



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

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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

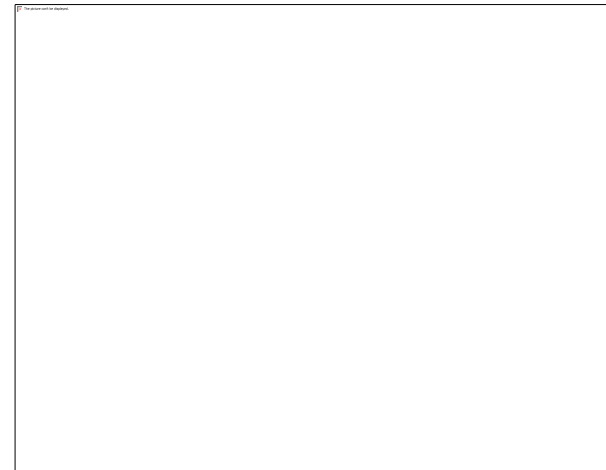
Before 1928

Before the discovery of antibiotics, bacteria infections often ended lethally (Bubonic plague, typhus, cholera or even a harsh bronchitis could kill you)

Discovery in 1928

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

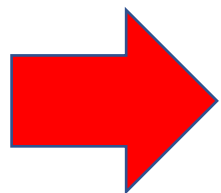
Returning from a holiday, he noticed that mould growing on a Petri dish of Staphylococcus bacteria seemed to be preventing the bacteria around it from growing with "mould juice" – penicillin !



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

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CORID
(amprolium)
9.6% Oral Solution Coccidiostat

Net Contents: 16 oz (473 mL)
Active ingredient: amprolium... 9.6%
Approved by FDA under NADA # 013 149

Manufactured by:
Huvepharma, Inc.
Parsippany, NJ, USA 07054

Amprolium Kit for Calves, Pigs & Herds
The Art of Animal. Built with Purpose.

4.5 ★★★★★ 15 ratings
100+ bought in past month

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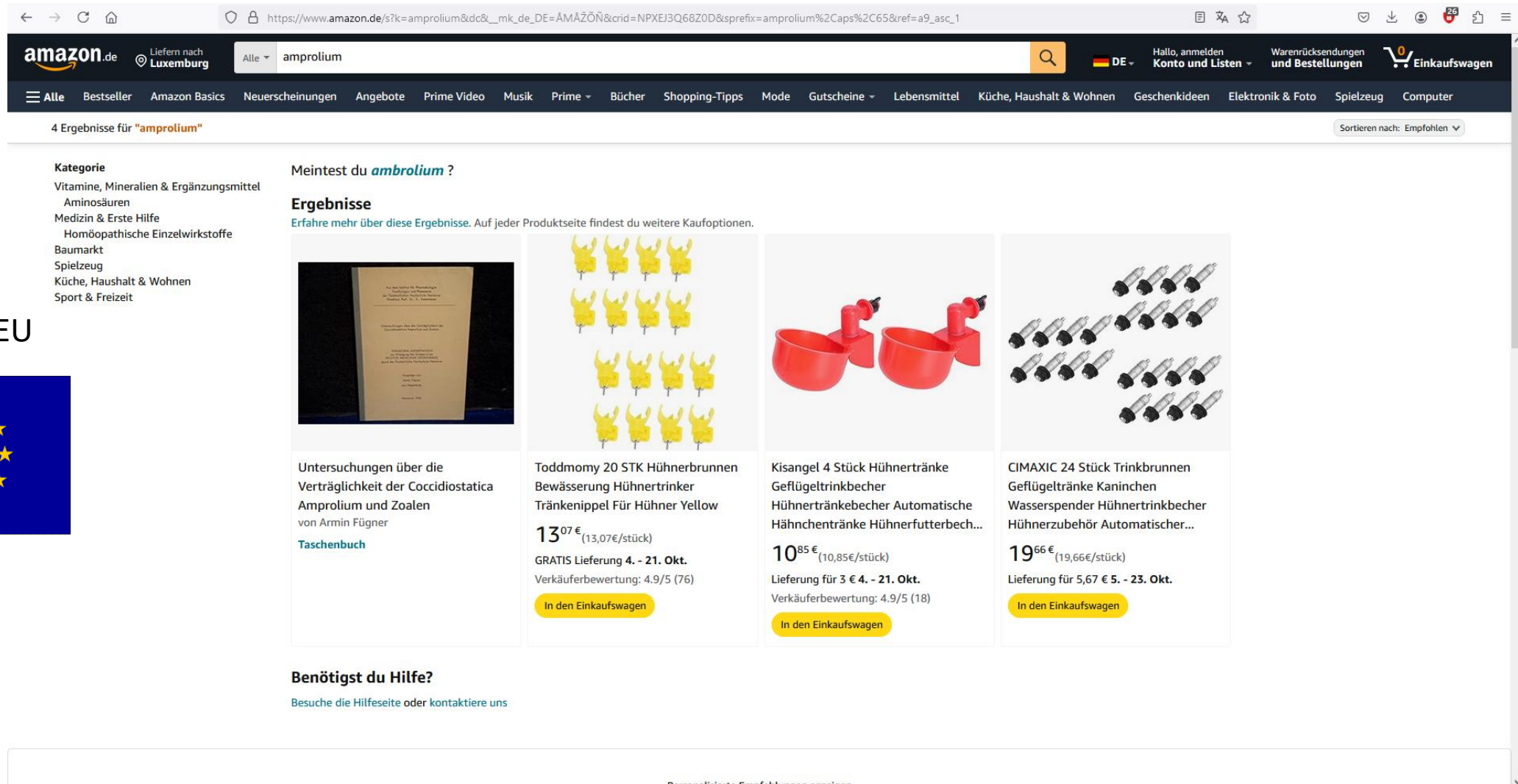
Product Description

CORID Liquid Amprolium Veterinary Kit
Oral Solution in Groups, Pens & Herds

CORID[®]



Antibiotics in agriculture



The screenshot shows an Amazon.de search for 'amprolium'. The search results are categorized into four items:

- Untersuchungen über die Verträglichkeit der Coccidiostatica Amprolium und Zoalen von Armin Fügner**: A pocket book (Taschenbuch) with a price of 13.07€ (13,07€/stück). It has a rating of 4.9/5 (76) and is available for free delivery from October 4th to 21st.
- Toddmomy 20 STK Hühnerbrunnen Bewässerung Hühnertrinker Tränkenippel Für Hühner Yellow**: A set of 20 yellow chicken waterers with a price of 10.85€ (10,85€/stück). It has a rating of 4.9/5 (18) and is available for free delivery from October 4th to 21st.
- Kisangel 4 Stück Hühnertränke Geflügeltrinkerbecher Hühnertränkebecher Automatische Hähnenchetränke Hühnerfutterbech...**: A set of 4 red automatic chicken waterers with a price of 19.66€ (19,66€/stück). It has a rating of 4.9/5 (18) and is available for free delivery from October 5th to 23rd.
- CIMAXIC 24 Stück Trinkbrunnen Geflügeltränke Kaninchen Wasserspender Hühnertrinkerbecher Hühnerzubehör Automatischer...**: A set of 24 silver automatic chicken waterers with a price of 19.66€ (19,66€/stück). It has a rating of 4.9/5 (18) and is available for free delivery from October 5th to 23rd.

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 efficacy as resistant bacteria arises.
- Toxicity: «Dose makes the poison» - Paracelsus
- Allergies (Beef or Pork containing penicillines residues can cause anaphylactic reaction)
- Carcinogenicity (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 20th 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 reproducibility.



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


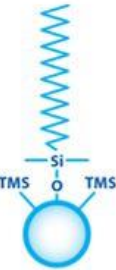
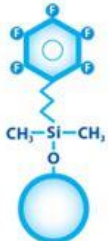
The aim of this study was the development of a multiresidue method based on solid-liquid extraction and UHPLC-MS/MS detection, suitable for the required MRLs set in European legislation 2024/1229, which are relatively strict.



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 quadrupole Mass spectrometer, with a Qtrap. This adds a huge layer of depths to the method as you considerably boost sensitivity and selectivity.

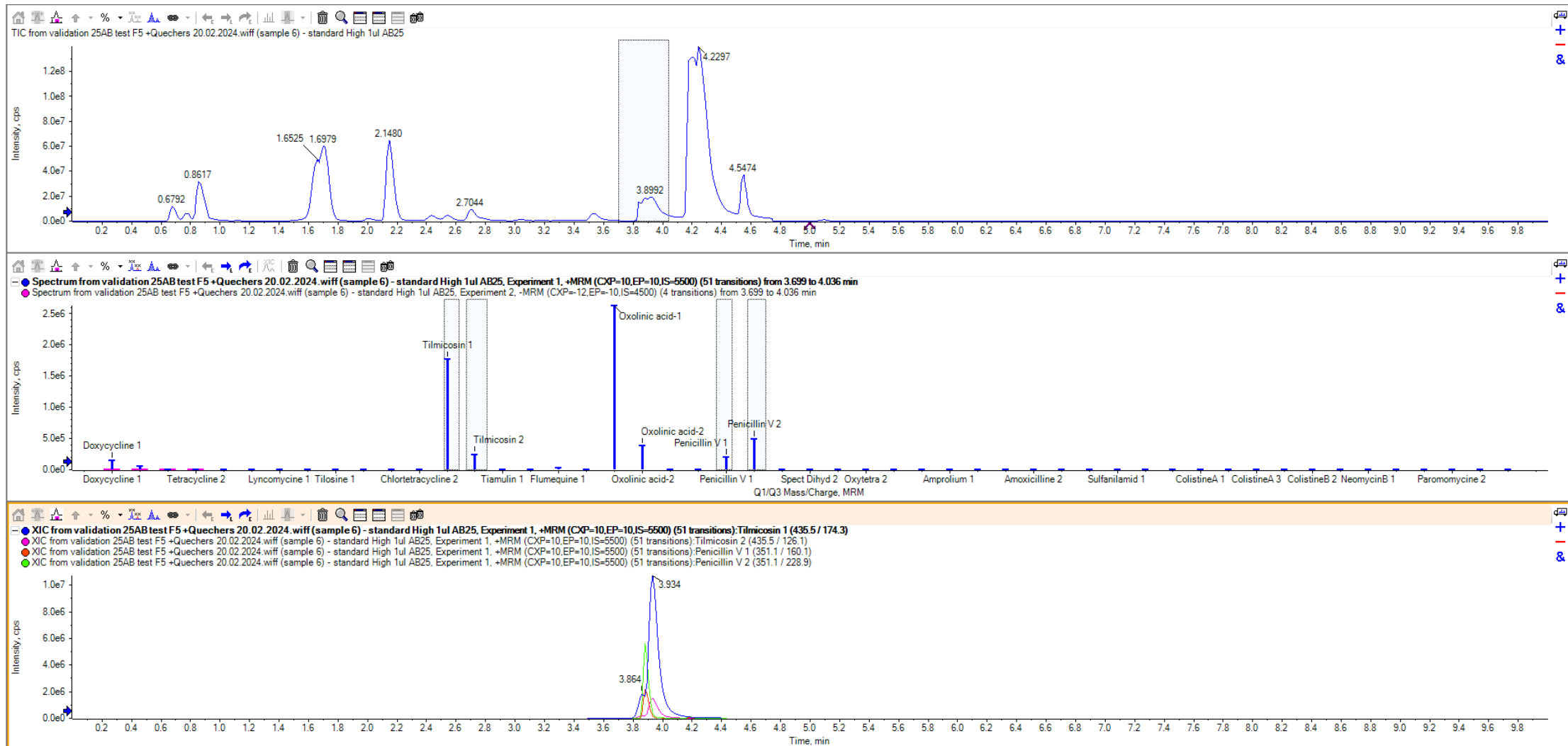


Chromatograms

F5 column:

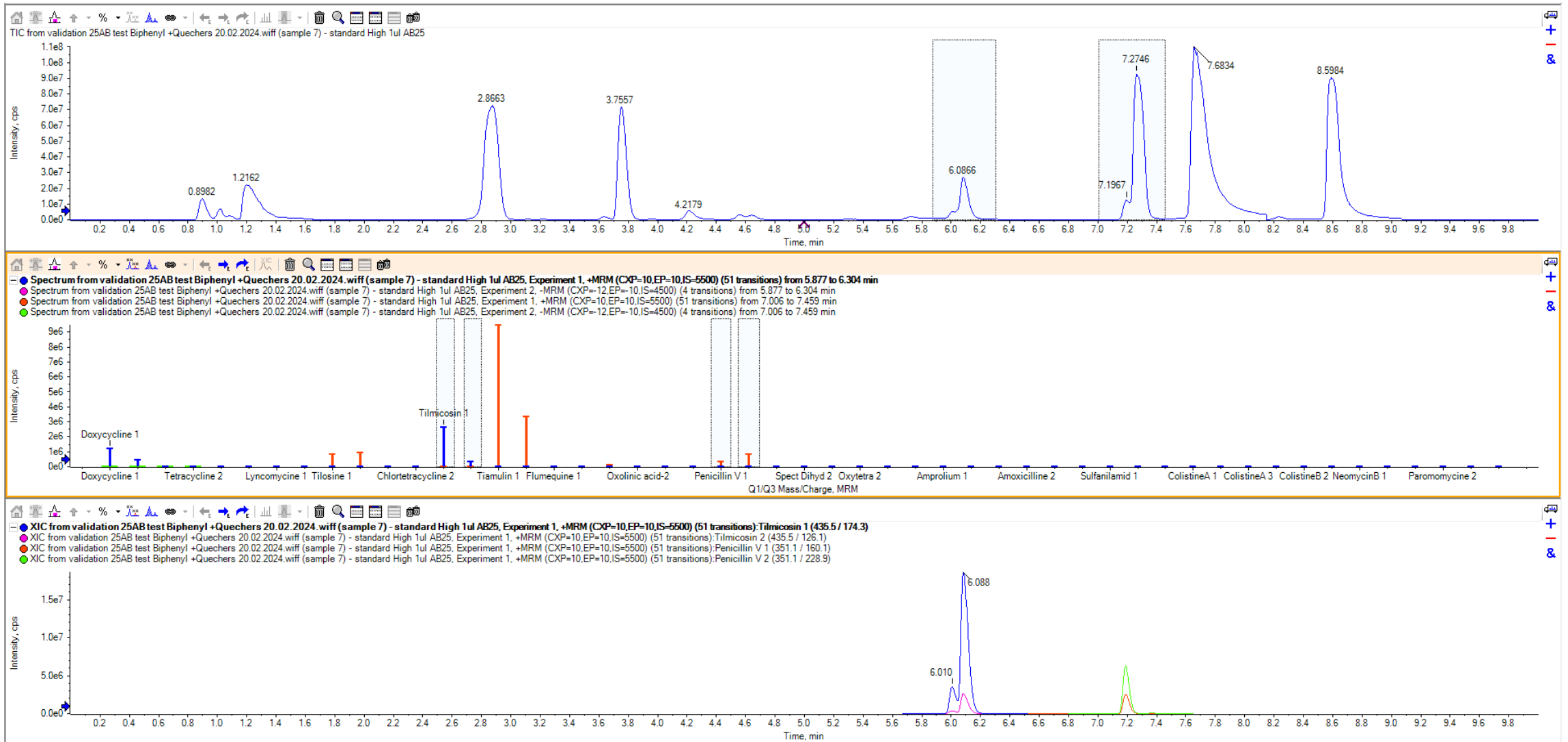
A:Water B:MeOH

Start 10%B Gradient to B:100% in 10 minutes



Chromatograms

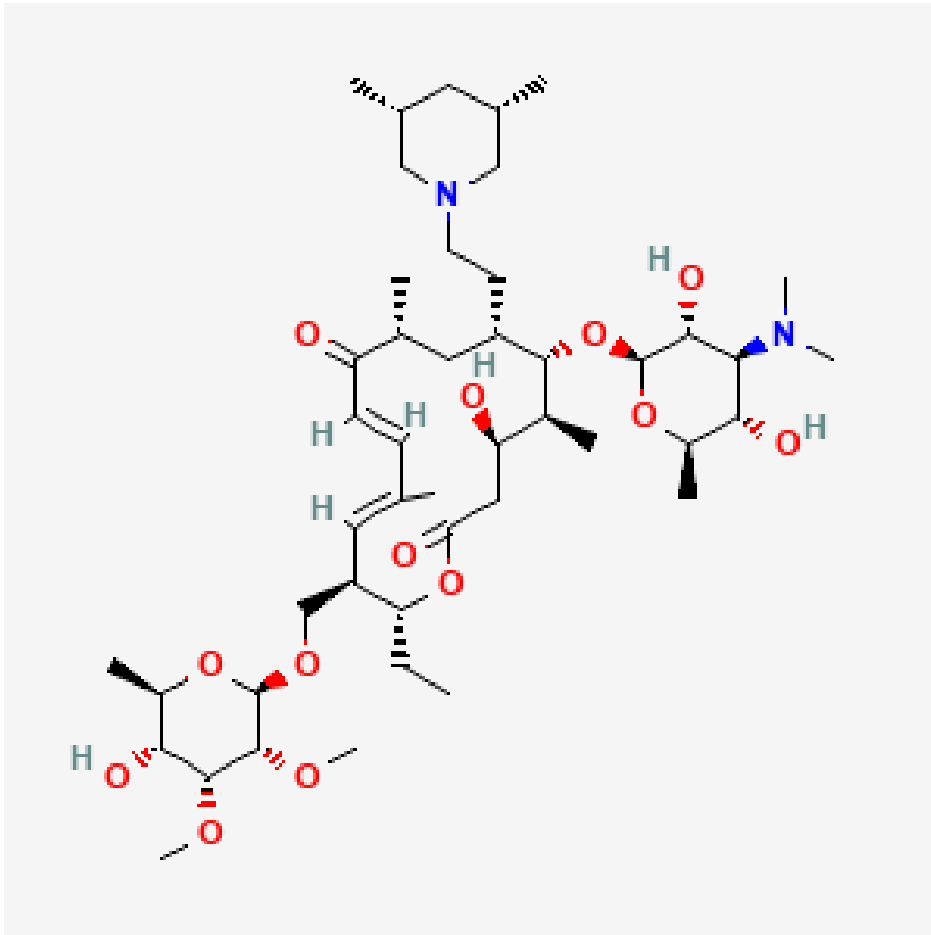
Biphenyl column: same LC parameters as F5



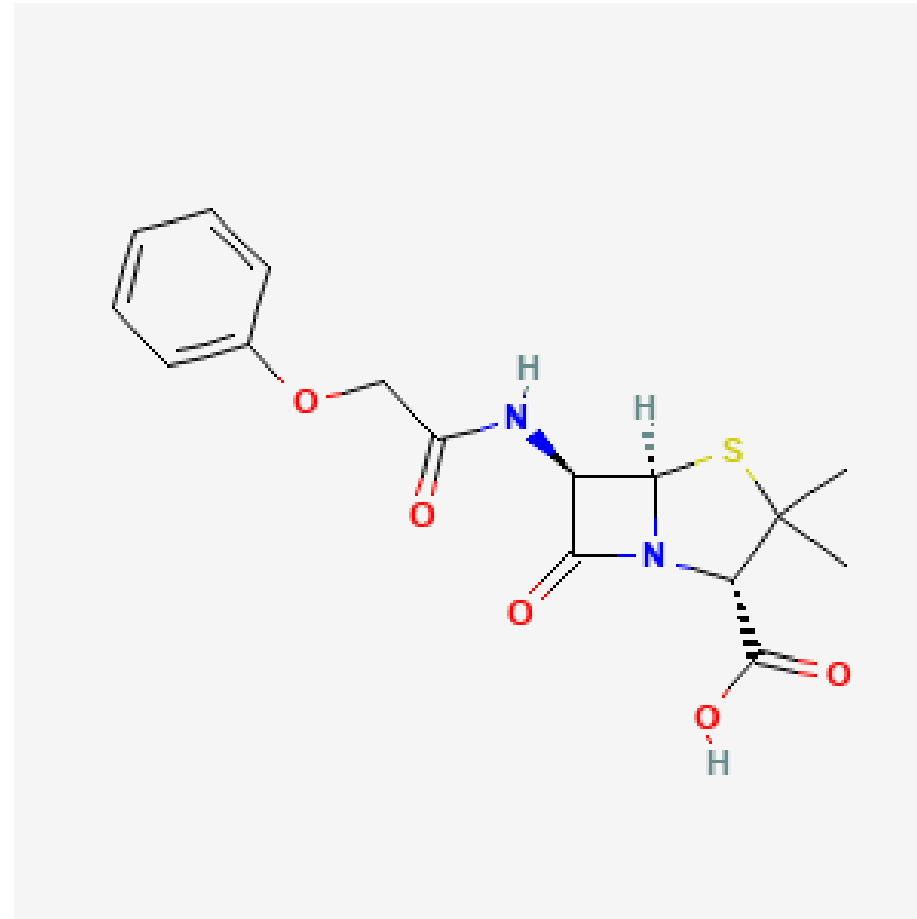
Chromatograms

To contextualize

Tilmicosin



Penicillin V



Quantification/ results

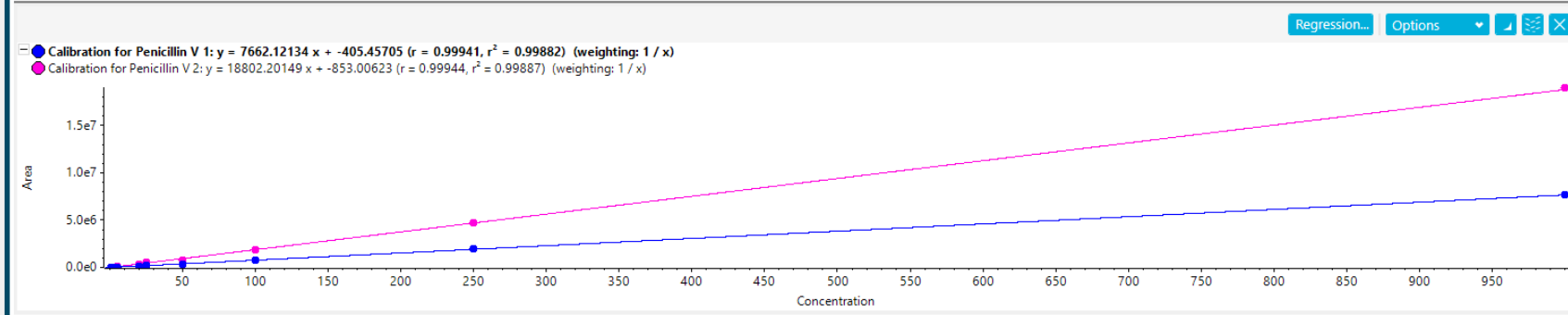
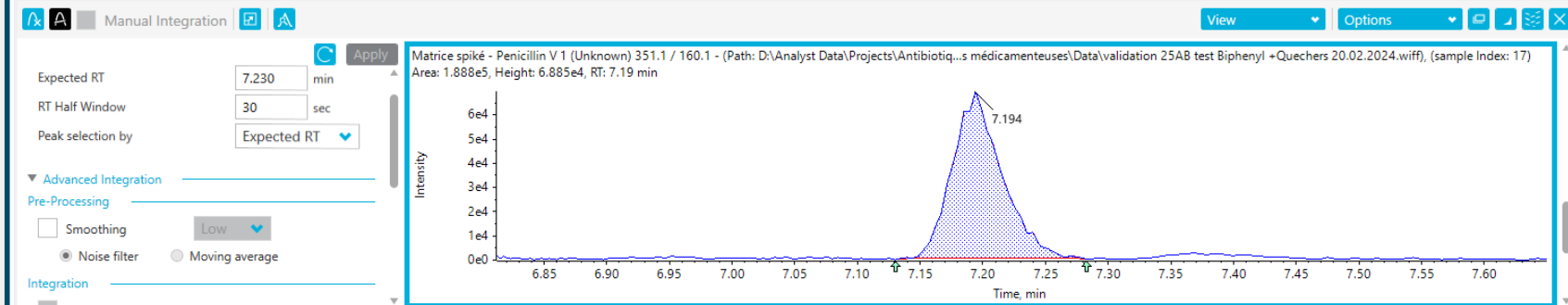
Project: Antibiotiques et substances medicamenteuses | Projects | Results | Reporting | Views | Process Method

- Samples | Components and Groups
- Options
- All Components
 - Doxycycline Group
 - Tetracycline Group
 - Trimethoprim Group
 - Lyncmicycine Group
 - Tilosine Group
 - Chlortetracycline Group
 - Tilmicosin Group
 - Tiamulin Group
 - Flumequine Group
 - Oxolinic Group
 - Valnemulin Group
 - Penicillin V Group**
 - Spect Dihyd Group
 - Oxytetra Group
 - Spectino sulfate Group
 - Amprolium Group
 - Amoxicilline Group
 - Apramycine Group
 - Sulfanilamid Group
 - ColistineA Group
 - ColistineB Group
 - NeomycinB Group
 - Paromomycine Group
 - Tylvalosine Group
 - Flor Group
 - Thiamphen Group
 - (Empty Group)
 - Doxycycline 1
 - Doxycycline 2
 - Tetracycline 1
 - Tetracycline 2

[AutoPeak] Results Table (AB 2024 12.04.2024.qsession) | [AutoPeak] Results Table (AB 2024 13.03.2024.qsession) | [AutoPeak] Results Table (Validation 25AB Biphenyl + Quechers 20.02.2024.qsession)

42 rows | Filters: 0 | Qualify for Rules Filters

Index	Sample Name	Sample T...	Component Name	Component Type	Component Group Name	Actual Concentr...	Expected RT	Area	Retent... Time	Retenti... Time D...	U...	Calculated Concentrat...	Adduct / Charge	Accuracy	Formula	Precursor Mass
752	Blanc solvant	Unknown	Penicillin V 2	Qualifiers	Penicillin V	N/A	7.23	N/A	N/A	N/A	<input checked="" type="checkbox"/>	N/A		N/A		351.100
807	Blanc solvant spiké	Unknown	Penicillin V 1	Quantifiers	Penicillin V	N/A	7.23	2.103e5	7.20	0.03	<input checked="" type="checkbox"/>	2.750e1		N/A		351.100
808	Blanc solvant spiké	Unknown	Penicillin V 2	Qualifiers	Penicillin V	N/A	7.23	5.031e5	7.20	0.03	<input checked="" type="checkbox"/>	2.680e1		N/A		351.100
863	Matrice (23189)	Unknown	Penicillin V 1	Quantifiers	Penicillin V	N/A	7.23	1.354e3	7.35	0.12	<input checked="" type="checkbox"/>	2.296e-1		N/A		351.100
864	Matrice (23189)	Unknown	Penicillin V 2	Qualifiers	Penicillin V	N/A	7.23	7.336e3	7.37	0.14	<input checked="" type="checkbox"/>	4.356e-1		N/A		351.100
919	Matrice spiké	Unknown	Penicillin V 1	Quantifiers	Penicillin V	N/A	7.23	1.888e5	7.19	0.04	<input checked="" type="checkbox"/>	2.469e1		N/A		351.100
920	Matrice spiké	Unknown	Penicillin V 2	Qualifiers	Penicillin V	N/A	7.23	4.558e5	7.19	0.04	<input checked="" type="checkbox"/>	2.429e1		N/A		351.100



Extraction methods

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

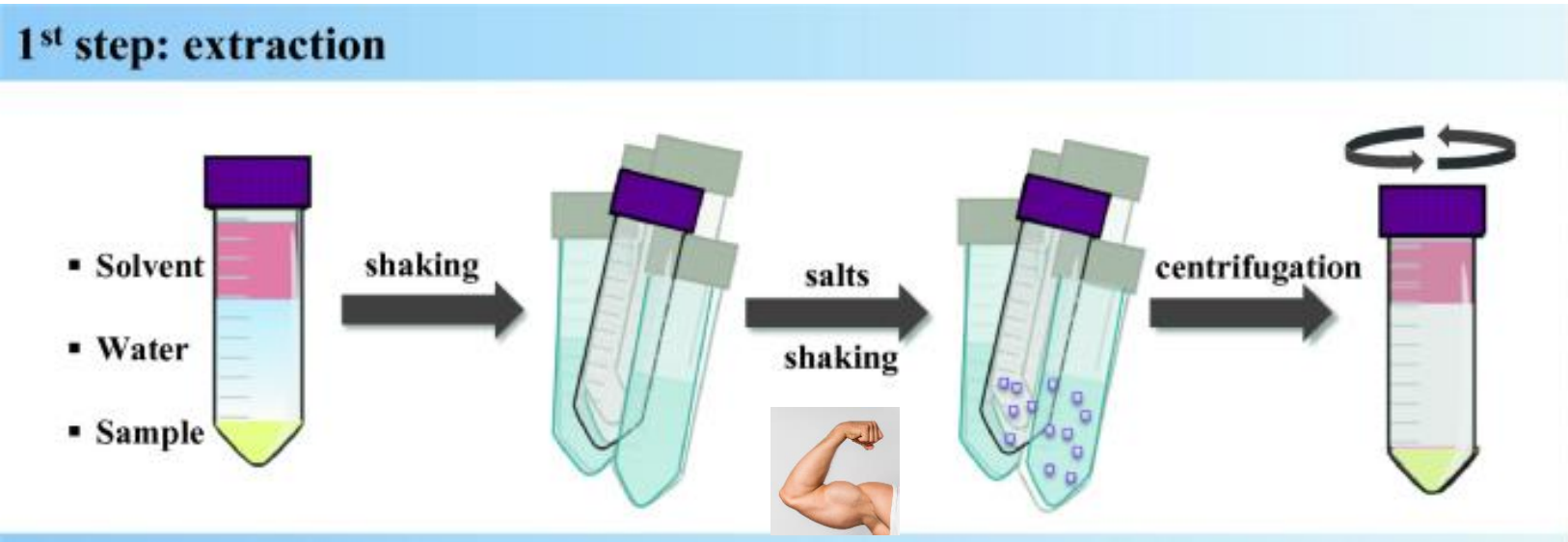
Quechers stands for **Quick, Easy, Cheap, Efficient, Rugged, Safe**



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/H₂O/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
Lincosamides	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
Cocciostates	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
	Spectinomycine sulf	<300	2.7	0.0
	Apramycine	<300	0.0	0.0
	Spectinomycin dihydr	100	1.3	0.0
	Pyrimidine	Trimethopine	<100	43.3

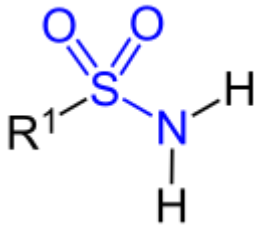


Conclusions

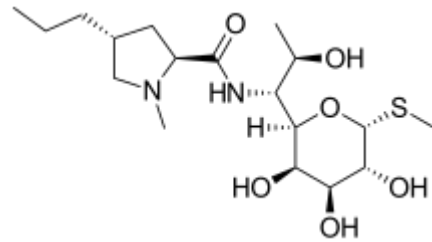
This is a challenging analysis, especially considering the diverse group of molecules. There are compromises that have to be done at every level of the method.

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

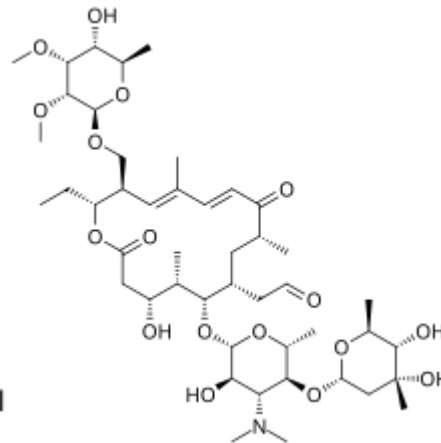
Sulfonamides



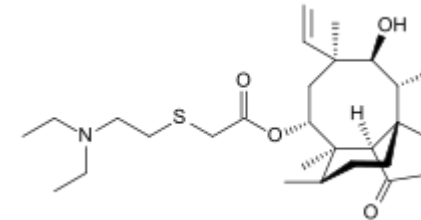
Lincosamides
(ex. Lyncomycin)



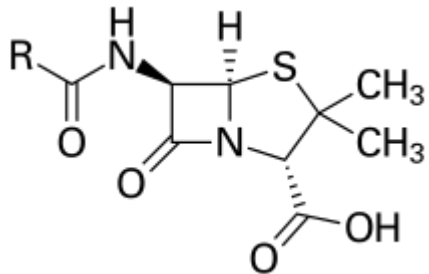
Macrolides



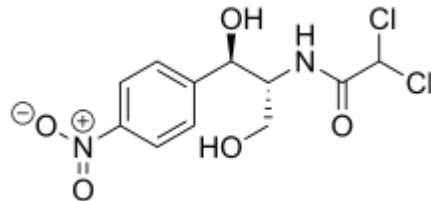
Pleuromutilin
(Tiamulin)



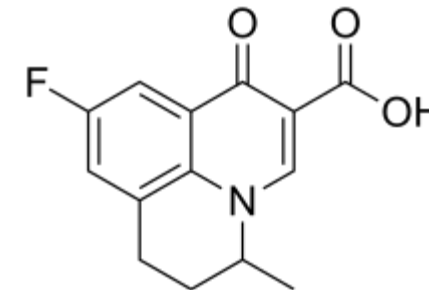
Penicillins



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) and 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.



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DE VOTRE ATTENTION

further questions: chimie@asta.etat.lu



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