Analysis of antibiotics in feedingstuffs by liquid chromatography and tandem mass spectrometry (LC-MS/MS)

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Layout

- Introduction
  - The Analytical Chemistry Platform in CRP-GL/EVA
  - Context of this study
  - Analytical methods
    - *Screening and confirmatory methods*
    - *Investigated molecules*
    - *Objectives and challenges*
- Method development and validation
  - LC-MS/MS method
  - Extraction protocol
- Conclusions
  - Method performance
  - Limits

Introduction

- EVA's Analytical Chemistry Platform
  - General figures
    - *Started in 2004 as a centralised analytical laboratory in the department Environment and Agro-biotechnologies (EVA)*
    - *Support activity, distinct form the research units*
    - *Users*
      - Own staff: 1 PF leader + 3 Full-time Engineers
      - Collaborators in EVA: +/- 10 researchers, technicians or students
  - Activities
    - *Management of analytical instruments*
      - Coordination and planning
      - Routine or advanced maintenance, calibrations, upgrades
      - Renewal and acquisition of new instruments
    - *Analyses for external partners*
      - Routine analyses
      - « Custom » analyses
      - Scientific collaborations
    - *Analytical support for Research Units*
      - Development and implementation of analytical methods
      - Optimization of sample preparation protocols
      - Training and support for users
      - If needed: Full analytical job (from sample to report)

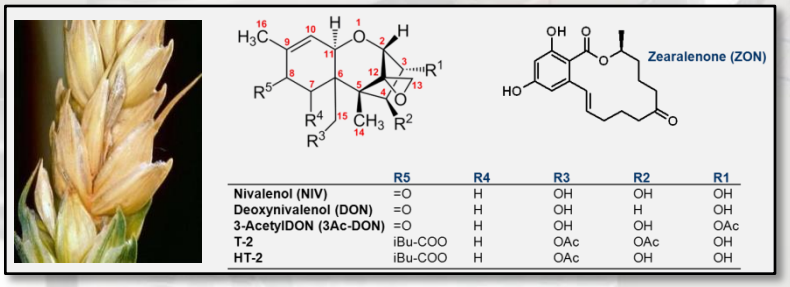
Analytical capabilities

Inorganic analysis

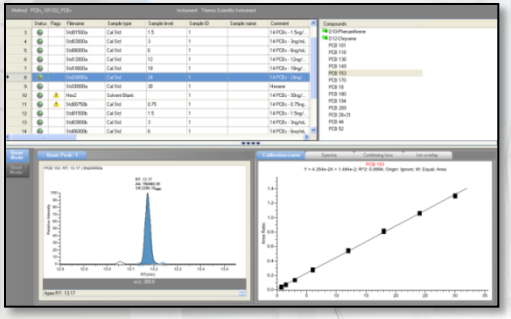
- Major inorganic anions and cations
- Heavy metals
- Rare earth elements

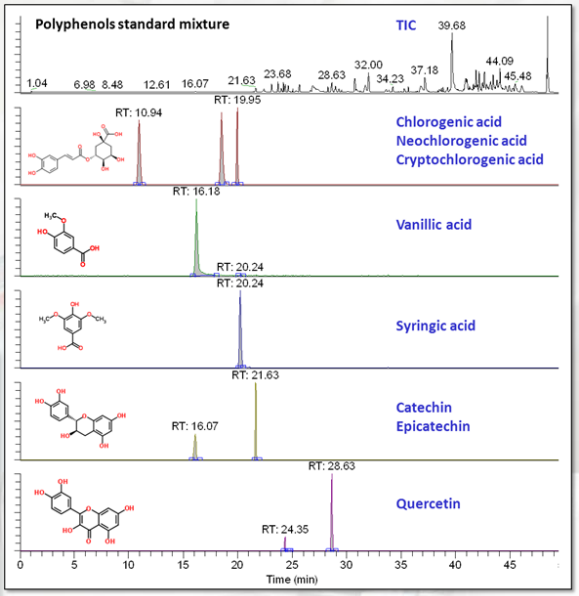
Organic analysis

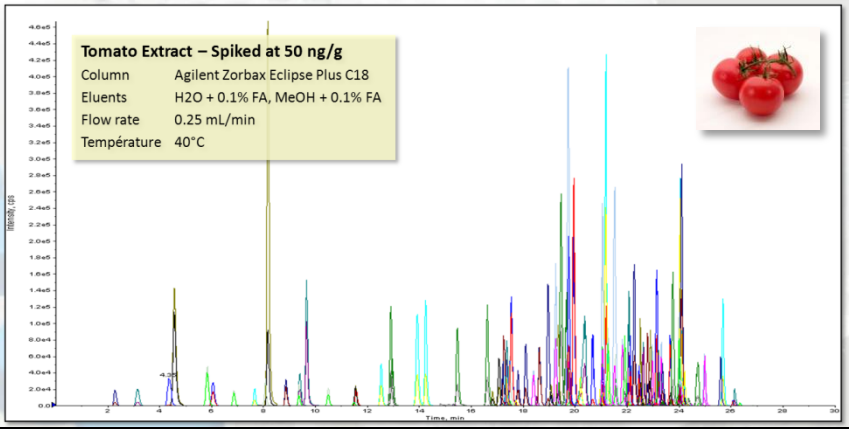
- Pollutants: Pesticides, PCBs, PAHs, Pharmaceuticals
- Natural compounds: Polyphenols, Carbohydrates, Toxins, Fatty acids, Aromas…

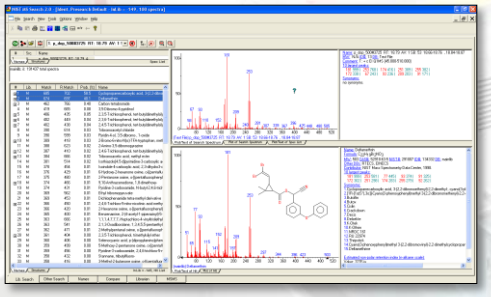


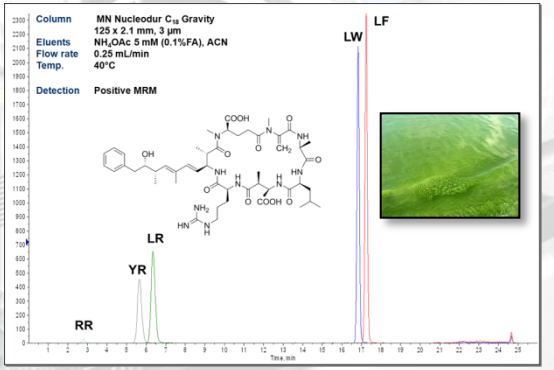
	R5	R4	R3	R2	R1
Nivalenol (NIV)	=O	H	OH	OH	OH
Deoxynivalenol (DON)	=O	H	OH	H	OH
3-AcetylDON (3Ac-DON)	=O	H	OH	OH	OAc
T-2	iBu-COO	H	OAc	OAc	OH
HT-2	iBu-COO	H	OAc	OH	OH











Context

- A large number of antibiotics have been used since the 50's to improve animal health and as growth promoters for intensive animal production
- The overuse/abuse of antibiotics can result in their presence in final products (meat, milk), to the increase of allergies or to the development of antibiotic-resistant bacteria
- Now, antibiotics have to be strictly used to ensure animal safety and health (prevention or treatment of diseases)
- However, antibiotics can still be present in animal feed (through illegal used or unintended cross-contamination), making necessary to implement dedicated analytical procedures

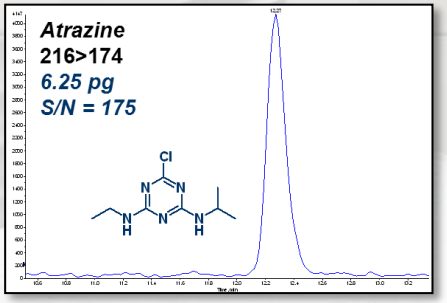
- Analytical methods for the control of antibiotics in animal feed
  - Screening method
    - *Quick and easily implemented*
    - *Covers a large range of antibiotics*
    - *Qualitative control*
    - ***Microbiology-based method***
  - Confirmatory method
    - *Only on positive samples*
    - *Specialised laboratories*
    - *Quantitative analyses*
    - ***Liquid Chromatography coupled to tandem mass spectrometry***
  - Both methods have been developed in CRP-GL, in the framework of the project "Antibiotics", based on a collaboration with ASTA

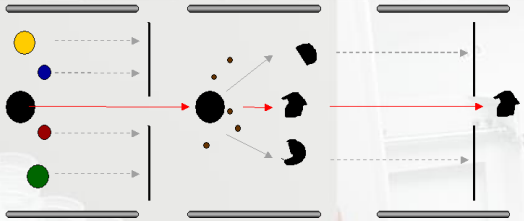
- Investigated molecules
  - *Penicillins*
  - *Tetracyclines*
  - *Polypeptides*
  - *Phenicols*
  - *Ionophores*
  - *…*
- Objectives and challenges
  - *Detect and quantify the widest range of antibiotics*
  - *Perform an accurate and sensitive quantitation*
  - *Develop a versatile extraction protocol*
  - *Be able to deal with complex and different matrices*

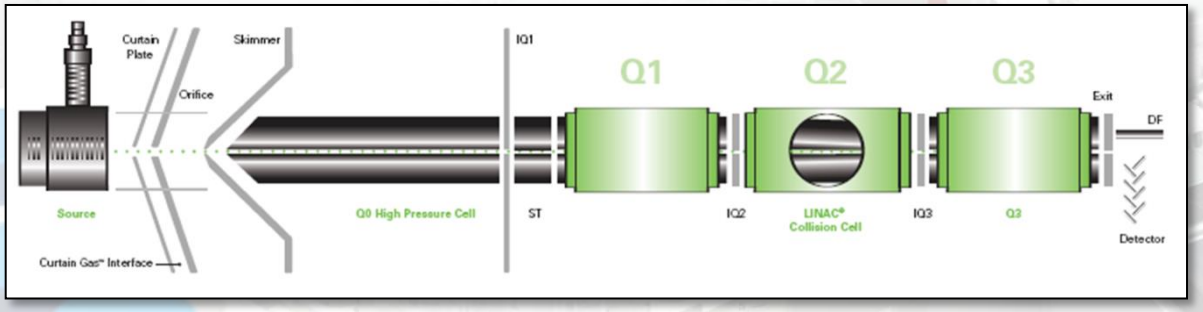
Name	*Group*
Amoxicillin	Penicillins
Bacitracin	Polypeptides
Carbadox	Quinoxalines
Chloramphenicol	Phenicols
Chlortetracycline	Tetracyclines
Ciprofloxacin	Quinolones
Doxycycline	Tetracyclines
Enrofloxacin	Quinolones
Flavomycine (Flavophospholipol)	Glycolipide
Florfenicol	Phenicols
Flumequine	Quinolones
Lasolacid	Ionophores coccidiostats
Lincomycin	Lincosamides
Maduramycin	Ionophores coccidiostats
Marbofloxacin	Quinolones
Monensin	Ionophores coccidiostats
Nalidixic acid	Quinolones
Narasin	Ionophores coccidiostats
Olinquadox	Quinoxalines
Oxolinic acid	Quinolones
Oxytetracycline	Tetracyclines
Penicillin G	Penicillins
Penicillin V	Penicillins
Robenidine	(coccidiostats)
Salinomycin	Ionophores coccidiostats
Spiramycin I	Macrolides
Sulfadiazine	Sulfonamides
Tetracycline	Tetracyclines
Tilmicosin	Macrolides
Trimethoprim	Diaminopyrimidines
Tylosin A	Macrolides
Valnemulin	Pleurotumilins
Virginiamycin M1	Streptogramins

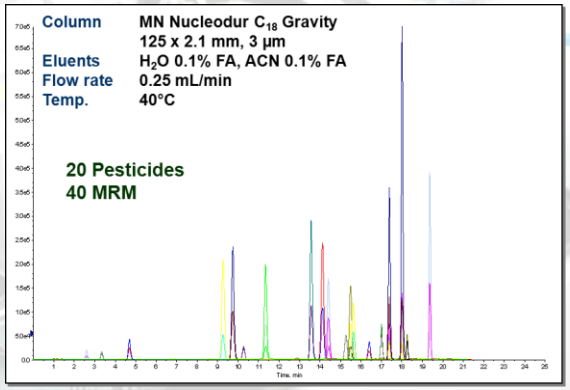
Method development and validation

- Liquid Chromatography and tandem Mass Spectrometry (LC-MS/MS)
  - Principle
    - *Separation of analytes by reverse-phase HPLC*
    - *Desolvataion and ionisation by Electrospray (+/-)*
    - *Selective detection in Multiple Reaction Monitoring*









- Developed methods
  - *2 Methods (polarity +/-)*
  - *3 Labelled internal standards (IS)*
  - *Detection limits 1-60 ng/mL*

Positive mode
27 Compounds
2 IS

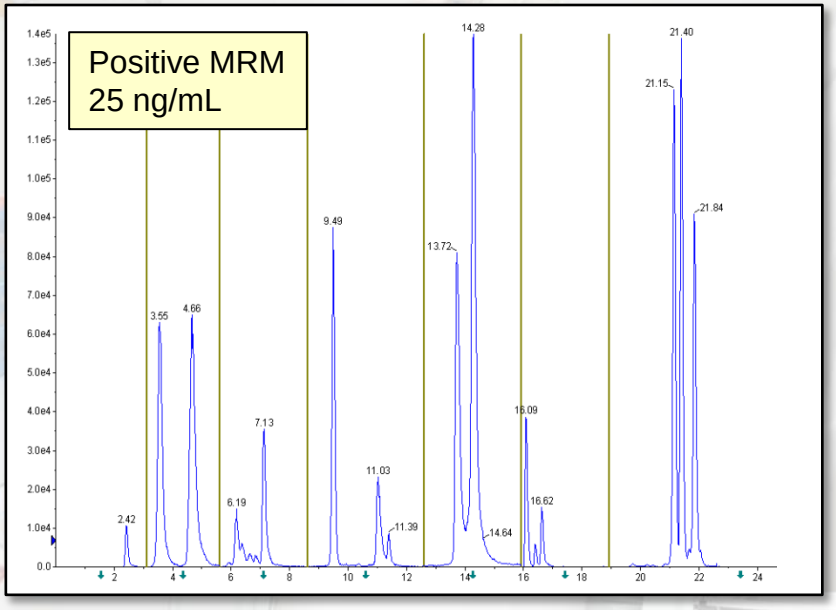
Quinolaxines
Lincosamides
Sulfonamides
Quinolones
Diaminopyrimidines
Tetracyclines
Macrolides
Polypeptides
Pleurotumilins
Streptogramins
Ionophores

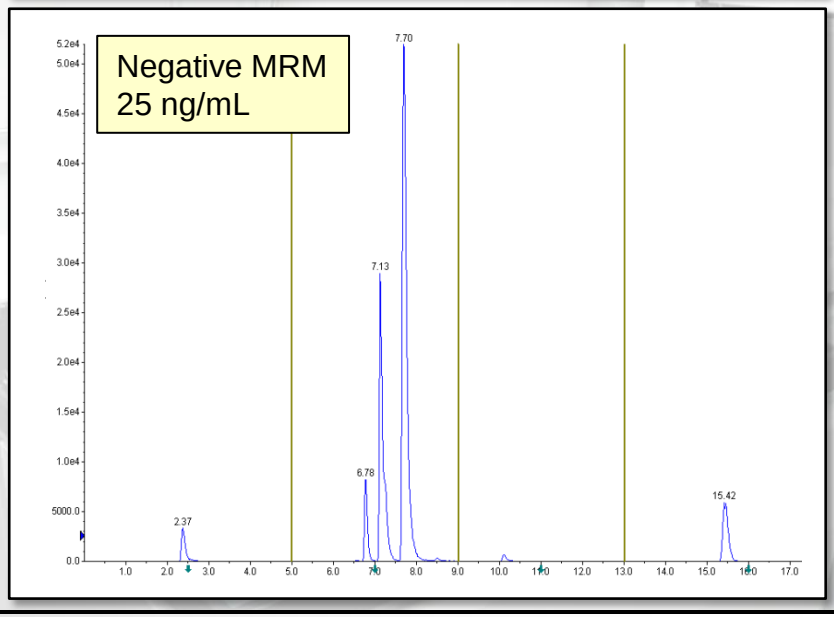
d_4-Sulfadiazin
$^{13}C_3$-Flumequine

Negative mode
7 Compounds
1 IS

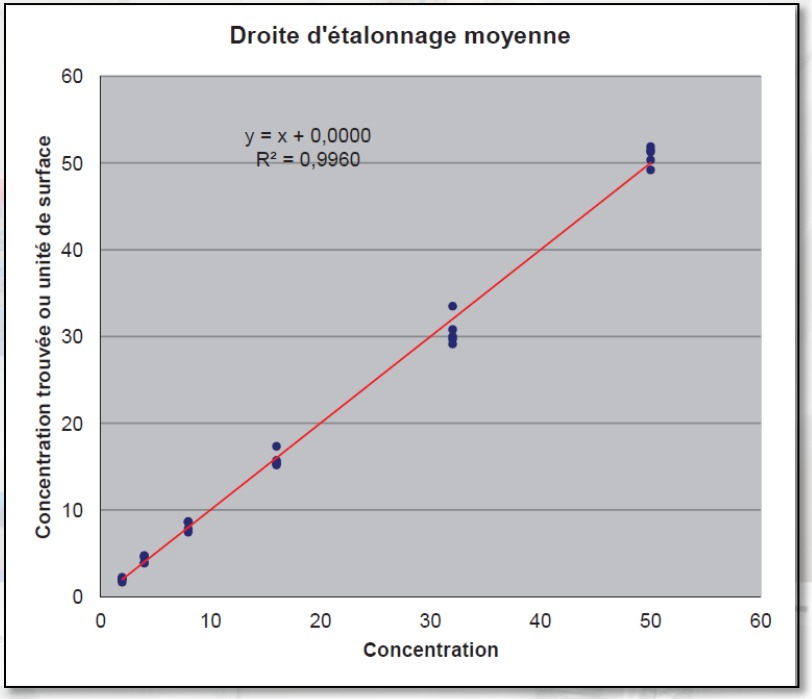
Penicillins
Phenicols
Flavomycin
Maduramycine

d_5-Chloramphenicol





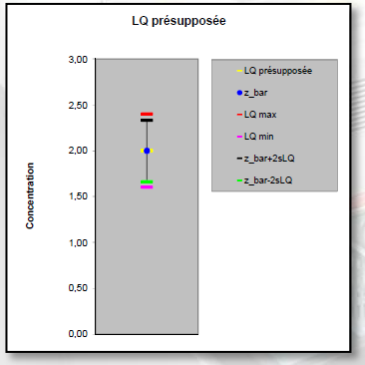
- Validation
  - *Linearity ranges*
    - Based on calibration replicates (5x6)
    - Example: Amoxicillin

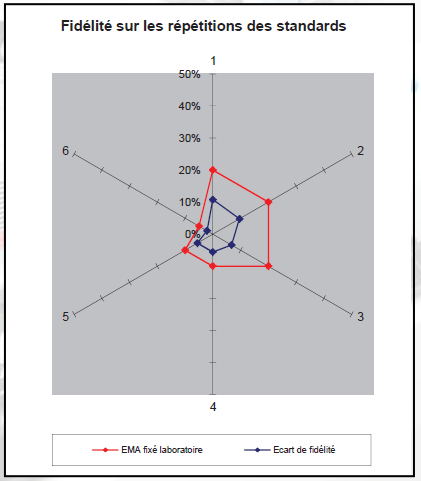


Plan d'étalonnage						
Niveaux	**1**	**2**	**3**	**4**	**5**	**6**
Concentrations / unités	2,000	4,000	8,000	16,000	32,000	50,000
Essai 1	2,156	3,939	7,472	15,722	33,495	49,215
Essai 2	2,274	4,791	8,688	15,211	29,132	51,904
Essai 3	1,687	3,908	7,838	17,377	30,847	50,343
Essai 4	2,063	4,696	8,653	15,356	29,745	51,487
Essai 5	2,098	4,506	8,587	15,505	30,013	51,292

- *Quantification limits*
  - Based on multiple injections at the estimated LoQs

Répétitions	R1	R2	moyenne	variance
Jour 1	2,16	1,92	2,038	0,028
Jour 2	2,27	2,05	2,162	0,025
Jour 3	1,69	1,93	1,810	0,030
Jour 4	2,06	1,88	1,969	0,018
Jour 5	2,10	1,91	2,005	0,017





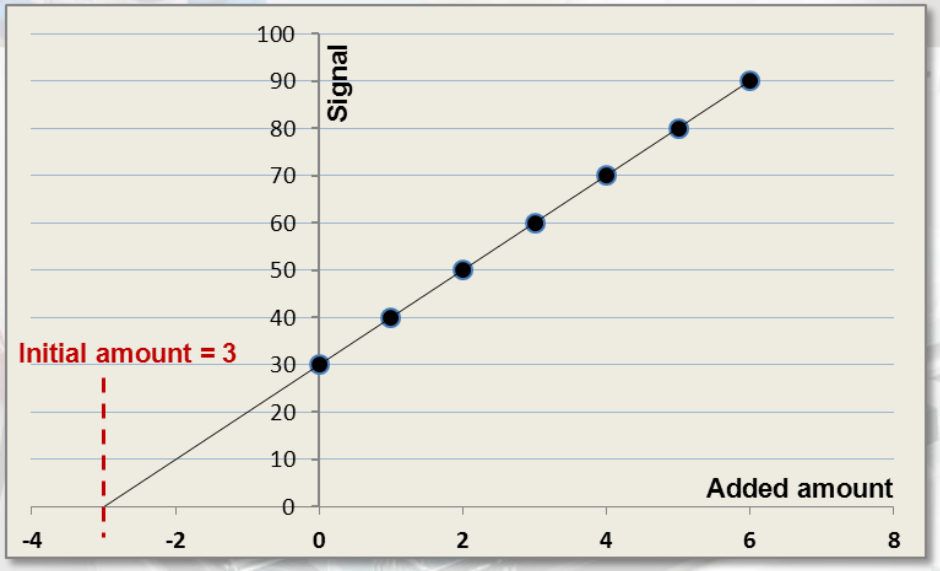
Extraction protocol

Challenges

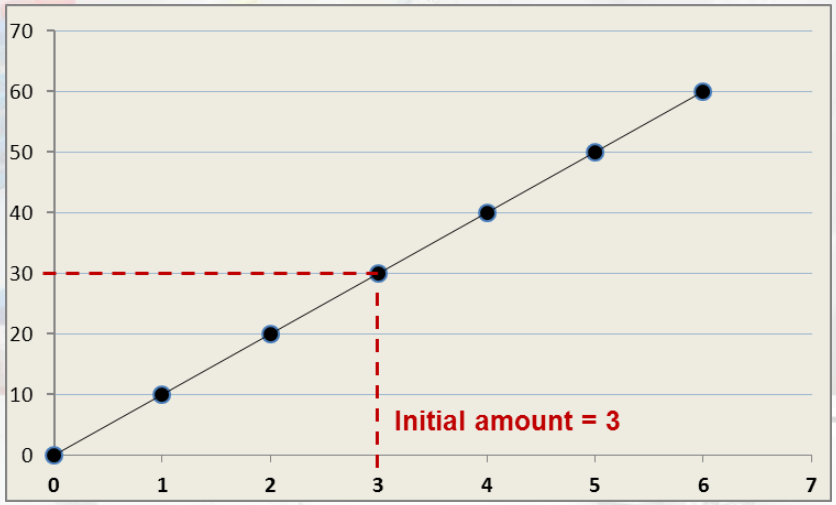
- *Extract the highest number of compounds of interest*
- *Obtain high and stable extraction yields*
- *Eliminate the most part of matrix compounds*
- *Deal with very different matrices*

Strategies

- *First approach: Matrix-matched calibration*
  - Based on standard addition in the sample
  - High sensitivity
  - Takes into account all the matrix effects
  - More lab work
  - **Very time-consuming**



- *Final protocol: Dilution/Cleanup and external calibration*
  - Based on classical quantification using separated standards
  - Lower sensitivity
  - Matrix effects reduced by dilution
  - Easy and robust protocol
  - Less time-consuming



Sample

Solid-Liquid Extraction
MeOH/ACN/McIlvaine buffer/EDTA 0.5M

Extract clean up
Primary-Secondary Amine solid sorbent

Dilution

LC-MS/MS analysis

Data treatment

Extraction yields

- Calculated based on spiked matrices (piglet feed)
- 3 Spiking levels analyzed in duplicates on 5 different days (30 values)
- Example: Amoxicillin

Determination of recovery yields and correction factors

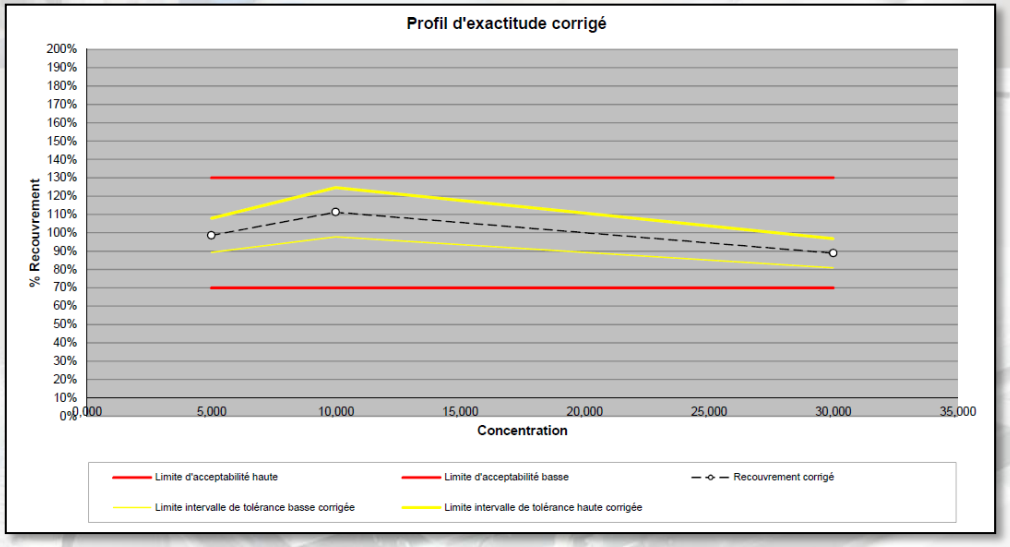
Valeurs de référence	A - bas		B - moyen		C - haut	
Jour 1	5,00	5,00	10,00	10,00	30,00	30,00
Jour 2	5,00	5,00	10,00	10,00	30,00	30,00
Jour 3	5,00	5,00	10,00	10,00	30,00	30,00
Jour 4	5,00	5,00	10,00	10,00	30,00	30,00
Jour 5	5,00	5,00	10,00	10,00	30,00	30,00
Valeur de référence moyenne	5,000		10,000		30,000	

Valeurs retrouvées	A - bas		B - moyen		C - haut	
Jour 1	3,21	3,68	7,57	6,79	18,52	17,37
Jour 2	3,09	2,98	7,12	6,67	18,27	17,26
Jour 3	3,31	3,37	6,95	8,09	17,49	16,81
Jour 4	3,07	3,35	8,48	7,22	15,84	18,07
Jour 5	3,44	3,37	7,78	7,47	19,84	18,23
Valeur moyenne retrouvée	3,286		7,414		17,770	

Paramètres d'exactitude		A	B	C
Nombre de jours	n	5	5	5
Nombre de répétitions par jours	r	2	2	2
Variance de répétabilité	$S\ repet^2$	12,612	38,195	11,576
Variance des moyennes	$s(zi)^2$	11,022	14,229	7,459
Variance inter-séries	sB^2	4,716	0,000	1,671
Variance de fidélité intermédiaire	sFI^2	17,328	38,195	13,247
Ecart type de fidélité intermédiaire	sFI	4,163	6,180	3,640
	2 sFI	8,325	12,360	7,279
CV % de fidélité intermédiaire	sFI	6,3%	8,3%	6,1%
Rendement par niveau %	Rdt	**65,7**	**74,1**	**59,2**

Rendement moyen %	f corr
66,4	1,51

Accuracy profile (stability of extraction yields over the analytical range)



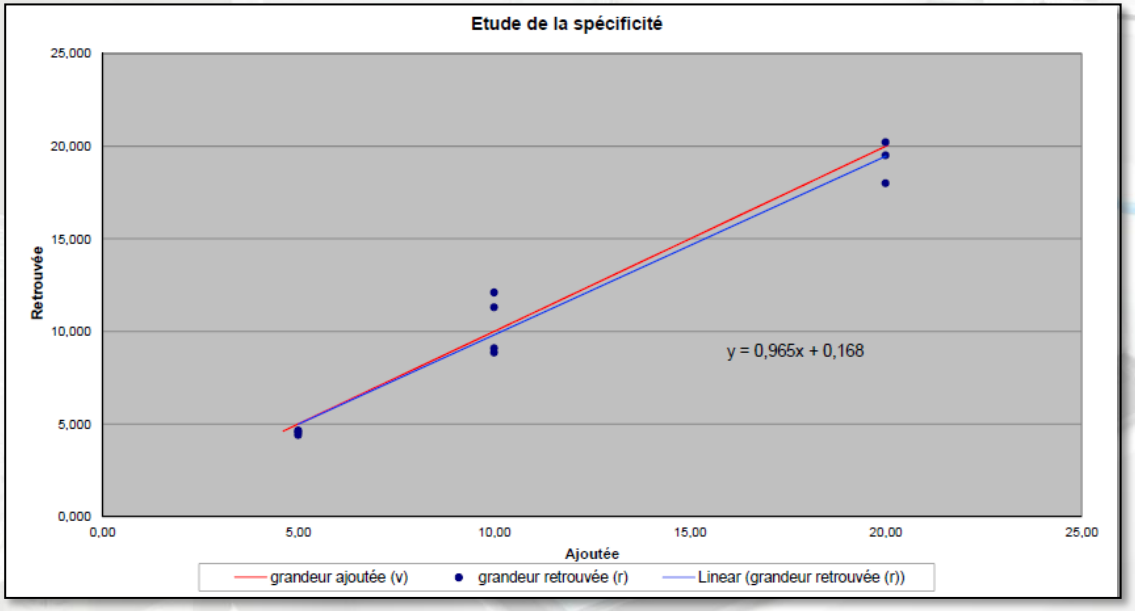
Specificity

- Checks the ability of the method to be applied on different matrices
- Analysis of spiked samples based on various feedstuffs (piglet, lamb, cattle)

- Example: Robenidine

Essais	désignation	teneur connue avant ajout	grandeur ajoutée (v)	grandeur mesurée	grandeur retrouvée (r)
Essai 1	Vormast DSM	0	20,00	20,20	20,20
Essai 2	Vormast DSM	0	10,00	9,09	9,09
Essai 3	Vormast DSM	0	5,00	4,64	4,64
Essai 4	Lammstar	0	20,00	19,50	19,50
Essai 5	Lammstar	0	10,00	12,10	12,10
Essai 6	Lammstar	0	5,00	4,55	4,55
Essai 7	K11	0	20,00	18,00	18,00
Essai 8	K11	0	10,00	11,30	11,30
Essai 9	K11	0	5,00	4,40	4,40
Essai 10	Vormast DSM	0	10,00	8,85	8,85

Paramètres	valeurs observées	valeurs critiques
Nombre de mesures :	10	
Sensibilité (pente de la droite) c1 :	0,965	1,000
Ecart type sur la sensibilité s(c1) :	0,066	
Blanc (ordonnée à l'origine) c0 :	0,168	0,000
Ecart type sur le blanc s(c0):	0,853	
Test de signification de la pente :	0,535	3,355
	Acceptable	
Test de signification de l'ordonnée à l'origine :	0,197	3,355
	Acceptable	
La méthode est spécifique		



Overall results

- From the original list of 34 targeted antibiotics, 7 have not been validated
- 27 Validated compounds, with quantification limits from 0.02 to 1.2 µg/g

	Not validated	*Validated*
Linearity	*Flavophospholipol*	*33*
Limits of quantification	*Danofloxacine*	*32*
Extraction yields, accuracy	*Tetracyline* *Oxytetracycline* *Chlortetracycline*	*29*
Specificity	*Tilmocosin* *Salynomycin*	*27*

Antibiotique	Domaine de Linéarité (ng/mL)	Limite de quantification (µg/g)
Amoxicillin	[2-50]	0,05
Bacitracin (Zinc)	[45-200]	0,50
Carbadox	[2-20]	0,04
Chloramphenicol	[1-32]	0,02
Ciprofloxacin	[60-200]	1,20
Doxycycline	[4-32]	0,08
Enrofloxacin	[60-200]	1,20
Florfenicol	[1-32]	0,02
Flumequine	[1-16]	0,02
Lasalocid A	[1-16]	0,02
Lincomycin	[1-20]	0,02
Maduramicin	[1-32]	0,02
Marbofloxacin	[60-200]	1,20
Monensin	[1-16]	0,02
Nalidixic acid	[1-16]	0,02
Narasin	[1-16]	0,02
Olaquindox	[2-20]	0,07
Oxolinic acid	[1-16]	0,02
Penicillin G	[2-50]	0,06
Penicillin V	[2-32]	0,05
Robenidine	[2-20]	0,02
Spiramycin I	[60-200]	0,80
Sulfadiazin	[2-20]	0,04
Trimethoprim	[2-20]	0,07
Tylosin	[1-16]	0,02
Valnemulin	[2-20]	0,04
Virginamycin M1	[2-20]	0,07

Conclusions

- Method performance
  - Highly sensitive and specific method for the analysis of 27 antibiotics, over a wide range of chemical families
  - Quantitative measurement
  - Robustness demonstrated through the validation process
  - Versatile and efficient extraction protocol
  - Interesting complement to screening methods based on microbiology
- Limits
  - Extraction yields needs to be evaluated for each unknown matrix
  - Equipment needed (LC-MS/MS)
  - Remains time-consuming and expensive for a low number of samples
  - Hard to implement for punctual unexpected controls