agreement between a test result and the accepted reference value. It is determined by determining trueness and precision ».

> **Trueness** (bias) means the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value.

> **Precision** means the closeness of agreement between independent test results obtained under stipulated conditions. The measure of precision usually is expressed in terms of imprecision and computed as standard deviation of the results.

Recommended accuracy for quantification methods will vary depending upon the concentration of the analyte.

<table>
<thead>
<tr>
<th>ANALYTE CONCENTRATION</th>
<th>ACCEPTABLE RANGE</th>
</tr>
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<tbody>
<tr>
<td>&lt; 1 µg/kg</td>
<td>- 50 % to + 20 %</td>
</tr>
<tr>
<td>≥ 1 µg/kg &lt; 10 µg/kg</td>
<td>- 40 % to + 20 %</td>
</tr>
<tr>
<td>≥ 10 µg/kg &lt; 100 µg/kg</td>
<td>- 30 % to + 10 %</td>
</tr>
<tr>
<td>≥ 100 µg/kg</td>
<td>- 20 % to + 10 %</td>
</tr>
</tbody>
</table>
2 Validation of analytical methods for residue controls

2.4 Interpretation of Results

« The result of an analysis shall be considered non-compliant (positive) if the decision limit (CC $\alpha$) of the confirmatory method for the analyte is exceeded.

⇒ If no MRL has been established for a substance:
   CC $\alpha$ is the lowest concentration level at which a method can discriminate with a statistical certainty of 1- $\alpha$ that the particular analyte is present ($\alpha = 1\%$).

⇒ If a MRL has been established for a substance:
   CC $\alpha$ is the concentration above which it can be decided with a statistical certainty of 1- $\alpha$ that the permitted limit has been truly exceeded ($\alpha = 5\%$) »
With the increased awareness of the safety and quality it becomes imperative to maintain the safety standards of the food for human consumption.

The active involvement of livestock producers, veterinarians, toxicologists, pharmacologists, and microbiologists in food safety risk assessment and risk management is imperative.