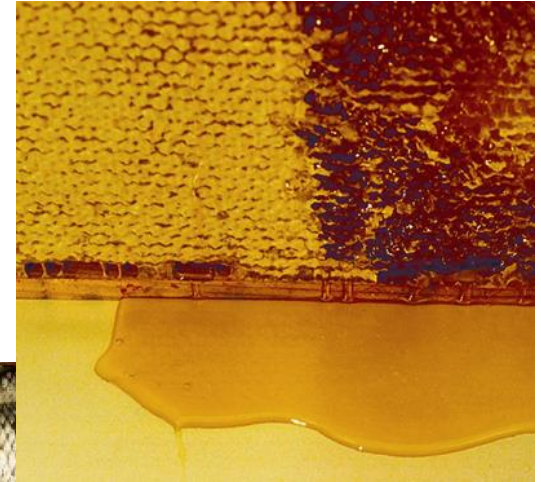
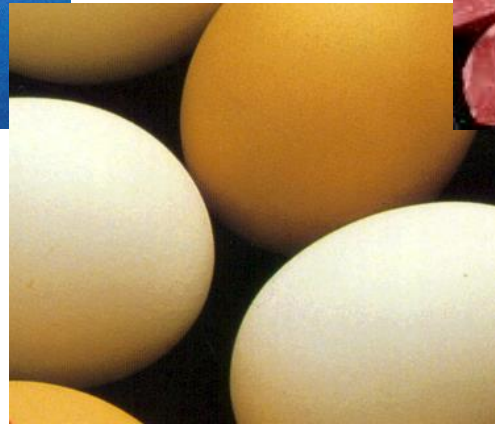
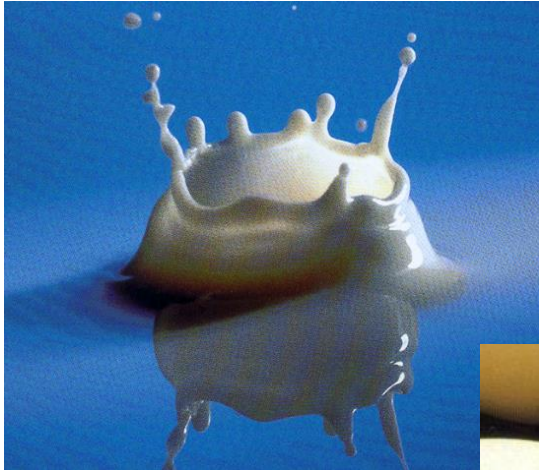


SCREENING FOR ANTIBIOTIC RESIDUES: FROM A HISTORICAL OVERVIEW TO FUTURE PERSPECTIVES



Dr. Wim Reybroeck

TRACES 2025, Gent, June 6

ILVO

Flanders research institute for
agriculture, fisheries and food

ANTIMICROBIALS IN FOOD OF ANIMAL ORIGIN

% non-compliant results related to antimicrobials (B1)

Animal/product categorie	# of targeted samples	% non-compliant results
aquaculture	1558	0.13
bovines	17,232	0.25
eggs	3108	0.13
honey	1568	0.70
milk	7406	0.04
pigs	35,822	0.08
poultry	17,503	0.05
sheep/goats	3161	0.19



EFSA 2025: Report for 2023 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products

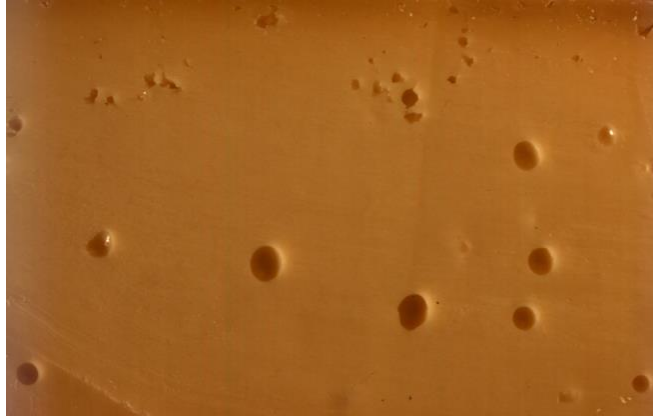
ANTIMICROBIALS IN FOOD OF ANIMAL ORIGIN

These low % of non-compliant results related to antimicrobials could only be obtained by continuous and frequent testing by

- food business operators using **screening tests**
- food authorities (low number of samples)

LONG HISTORY IN TESTING FOR RESIDUES OF ANTIMICROBIALS

- Testing to ensure technological food safety, to prevent problems with the production of fermented products



- 50 years ago, the dairy industry applied the yoghurt test (or coagulation test)
 - Microbiological screening test with *Streptococcus salivarius* spp. *thermophilus* as test organism

WHY TESTING RESIDUES OF ANTIMICROBIALS?

- Testing to ensure technological food safety
- To protect the consumer (ADI, MRL)
 - direct toxicity
 - allergic reactions (penicillins)
 - inhibition of bone growth (tetracyclines)
 - aplastic anaemia (chloramphenicol)

Council Regulation (EEC) No 2377/90 and amendments



Commission Regulation (EU) 37/2010 and amendments



HAZARD ANALYSIS AND CRITICAL CONTROL POINT (HACCP)

HACCP is a way of managing food safety hazards

→ identifying any critical control points

- presence of antimicrobial residues in primary products = CCP
- continuous monitoring

For this monitoring food business operators rely on **screening tests**, mainly on **rapid tests**

Dairies have to perform autocontrol programmes (Regulation (EC) No 853/2004 laying down specific hygiene rules for food of animal origin).

REGULATION (EC) No 853/2004

Food business operators in the dairy sector are not allowed to place on the market raw milk containing levels of antibiotic residues >MRL.

⇒ In practice: performance of **(rapid) screening tests** on (incoming) **tanker milk**
Screening for **β-lactam** residues (penicillins and cephalosporins)

US: the testing of all incoming shipments of milk for β-lactam antibiotics is mandatory since January 1, 1992.

Spain: Real Decreto 1728/2007: besides β-lactam also tetracyclines (one test per 5 tanker loads)

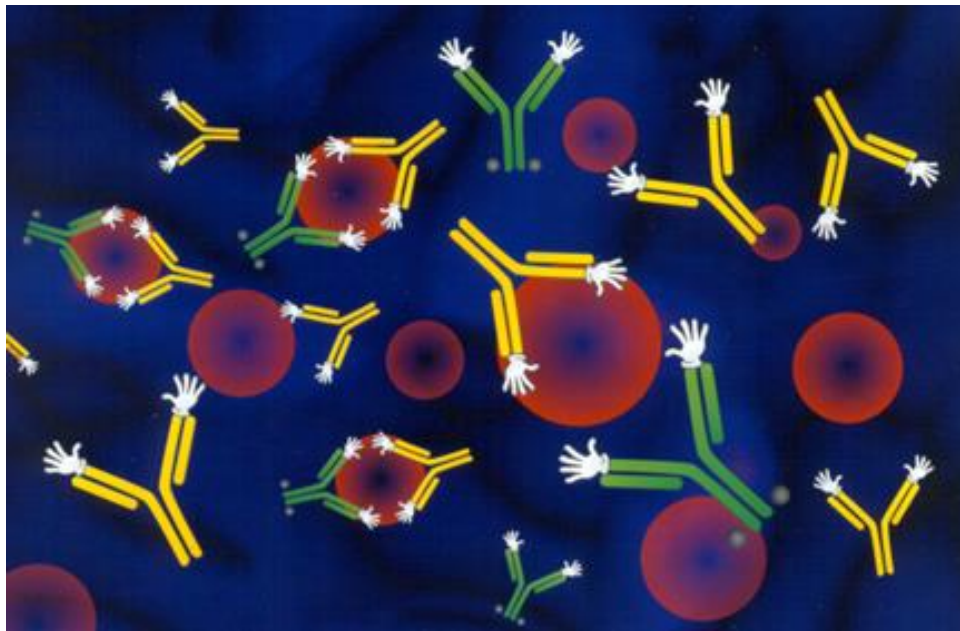
Ex-farm milk: a representative number of raw milk samples, collected from milk production holdings taken by random sampling, must be checked on the presence of antibiotic residues for that the raw milk placed on the market is not containing residues in a quantity >MRL

⇒ In practice: in many countries each delivery is tested. BE: >800,000 tests in 2024

ANALYTICAL METHODS (COMMISSION IMPLEMENTING REGULATION (EU) 2021/808)

Commission Implementing Regulation (EU) 2021/808 of 22 March 2021 on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling.

Screening methods



Confirmatory methods



SCREENING METHOD: DEFINITION

(Commission Implementing Regulation (EU) 2021/808)

‘Screening method’: method used for screening of a substance or class of substances at the level of interest;

‘Level of interest’: concentration of a substance or analyte in a sample that is significant to determine its compliance with the legislation as regards:

(a)- maximum residue level (MRL) for authorized substances (VMPs)
(Regulation 37/2010 and amendments)

- maximum level (ML) for coccidiostats & histomonostats due to the unavoidable carry-over of these substances in non-target feed (Reg. 124/2009 and amendments (Regulation 610/2012 & Regulation 2020/499));

(b) reference points for action for prohibited or unauthorised substances
(Regulation 2019/1871 and amendments (Reg. 2023/411 & 2024/2858));

(c) a concentration as low as analytically achievable for prohibited or unauthorized substances, for which no reference point for action is established.

REQUIREMENTS FOR SCREENING TESTS

The rapid test used should be able to detect the marker residues at least in 95% of the cases:

- at or below the MRL (authorized substances)
e.g. $\leq 4 \mu\text{g}/\text{kg}$ benzylpenicillin in milk; $\leq 50 \mu\text{g}/\text{kg}$ benzylpenicillin in meat
or
at or below the ML (for coccidiostats & histomonostats)
e.g. $\leq 2 \mu\text{g}/\text{kg}$ diclazuril in eggs; $\leq 40 \mu\text{g}/\text{kg}$ diclazuril in liver and kidney
- at or below the reference point for action (non-authorized substances)
e.g. $\leq 0.15 \mu\text{g}/\text{kg}$ chloramphenicol
or
- at or below the minimum method performance requirement (MMPR) (EURL Guidance on MMPRs, June 2022))
e.g. $\leq 5 \mu\text{g}/\text{kg}$ dapson in milk and meat

Maximum percentage of false negative results: 5%

Maximum percentage of false positive results: not fixed, but in general this percentage should be as low as possible

USE OF SCREENING METHODS

Screening methods:

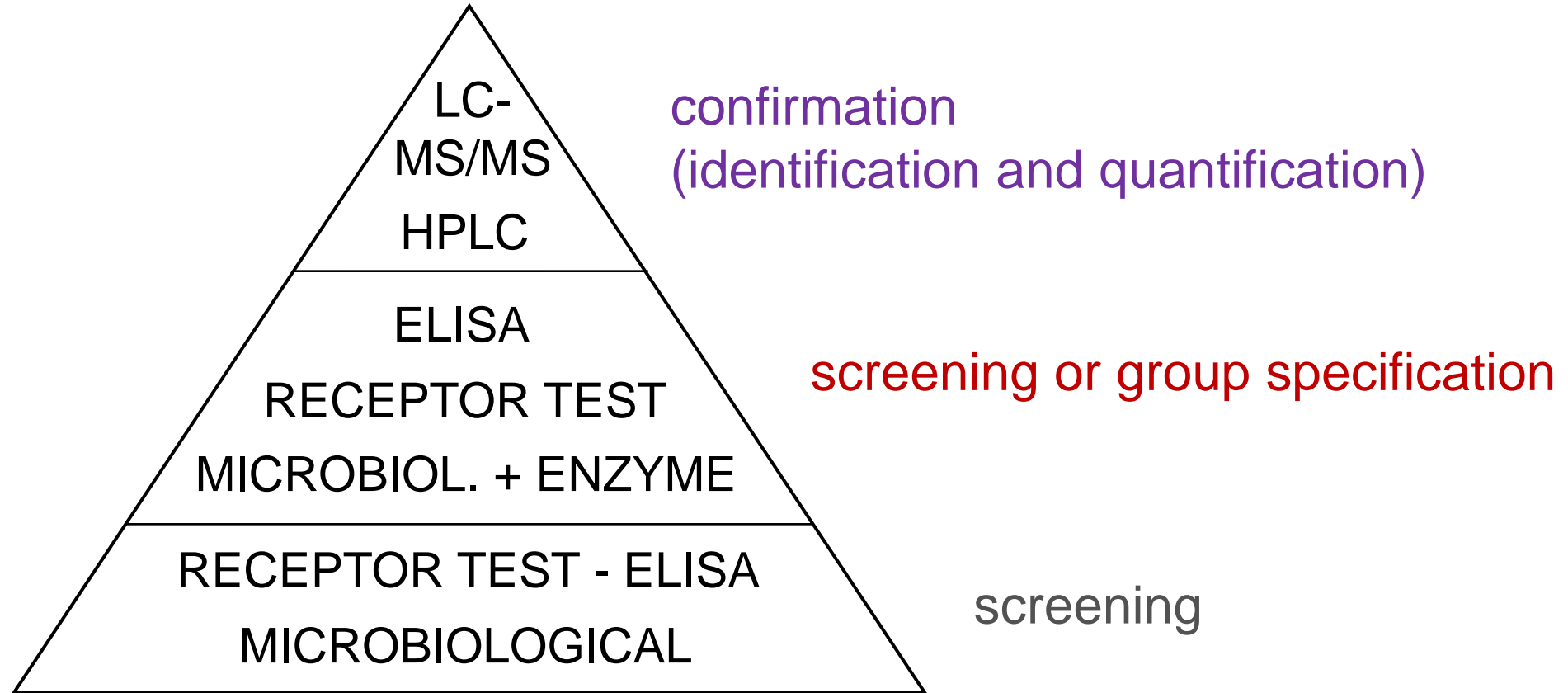
- capability for a high sample throughput
 - used to sift large numbers of samples for potential non-compliant results
 - specifically designed to avoid false compliant results
-
- **validated**
 - **$CC\beta \leq$ regulatory limit (MRL/ML/RPA)**
 - **false compliant rate of $\leq 5\%$**

Official tests: suspected non-compliant result → confirmed by a confirmatory method

(Commission Implementing Regulation (EU) 2021/808)

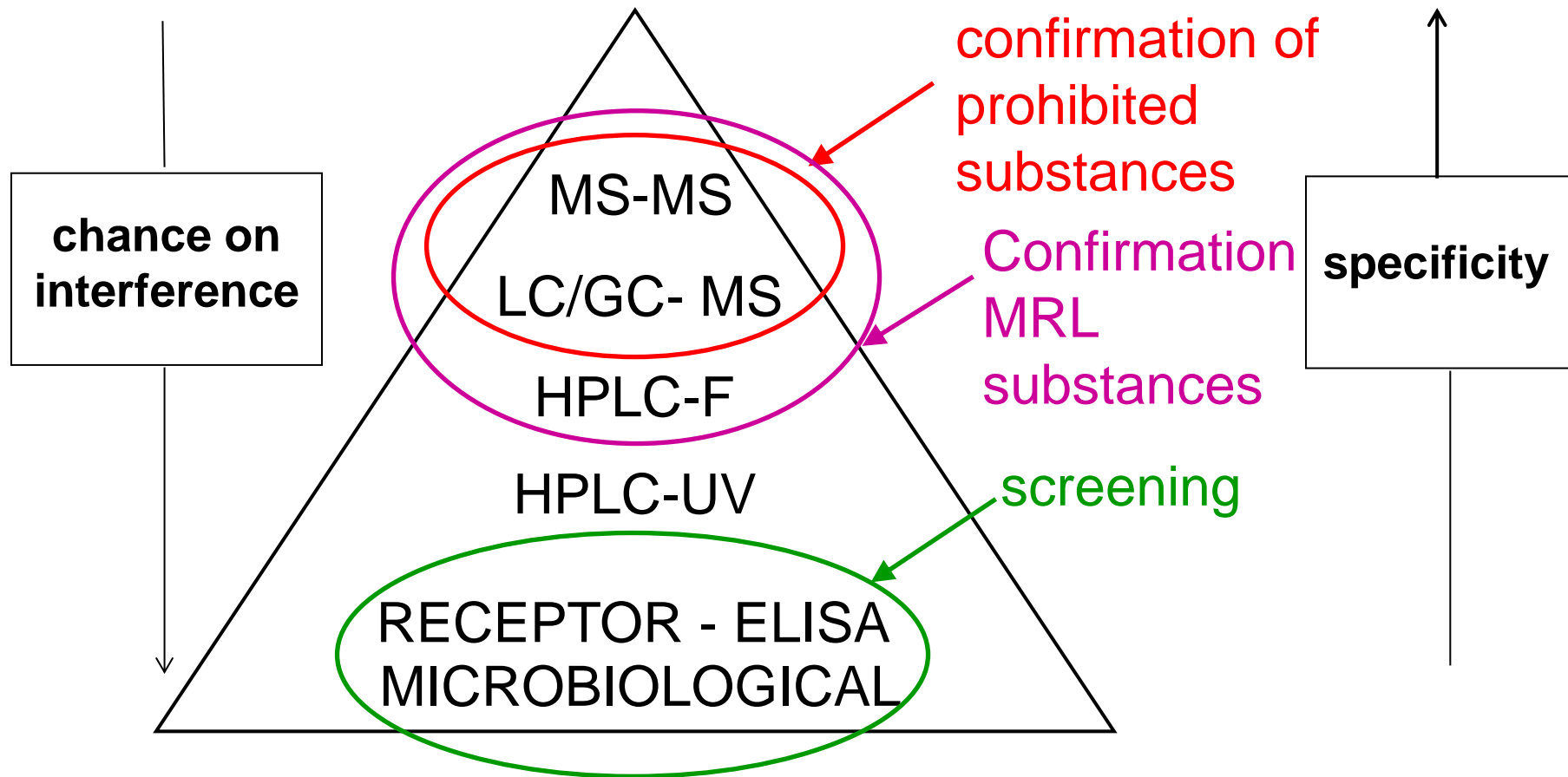


GENERAL APPROACH FOR MONITORING FOR RESIDUES OF ANTI-INFECTIOUS AGENTS



MOST COST-EFFECTIVE APPROACH

GENERAL APPROACH FOR MONITORING FOR RESIDUES OF ANTI-INFECTIOUS AGENTS



REQUIREMENTS OF A SCREENING TEST

Sensitive

Reliable (**false compliance rate of ≤ 5 %**)

High sample throughput

Easy, could be performed outside a laboratory

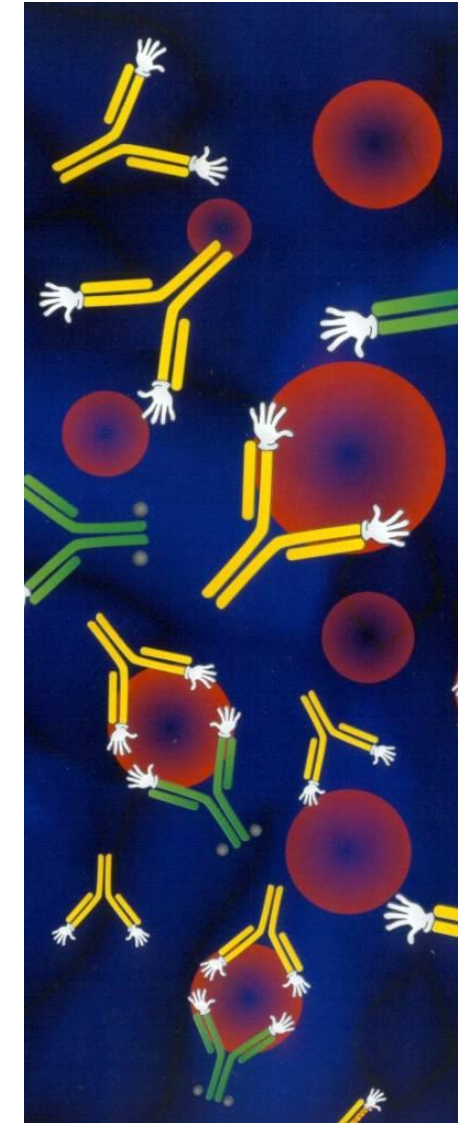
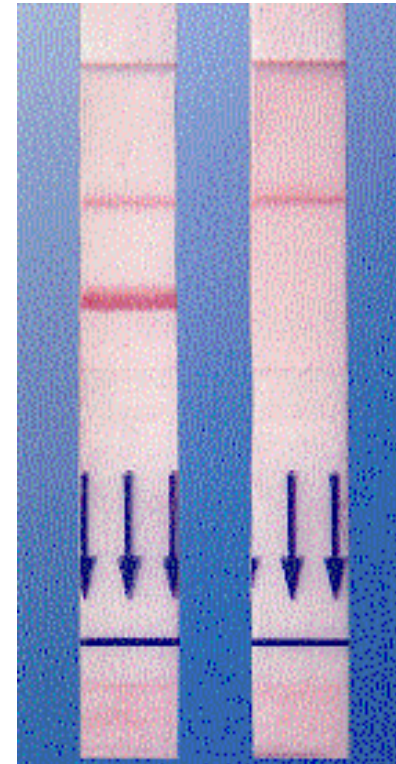
Cheap

(Fast)



SCREENING TESTS

- MICROBIOLOGICAL INHIBITOR TESTS
- ENZYMATIC TESTS
- RECEPTOR ASSAYS
- IMMUNOLOGICAL TESTS
- CHIP-BASED METHODS



MICROBIOLOGICAL INHIBITOR TESTS

- disc assays

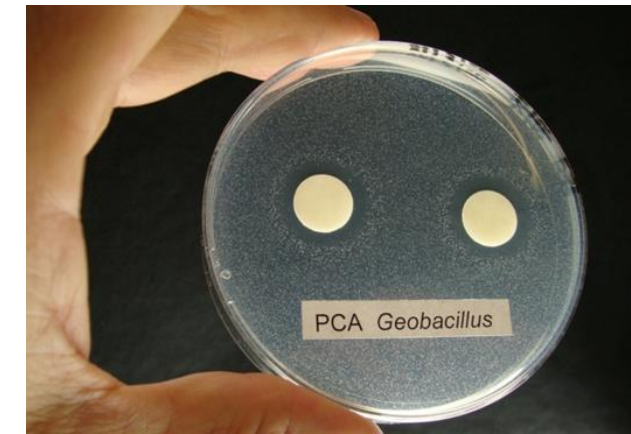
- . filter disc + milk/extract on agar medium in Petri dish
- . criterion = inhibition of growth of test organism
- . diameter inhibition zone ~ log concentration



Milk: 'calidolactis test' based on *Geobacillus*
stearothermophilus var. *calidolactis* C953

Test organism selected because its sensitivity for benzylpenicillin, in the '70s and '80s the only compound with a MRL in milk.

'calidolactis' test was longtime the official test in milk payment programmes in many countries



MICROBIOLOGICAL INHIBITOR TESTS – disc assays

Meat: use of different test media (pH) and different test organisms

Test organisms

Geobacillus stearothermophilus var. *calidolactis*

Bacillus cereus (tetracyclines)

Escherichia coli (quinolones)

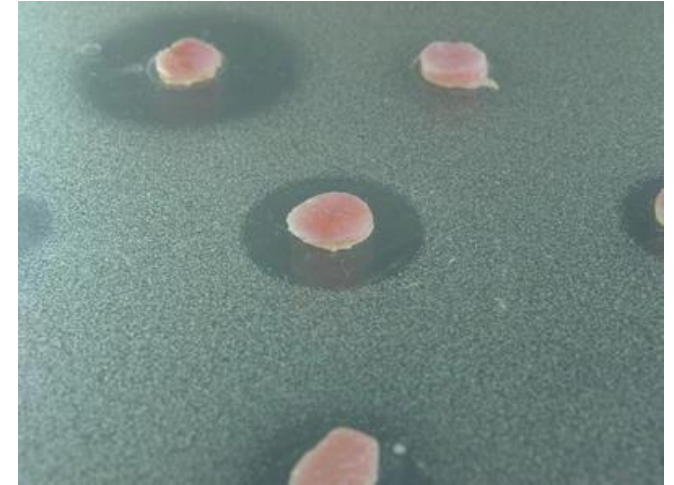
Bacillus subtilis

Kocuria rhizophila

...

Example

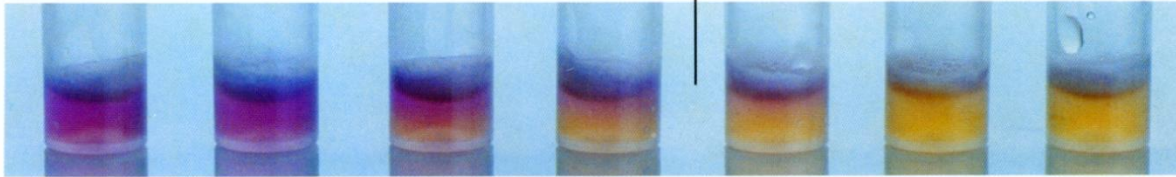
STAR (Screening Test for Antibiotic Residues) protocol - Five Plate Test (Gaudin *et al.*, 2010)



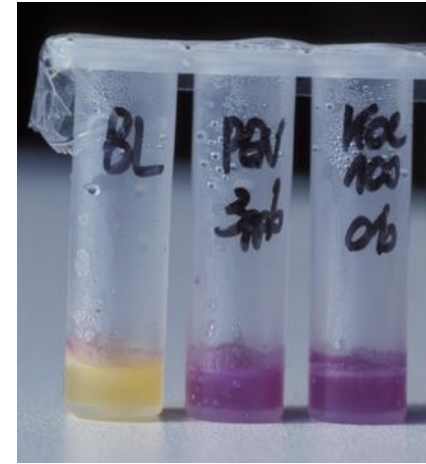
MICROBIOLOGICAL INHIBITOR TESTS

- agar diffusion tests

- . milk/extract on top of agar in ampoules or wells of microtiter plates
- . criterion = acid production (pH-measurement) (purple to yellow)
or reduction of redox indicator brilliant black (blue to yellow)



- . visual interpretation: purple - yellow or blue - yellow
- . originally also based on *Geobacillus stearothermophilus* var. *calidolactis*
- . now colour measurement with flatbed scanner or spectrophotometer



MICROBIOLOGICAL INHIBITOR TESTS – agar diffusion tests

Milk (broad spectrum)

- Delvotest (DSM-Food Specialties, NL)

Delvotest P	Delvotest SP	Delvotest SP-NT	Delvotest T	DAS (Smart)
1980 Van Os/Beuckers	+ trimethoprim → sulfonamides	no longer nutrient tablet; improved detection + Delvoscan	detection of tetra's↑	Automated incubation and reading

- Alternatives:

Delvotest Accelerator Smart

- . Eclipse (Zeulab, ES)

Latest development: Eclipse FARM 4G & COMET 4 (see poster)

Automated incubation and reading. Results automatically sent to Smartphone or Cloud account

- . Brilliant Reduction Test, BRT MRL-Such test & BRT hi-sense (Analytik in Milch, DE)

- . Charm Blue Yellow II (Charm Sciences, US)

MICROBIOLOGICAL INHIBITOR TESTS – agar diffusion tests

Meat (broad-spectrum)

- Premitest (R-Biopharm AG, DE)

Enhanced detection capability by a solvent extraction (Stead, 2005)

Kit version for detection in eggs and in urine

- Alternatives:

- . Explorer 2.0 (Zeulab, ES)

e-Reader or photometric reading

INHIBITORY SUBSTANCES OR INHIBITORS

substances which prevent growth of micro-organisms temporarily or permanently, partially or totally.

- Occur:
- naturally (natural inhibitors)
 - as a result of drug administration (targeted)
 - by contamination (unintentionally)

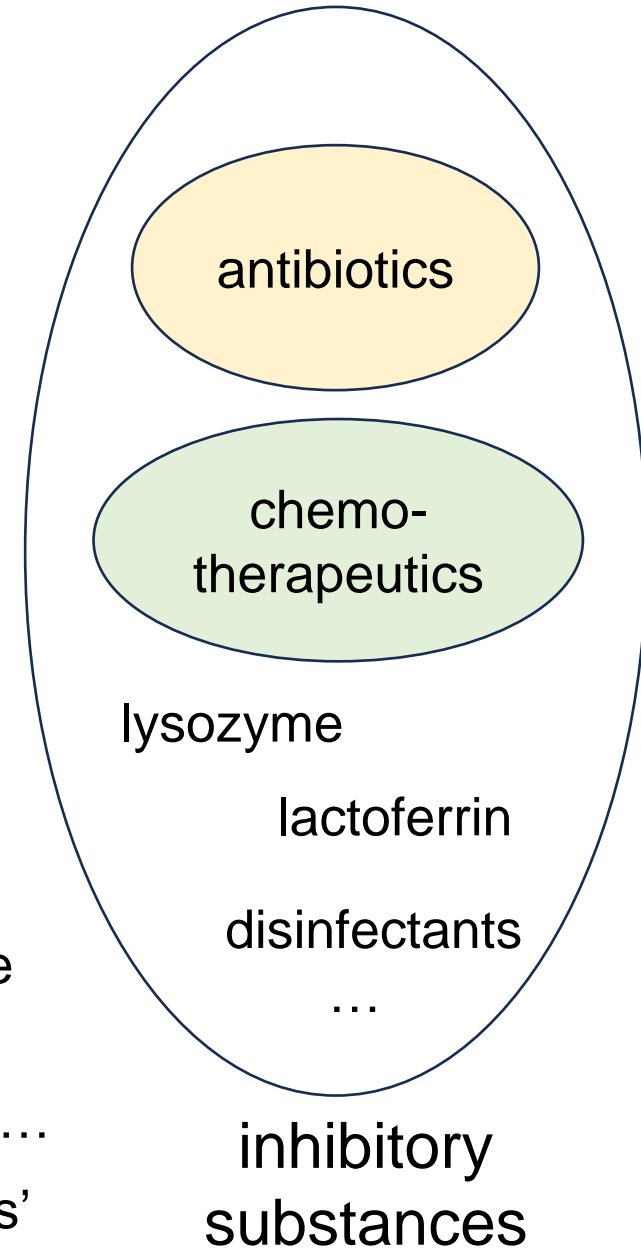
Natural inhibitors: E.g. in mastitic or colostrum milk

- lactoferrin
- lysozyme
- lactoperoxidase
- somatic cells
- cyclic lipodepsipeptides (< *Pseudomonas*)
- free fatty acids (e.g. capric acid)

Could interfere in microbiological inhibition assay methods causing false positive results.

Also interference possible by residues of disinfectants, pH, salt content,...

In near future publication IDF/FIL document 'Frequently asked questions'



MICROBIOLOGICAL INHIBITOR TESTS

In general broad-spectrum, but also group-specific tests possible

- Some test-organisms are relatively sensitive for a specific antibiotic family

E.g. *Bacillus cereus* (tetracyclines)

Escherichia coli (quinolones)

- Use of chemicals to indicate the antibiotic group or family

E.g. Penase (SR0129, Difco): neutralises natural penicillins (benzylpenicillin) & aminopenicillins
(ampicillin and amoxicillin)

Beta-lactamase ES (BELA-70.1461, Sekisui) neutralises all penicillins & cephalosporines

PABA: sulfonamides

For example: sample positive; sample +penase negative → natural or aminopenicillin

NEED FOR SPEED

Food business operators want to take a fast decision

Microbiological tests take a long time:

- mesophilic germs require an overnight incubation (16-24h)
- other strains: 2 to 3.5 hour

'Rapid' tests developed

- Lumac Rapid Antibiotic Test: 90 minutes (Lumac, NL)
- Penzym: 20 minutes (UCB Bioproducts, BE)
- 1991: Charm II tests: 45-90 minutes

- 1992: LacTek B-lactam test: 7 minutes (Idexx Laboratories, US)
- Parallax: 5 minutes (Idexx, US); β beta-s.t.a.r.: 5 minutes (UCB, BE & Neogen, US)
- Charm MRL ROSA: 8 minutes (Charm Sciences, US)
- SNAP: 9 minutes (Idexx Laboratories, US); ...

RAPID SCREENING TESTS

Formats

- Strips (LFA)
- microplate
- cartridges
- biochip

Spectrum

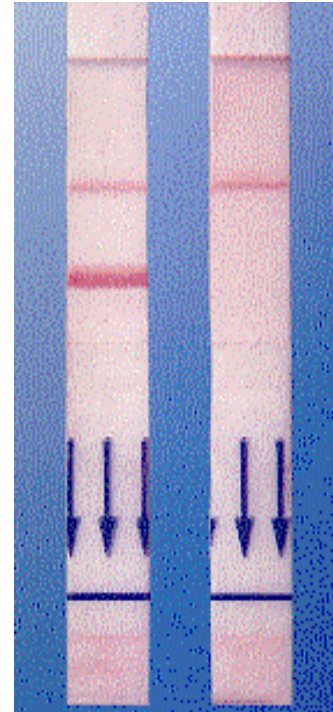
- single compound
- single family
- several families

Test time

1 - 10 minutes (dipsticks) to 45 minutes

Equipment

- incubators
- readers
- software



ELISA TESTS

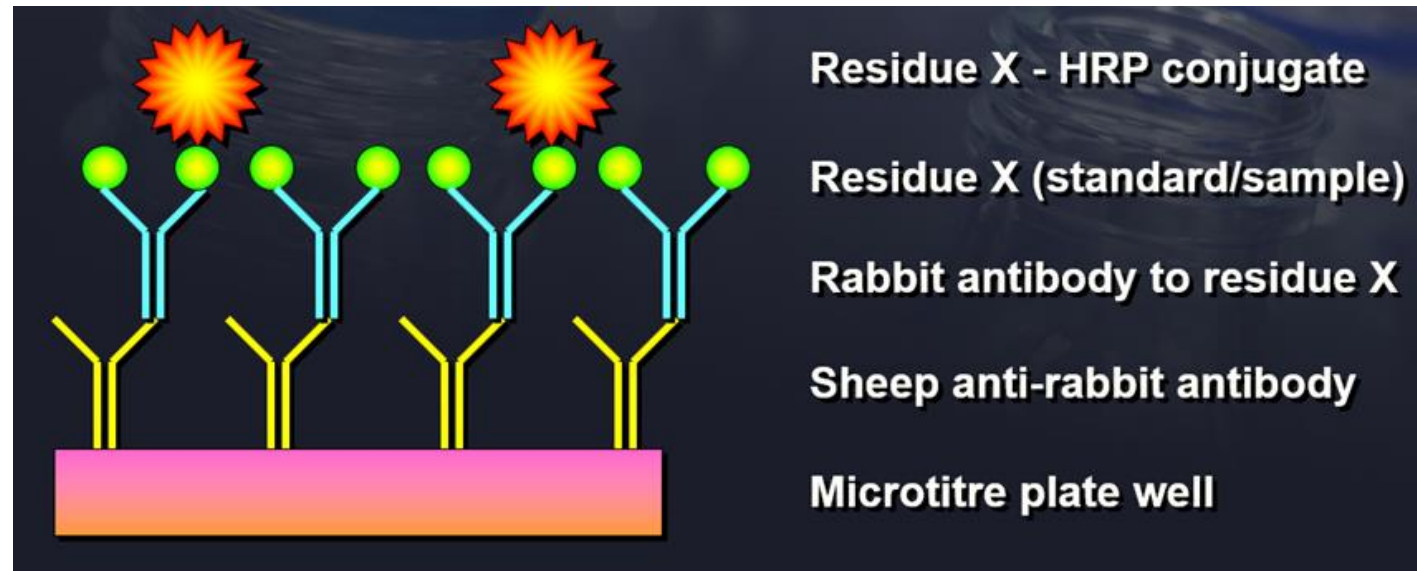


Figure: format of a competitive ELISA.
Residue X = target molecule,
HRP = horseradish peroxidase enzyme

- Microtiterplate
- Single compound or family
- Semi-quantitative

TRENDS IN RESIDUE SCREENING

Faster

- up to 1 minute: e.g. Charm MRLBL1

Easier in use

- one step test protocol: e.g. Quantum BT-CEF (Prognosis, GR)(see poster)
- no incubator (ambient temperature) e.g. SNAP BL ST-Plus
- combination incubator/reader
- automation e.g. Aurox (Unisensor) (see poster)
 - no pipetting
 - no manual mixing



TRENDS IN RESIDUE SCREENING

Broader spectrum:

. several test lines/dots on 1 strip

- combination β -lactam - tetracyclines - (sulfonamides)
- combination β -lactam - quinolones - tetracyclines - sulfonamides
- combination macrolides/gentamicin - aminoglycosides – quinolones

- EXTENSO (Unisensor, BE): 17 dots on 1 strip
>100 compounds (→ lecture VDRA 8, 2018)

. cassette with different strips

- 4 strips, 4 test lines per strip (16in1 Rapid Test for Milk, Bioeasy, CN)
: 16 compounds, - 13 quinolones, - >18 sulfonamides
- 5 strips (iSCAN, Season, KOR): 58 compounds

TRENDS IN RESIDUE SCREENING

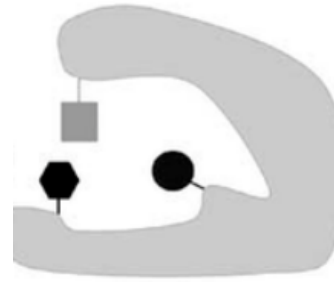
Adapted to certain markets

- US (detection of 6 β -lactams at US Safe Level)
- Russian Federation (BL, tetra (10 ppb), Strep, CAP)
- Australia/New Zealand: Charm ROSA SL KIWI (cefalonium)

BIOSENSORS

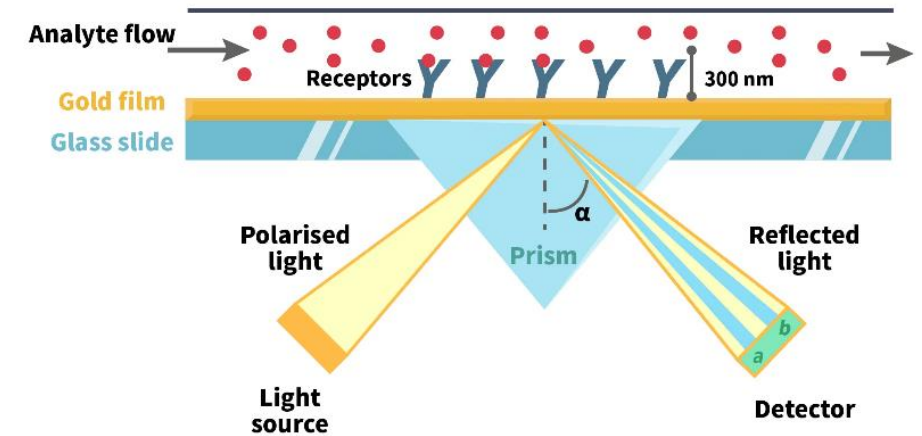
Bioreceptors

- Antibodies
- Enzymes
- Molecular Imprinted Polymers (MIPS)
- Whole-cell biosensors



Transducers

- Mass sensitive
- Optical
 - Surface Plasmon Resonance (Biacore (GE Healthcare Europe GmbH))
 - chemiluminescence (Evidence Investigator (Randox Lab., UK), MCR3 (R-Biopharm))
 - bioluminescence (Infiniplex for milk, 130 compounds)
 - flow cytometric (BeadXPlex (Unisensor, BE))
- Electrochemical
 - potentiometric
 - amperometric



REPORTING OF RESULTS OF A SCREENING METHOD

- Negative result:
 - 'no presence of antimicrobial residues'
 - 'compliant' in case $CC\beta$ for **all** compounds \leq MRL (Regulation 37/2010)
 - Positive result:
 - 'residues of antimicrobial residues detected', 'suspect'
 - no judgement about compliancy
- . Food business operator (self control): no obligation for confirmatory test but food is deemed to be unsafe (Decision 2006/694/EC) and cannot be processed
- . National control plan: confirmatory test (identification and quantification) is obligatory

PRESENT AND FUTURE

- National control plans: less and less space for screening tests but labs use confirmatory tests in screening mode
- Testing of non-food matrices (see further)
- Testing of farm silo milk at the farm instead of testing the commingled tanker milk at the reception of the dairy plant
- Use of smartphone camera (with app) to read dipsticks
- Testing by the consumer

ANTE-MORTEM SCREENING OF NON-FOOD MATRICES

- faeces / droppings

J. Cornejo (University of Chile, CL)
microbiological plate tests
6 families of antibiotics



- urine

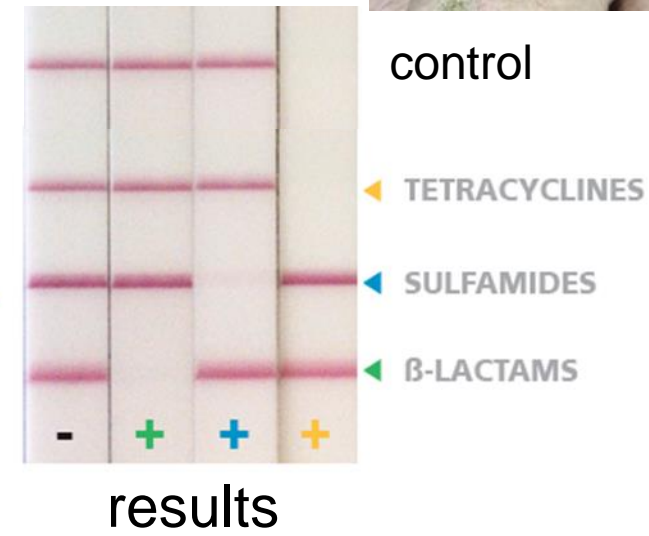
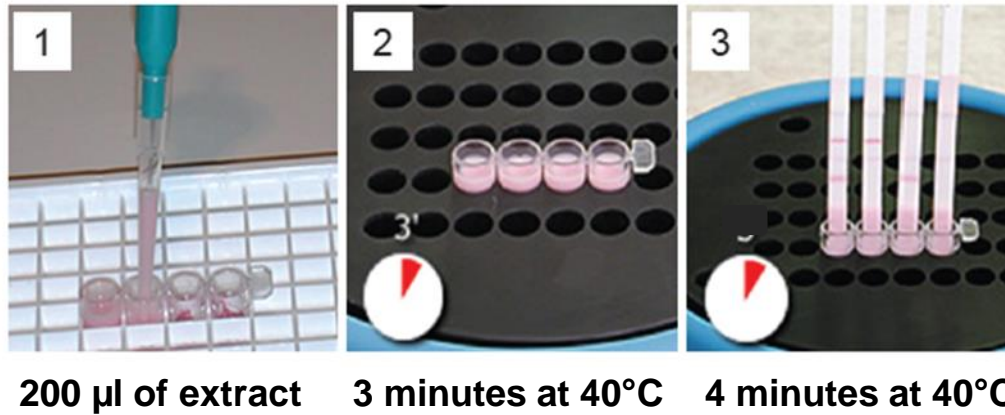


ANTE-MORTEM SCREENING OF NON-FOOD MATRICES

- saliva of pigs (ILVO, BE)
 - . sampling of saliva with a rope
 - . small extraction (milk or buffer)
 - . rapid testing (7 minutes)



TriSensor Milk & Tylosensor Milk

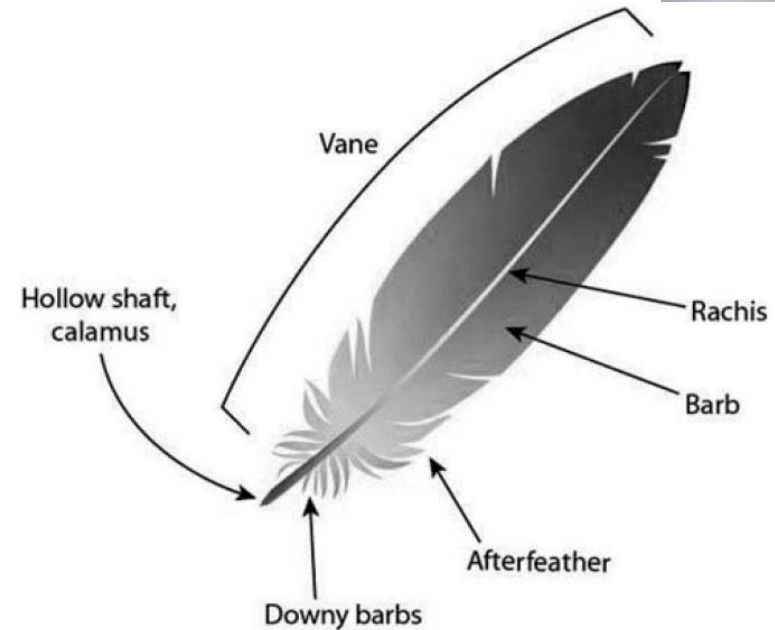


Non-invasive, smart sampling
On-site testing

(Lecture EuroResidue VIII, 2016)

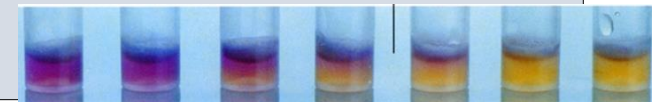
ANTE-MORTEM SCREENING OF NON-FOOD MATRICES

- feathers of chicken (WFSR, NL)
fluoroquinolones
(Charm Quinolone test, Charm Sciences)



PRO'S AND CON'S OF SCREENING TESTS

PRO's	CON's
cost effective: reagents, instrumentation & labour costs	normally no identification of the residue
tests ready for use	no quantitative result: MRL exceeded?
userfriendly, simple: no qualified people - onestep tests - no sample pretreatment - on-site testing	detection capabilities not always close to MRL: > MRL or <<MRL
automation: robot pipetting - scanner	possibility of false positive results - natural inhibitors - matrix effects
small sample volume	sometimes too slow for entrance check
high sample throughput	sometimes no detection of the metabolites
rapid : 1 minute to 45 minutes	not all compounds detectable at MRL
inhibitor tests mostly broad-spectrum, multiple families tests	no screening methods for difficult matrices or for some compounds
in general good detection capability - could be improved by sample solvent extraction	batch differences
allows direct action (critical control point-HACCP)	limited shelflife (e.g. test plates)
	subjective visual reading



VALIDATIONS REQUIREMENTS

Commission Implementing Regulation (EU) 2021/808

Method	Confirmation		Screening		
	Qualitative	Quantitative	Qualitative	Semi-Quantitative	Quantitative
Substances	A	A,B	A,B	A,B	A,B
CC α	x	x			
CC β	x		x		x
Trueness	-	x			x
Precision		x		(x)	x
Relative matrix effects/absolute recovery		x			x
Selectivity/Specificity		x	x	x	x
Stability *		x	x	x	x
Ruggedness		x	x	x	x

A: prohibited or unauthorised substances

B: authorised substances

* reference to literature possible

ANALYTICAL METHODS FOR THE DETERMINATION OF RESIDUES OF PHARMACOLOGICALLY ACTIVE SUBSTANCES

VALIDATION GUIDELINES – Screening methods

- EURL Guidance document on screening method validation. Version 1.1, 21 September 2023.

IDF/TS23758:2021(E) (IDF/RM251:2021(E)) Guidelines for the validation of qualitative screening methods for the detection of residues of veterinary drugs in milk and milk products

(→ Lecture at EuroResidue IX, 2022)

PRIMARY VALIDATIONS AT ILVO (as of June 2025)

Antibiotics in milk:

- 112 lateral flow test kits
- 11 microbiological inhibitor test kits

Antibiotics in tissue (meat/fish)

- 5 Charm II receptor test kits
- 3 microbial inhibitor test kits

Antibiotics in honey

- 2 lateral flow test kits

Aflatoxin M1 in milk

- 16 lateral flow test kits (semi-quantitatively)
- 1 ELISA kit (semi-quantitatively)

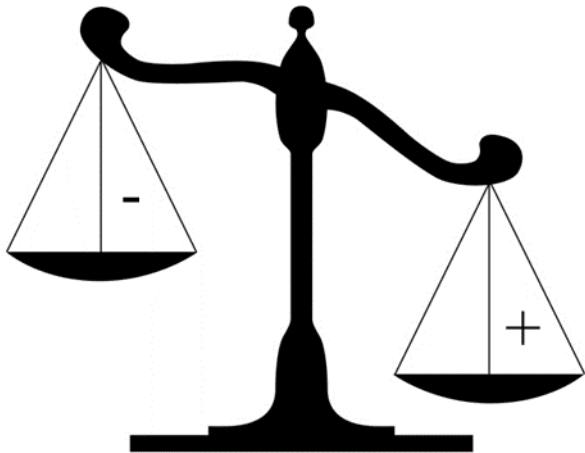


[Website ILVO \(link\): Validation of commercial screening tests at ILVO - ILVO Vlaanderen](#)

CONCLUSIONS

Screening tests and especially rapid tests remain very useful and even crucial for the food industry due to following characteristics:

- easy - cheap - sensitive - fast - high sample throughput
- reliable (false compliance rate of $\leq 5\%$)
- pro-active





Thanks to my team members:

Annelies

Caroline

Eline

Katleen

Katrien

Sigrid

Veroniek

Veronique

Thanks to ILVO colleagues Els D., Christof, Els VP. & Ilse
Thanks to the residue analysis community worldwide

Now time for....



W.Reybroeck@hotmail.com