MINISTÈRE DE L'AGRICULTURE

DE L'ALIMENTATION ET DE LA VITICULTURE

# Development of a multiresidue method for the quantitative determination of antibiotic residues in feed

**David Schuh, Nadine Daleiden, Danielle Ruckert and Claude Schummer**

**Administration des Services Techniques de l'Agriculture, Division des Laboratoires, Service d'analyse des engrais, aliments pour animaux et alcools. Ettelbruck**

LE GOUVERNEMENT
DU GRAND-DUCHÉ DE LUXEMBOURG
Ministère de l'Agriculture,
de l'Alimentation et de la Viticulture

# 1928: The discovery of antibiotics

## Before 1928

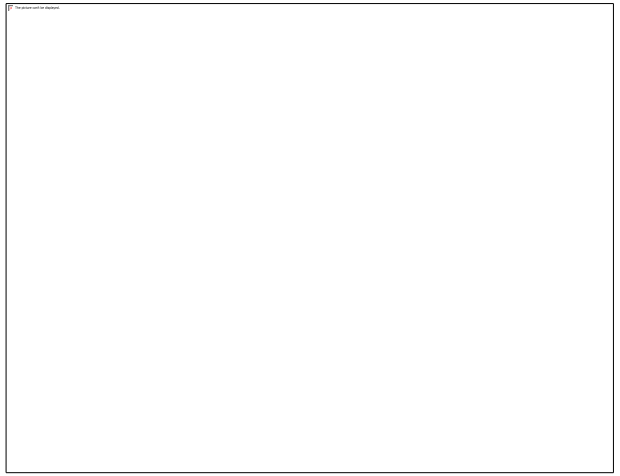
Before the discovery of antibiotics, bacteria infections often ended lethally

(Bubonic plague, typhus, cholera or even a harsch bronchitis could kill you )

## Discovery in 1928

The discovery of penicillin in 1928 by Sir Alexander Fleming marked the beginning of antibiotic revolution.

Returning from a holiday, he noticed that mould growing on a Petri dish of Staphylococus bacteria seemed to be preventing the bacteria around it from growing with “mould juice” – penicillin !



# Antibiotics in agriculture

- Today, antibiotics are used to cure bacterial infections in farm animals in order to treat, control and avoid the spread of diseases
- Antibiotics are also sometimes given preventively in the feed to avoid infections or stress-related diseases in very dense animal populations (chicken farm, …)
- Antibiotics also may be used at different dosages (usually lower) to help promote faster growth.

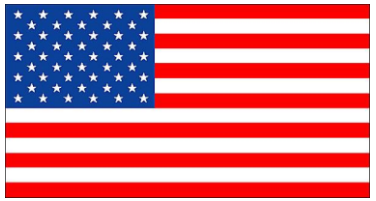
**Farmers have a moral and legal obligation to keep their animals healthy and ensure they receive appropriate treatment.**

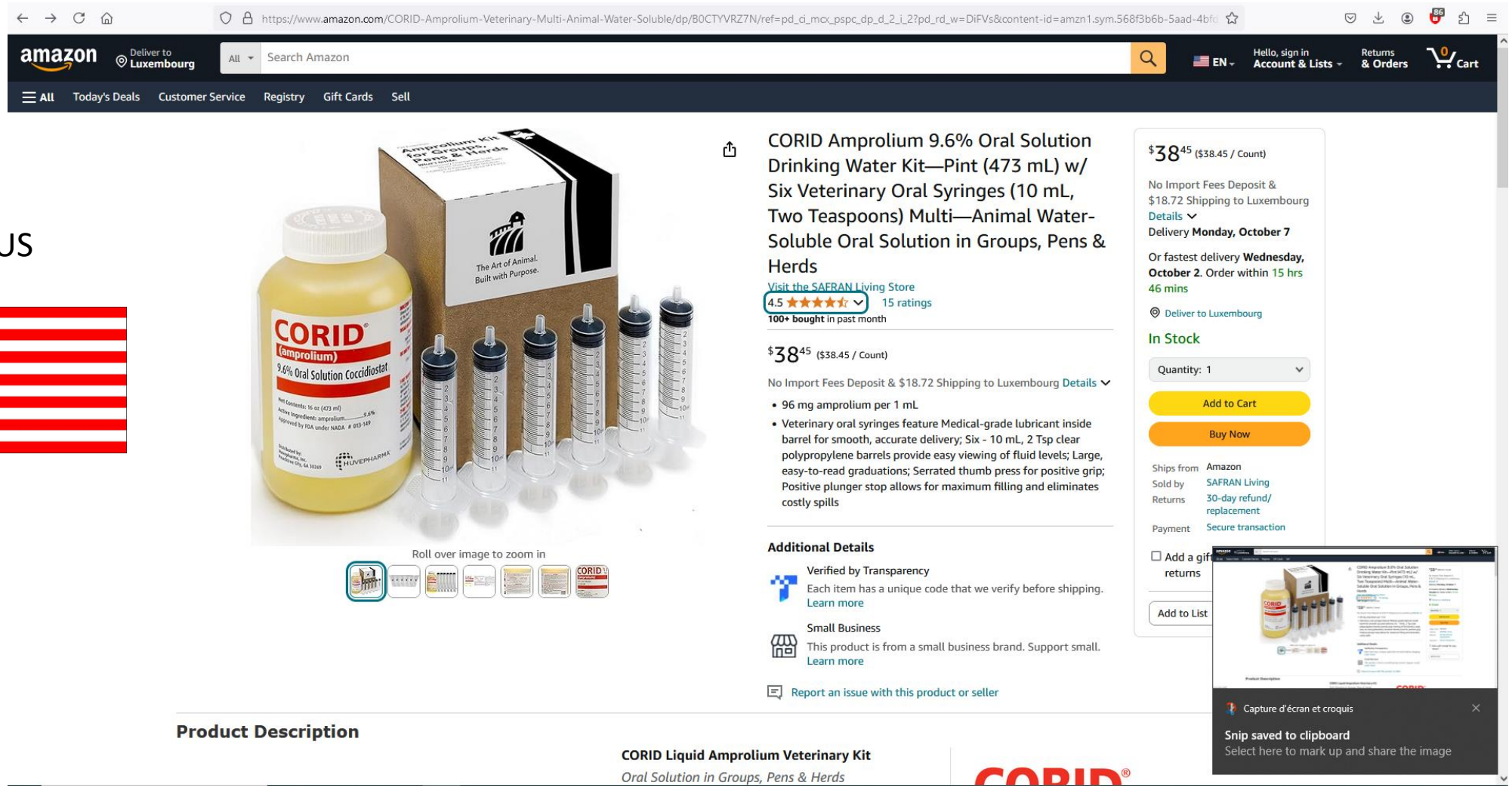
# Antibiotics in agriculture

- A multitude of antibiotics and anticoccidials are available today.
- Antibiotics are prescription-only medicines in Europe and are therefore only available for use by farmers following diagnosis by a veterinarian and after the provision of a veterinary prescription. This considerably limits the problem of overuse, at least in the EU.
- The proper use of antibiotics has been regulated in the EU regulation 1831/2003 on additives for use in animal nutrition
  - The aim is to ensure a proper use of antibiotics and to make sure that products that have been forbidden due to their toxicity, do not appear on the market

# Antibiotics in agriculture

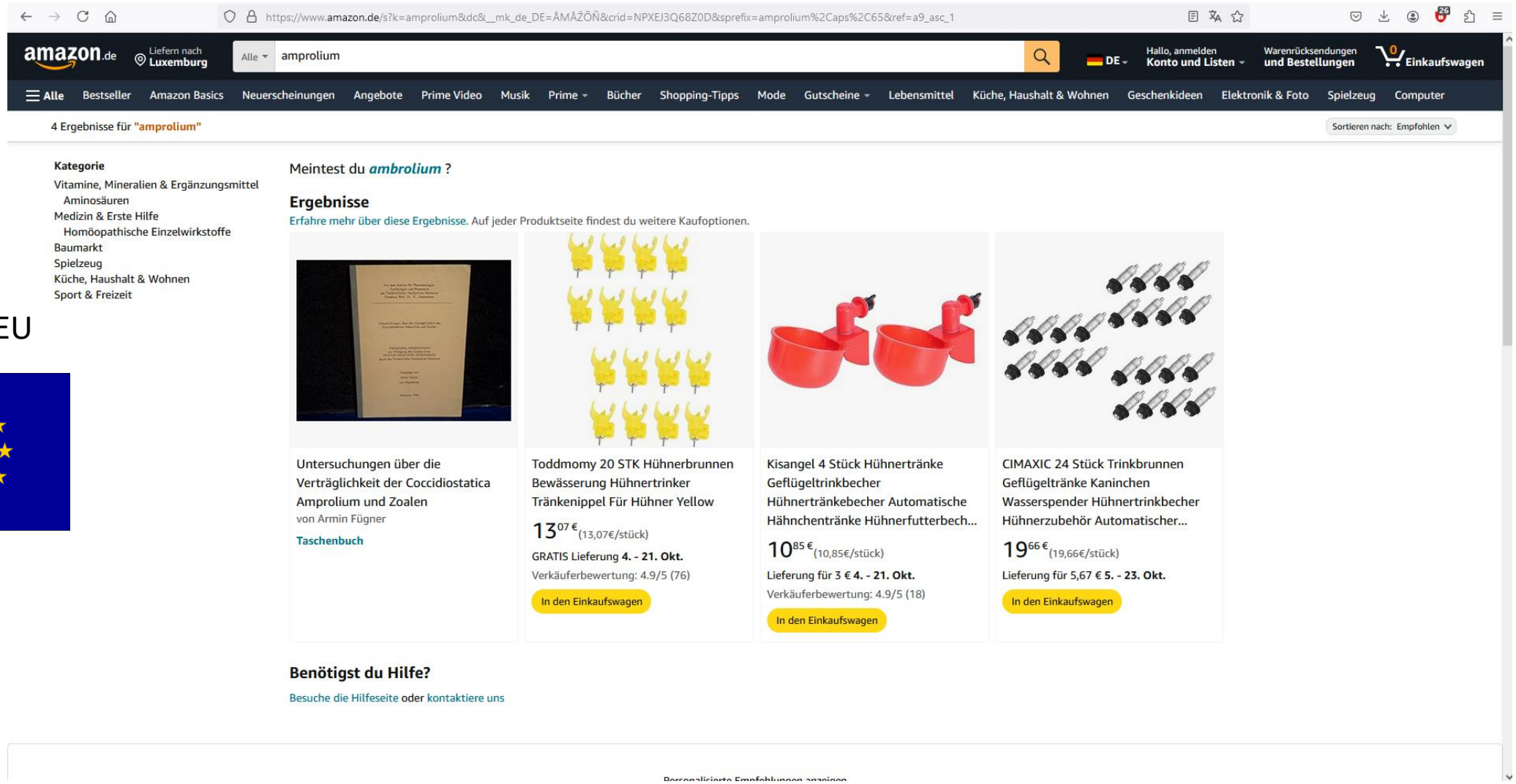
Amazon US





# Antibiotics in agriculture

Amazon EU



# Antibiotics in the food chain – what should be avoided

## 1. Excessive use of antibiotics

The FDA identified unnecessary long-term antibiotic treatment of food-producing animals as one of the major problems linked to antibiotics and tries to implement duration limits for all medically important antibiotics

## 2. Unnecessary use of antibiotics

WHO is recommending that farmers and the food industry stop using antibiotics routinely to promote growth and prevent disease in healthy animals

## 3. Cross contaminations

Cross-contaminations of feed (e.g.) due to blending in equipment previously used to spike feed with antibiotics, may lead to a long-term administration of low doses of antibiotics. Accidents and errors.

# Antibiotics in the food chain

- Antibiotics administered to animals can end up into the meat, in eggs or in the milk.
- Antibiotic traces can be found in irrigation Water due to inappropriate recycling process.
- Presence of antibiotics in manure which then accumulates in plants like cereals. (Pan and Chu 2017)
- It may interfere with food transformations, like fermentation.

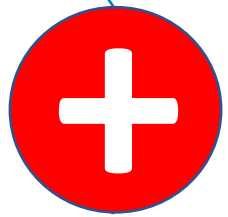

# Antibiotics in the food chain

Antibiotics given to animals

Antibiotics given to a person in hospital

Resistant bacteria spread to humans and other animals through poorly prepared food

Resistant bacteria spread to other people through poor hygiene and close proximity



Resistant bacteria spread to humans as contaminants in the food chain

Farm products can become contaminated when resistant bacteria from animal feces spread to them through irrigation water or fertilizers

# Effect of antibiotics on humans

- Antibiotic resistance, most known problem. Overuse of antibiotics makes them lose their efficacity as resistant bacterias arises.
- Toxicity: «Dose makes the poison» - Paracelsus
- Allergies (Beef or Pork containing penicillines residues can cause anaphylactic reaction)
- Carcinogenocity (ex Sulfamethazine in thyroid cancer)
- Teratogenicity (Alteration of DNA synthesis with Ciprofloxacin)
- Influence of human intestine microbiome

The issue of the presence of antibiotics residues in food is still intensely debated.

The 20'th of february 2024, the commission released a delegated regulation: 2024/1229, which gives clear maximum residues limits in feeds, which supplements regulation 2019/4.

WHO, together with national bodies, launched antibiotic reduction plans (Luxembourg: PNA – *Plan National Antibiotiques* 2018 – 2022. Extended to 2024).

# Monitoring antibiotic residues in feed

## Qualitative vs quantitative methods

### Most known screening methods:

### a. microbiological methods

Expensive and time consuming but very good specificity.

-> not suited for most MRL (*maximum residue limits*) set in European legislation

(2024/1229)

### b. enzymatic methods

Enzymatic methods are fast, sensitive and easy to perform, but normally limited to one family of compounds and have low reproductibility.

# Monitoring antibiotic residues in feed

## Screening methods are easy to operate and cheap but are mainly qualitative methods

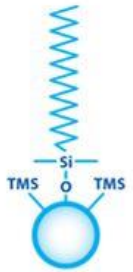
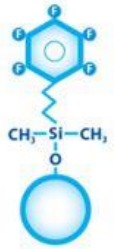
## State-of-the-art confirmatory analysis method for the detection of AB residues: UHPLC-MS/MS

The aim of this study was the development of a multiresidue method based on solid-liquid extraction and UHPLC-MS/MS detection, suitable for the required MRLs set in European legislation 2024/1229, which are relatively strict.

# UHPLC-M/MS method

## UHPLC

We tried different kind of columns: C18, F5 and Biphenyl.

| | Biphenyl | C18 | FluoroPhenyl |
|---|---|---|---|
| |  |  |  |
| **USP Phase Code** | L11 | L1 | L43 |
| **Stationary Phase Category** | Phenyl | C18, octadecylsilane | Pentafluorophenyl propyl |
| **Ligand Type** | Biphenyl | End-capped C18 | Fluorophenyl |
| **Particle Size** | 1.8 µm, 3 µm, or 5 µm fully porous | 1.8 µm, 3 µm, or 5 µm fully porous | 1.8 µm, 3 µm, or 5 µm fully porous |
| **Pore Size** | 100 Å | 100 Å | 100 Å |
| **Surface Area** | 300 $m^2/g$ | 300 $m^2/g$ | 300 $m^2/g$ |
| **Carbon Load** | 15% | 20% | 10% |
| **End-Cap** | yes | yes | no |
| **pH Range** | 2.0 to 8.0 | 2.0 to 8.0 | 2.0 to 8.0 |
| **Maximum Temperature** | 80 °C | 80 °C | 80 °C |

We are working with a very diverse set of molecules, so we are aiming for the most kind of interactions possible that could lead to potentially the best separation.

The choice of the mobile phase is important too:  Using acetonitril with the biphenyl phase will saturate and cancel electronic Phi bond electrons interactions. This limits orthogonal methods.

Technically they all worked. Best results were obtained with the biphenyl phase as the C18 was less efficient. The F5 worked great too but was more inconsistent (maybe Ionic reactions?). Examples later

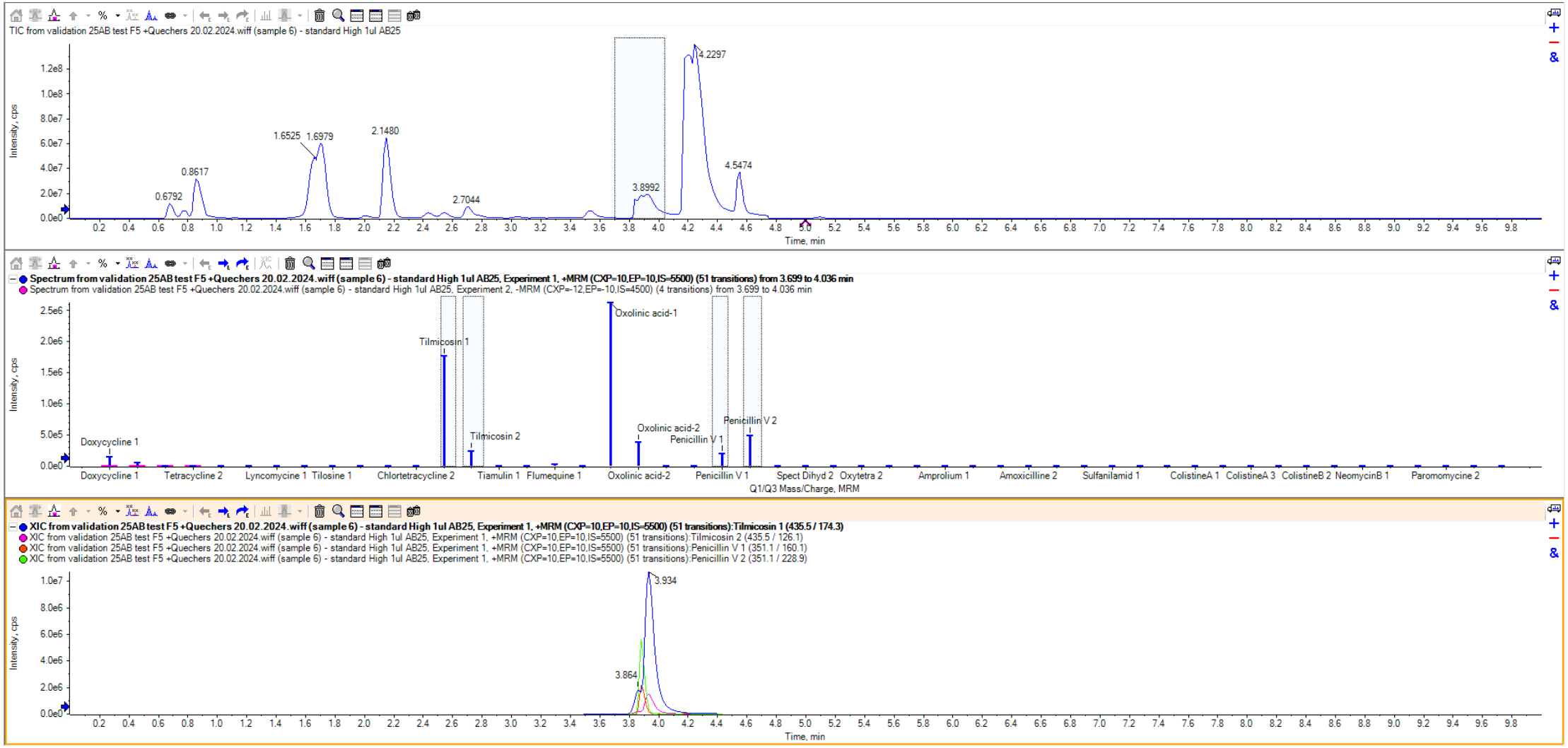
# UHPLC-M/MS method

## MS/MS

The detector was a 6500+ from Sciex, which is a triple quadripole Mass spectrometer, with a Qtrap. This adds a huge layer of depths to the method as you considerably boost sensitivity and selectivity.

# Chromatograms

F5 column: A:Water B:MeOH
Start 10%B Gradient to B:100% in 10 minutes



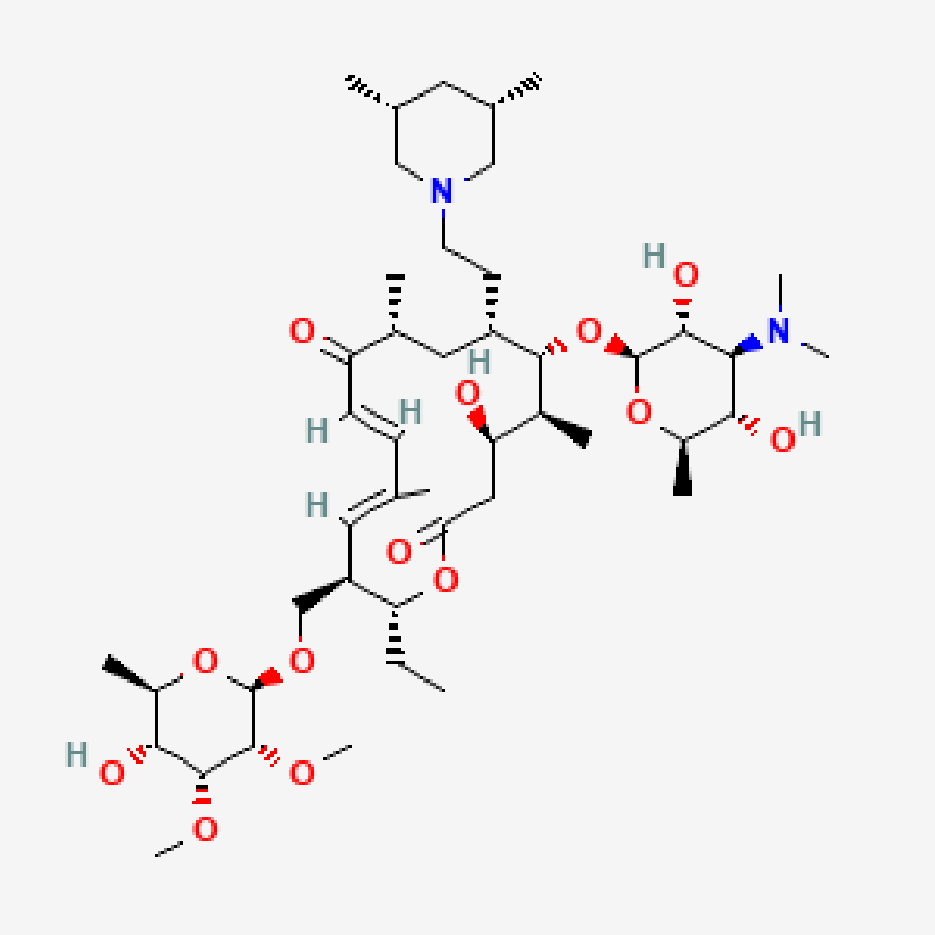
# Chromatograms

Biphenyl column: same LC parameters as F5

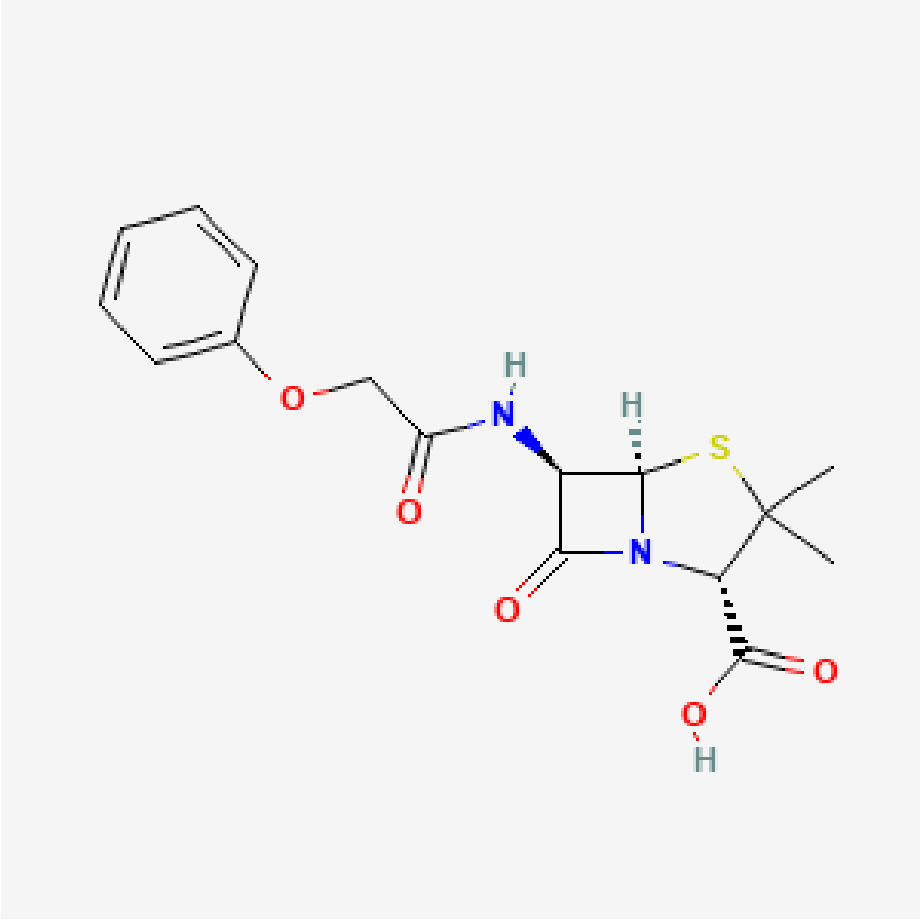
# Chromatograms

To contextualize

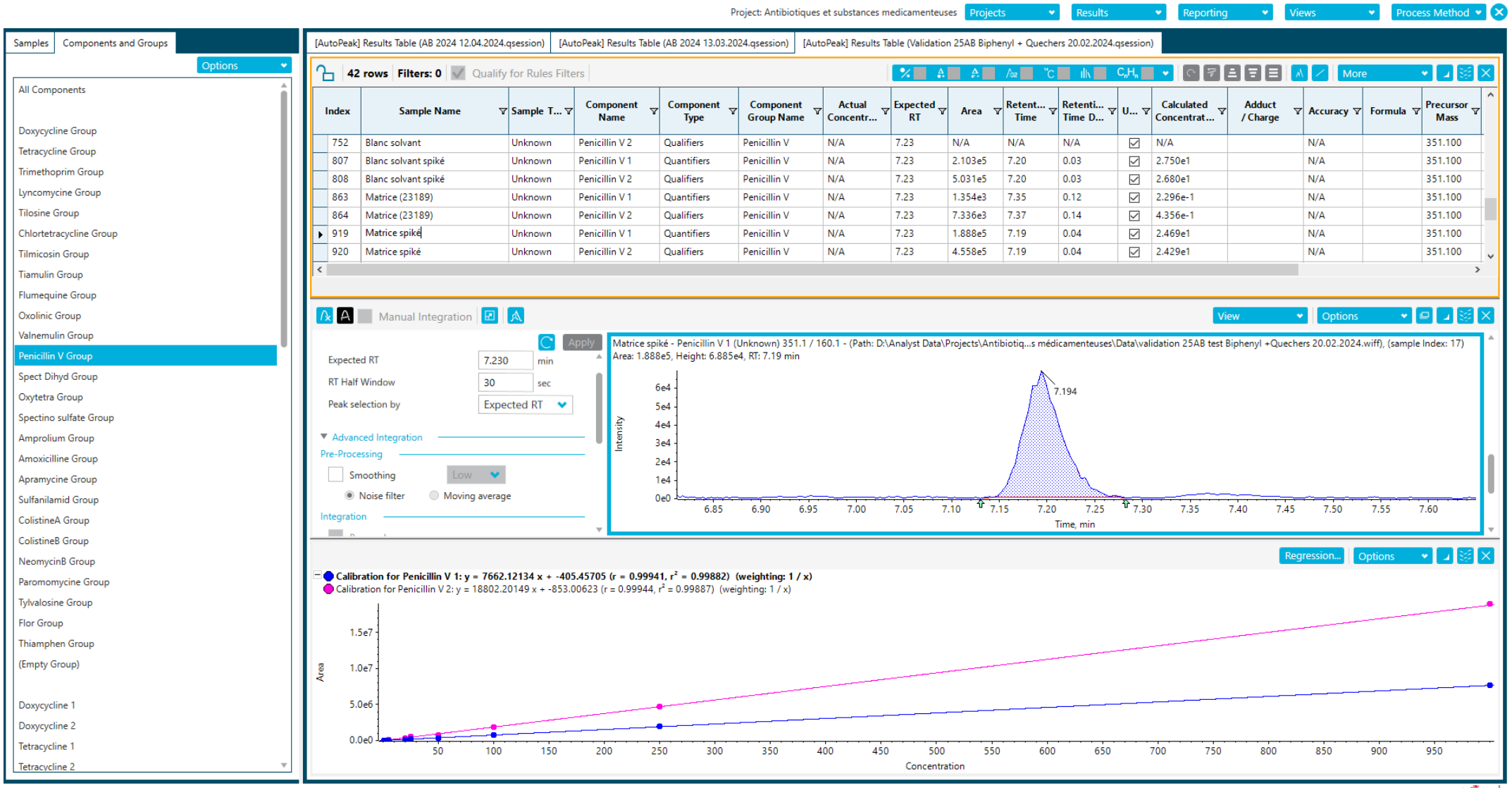
Tilmicosin



Penicillin V



# Quantification/ results



# Extraction methods

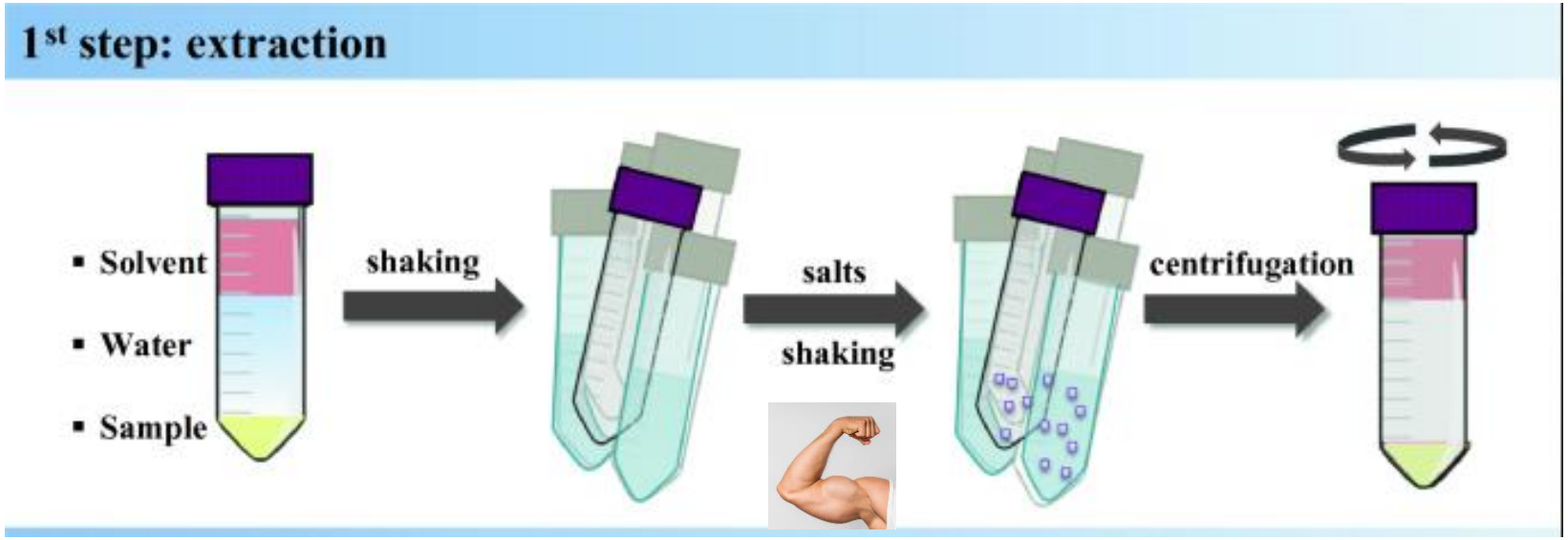
2 methods were tested: Quechers and «quick and dirty».

Quechers stands for **Qu**ick, **E**asy, **Ch**eap, **E**fficient, **R**ugged, **S**afe


# Extraction methods

5 grams of sample were put in a 50ml centrifuge tube.

The Quechers extraction was realized with ACN and water, shaken by hand, sonicated 15 minutes and then salts were added, with a ceramic bead to amplify the effect of shaking, then the tube was vigorously (!) shaken for 1 minute. We did filter the extract and directly injected it.



# Extraction methods

The Q&d method consist of mixing the sample in a 90/10/10 % solution of ACN/H20/MeOH on a shaker for 30minutes. Then centrifuge it after a 15 minute sonication.


# Results, summary

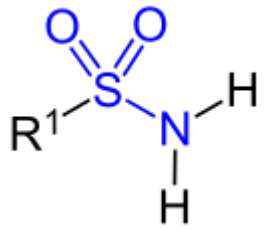
| Classe d'antibiotique | Antibiotique | projected LOD (ug/kg) | Recovery (%) Quick and not too dirty | Recovery (%) Quech |
|---|---|---|---|---|
| | | | | |
| Tetracyclines | Doxycycline | 100 | 81.5 | **109.1** |
| | Tetracycline | 100 | 31.0 | **10.7** |
| | Chlortetracycline | 200 | 42.9 | **8.4** |
| | Oxytetracycline | 100 | 23.8 | **30.0** |
| Sulfamides | Sulfanilamide | 5000 | 63.6 | **52.6** |
| Lyncosamides | Lyncomycine | <100 | 77.4 | **71.4** |
| Macrolides | Tilosine | 100 | 142.9 | **117.6** |
| | Tilmicosin | 100 | 83.3 | **65.7** |
| | Tylvalosine | 30 | 133.3 | **101.1** |
| Pleuromutilines | Tiamuline | <100 | 77.4 | **51.4** |
| | Valnemuline | <100 | 122.2 | **74.2** |
| Pénicillines | Amoxicilline | 100 | 67.4 | **52.5** |
| | Penicillin V | 100 | 92.6 | **80.0** |
| Amphéniciols | Florfenicol | <100 | 62.5 | **46.2** |
| | Thiamphenicol | <100 | 64.7 | **58.5** |
| Coccidiostates | Amprolium | <100 | 100.0 | **6.1** |
| Fluoroquinolones | Flumequine | <300 | 96.7 | **59.3** |
| | Acide oxolinique | <300 | 95.6 | **55.0** |
| Polypeptides | ColistineA | 15000 | 0.0 | **0.0** |
| | ColistineB | 15000 | 0.0 | **0.0** |
| Aminoglycosides | NeomycinB | 1200 | 0.0 | **0.0** |
| | Paromomycin | 600 | 0.0 | **0.0** |
| | Spectinomicine sulf | <300 | 2.7 | **0.0** |
| | Apramycine | <300 | 0.0 | **0.0** |
| | Spectinomycin dihydr | 100 | 1.3 | **0.0** |
| Pyrimidine | Trimethropine | <100 | 43.3 | **24.2** |

# Conclusions

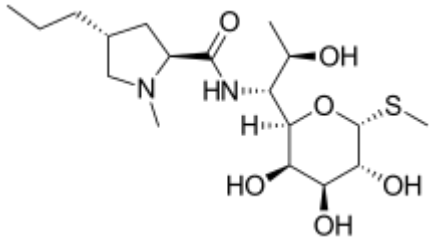
This is a challenging analysis, especially considering the diverse group of molecules. There are compromises that have to be done at every level of the method.

The molecules with a good LOD and retention (green on the tab) are already done in the routine by our laboratory.

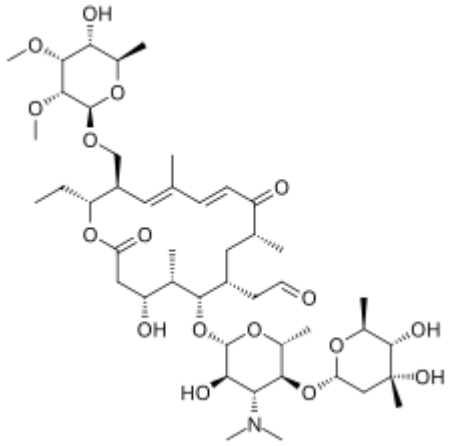
Sulfonamides



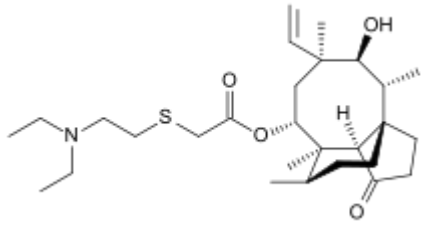
Lyncosamides (ex. Lyncomycin)



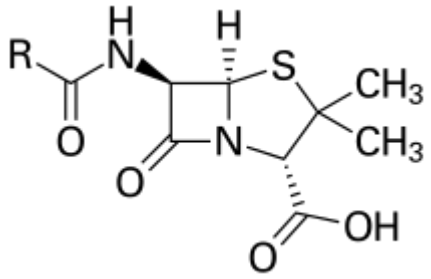
Macrolides



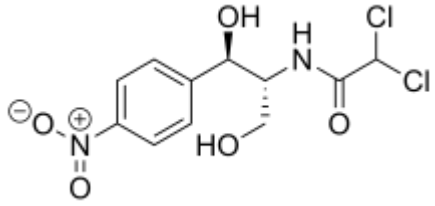
Pleuromutilin (Tiamulin)



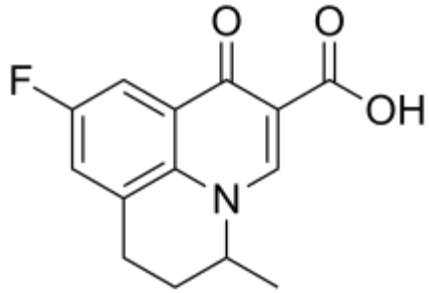
Penicillins



Amphenicols



Fluoroquinolones (Flumequin)



# Perspectives

Concerning the molecules with an average LOD and retention, we have the project to test a novel extraction method that should significantly improve the recovery :

In classic Quechers extraction, the salts that are used are buffers, magnesium sulfate and sometimes sodium chloride. What is known today is that bivalent ions like magnesium tends to form polar complexes with many analytes. It has been observed on Tetracyclines (!). So these formed compounds remains strongly in the watery phase and don't get extracted in the ACN.
To solve this issue, replacing these salts with phosphates (dipotassium hydrogen phosphate and potassium dihydrogen phosphate) ands avoiding sodium seems to improve greatly the recovery of many «problematic» molecules. It is called the P-Quechers variant.

Then the next challenge will then be to deal with the well known problem of aminoglycosides extraction. Worst case scenario is that a specific extraction will be needed for these analytes.

# MERCI

## DE VOTRE ATTENTION

*further questions: chimie@asta.etat.lu*

LE GOUVERNEMENT
DU GRAND-DUCHÉ DE LUXEMBOURG
Ministère de l'Agriculture,
de l'Alimentation et de la Viticulture