Development and validation of a multi-residue method for veterinary drugs using LC-HRMS

Eloi Marilleau[1,2], Yvan Gru[1], Ronan Colin[1], Renwei Hu[1], David Le Scornec[1], Thierry Paterour[1], Nicolas Cimetière[2]

[1] Inovalys, Chemistry R&D department, F-56000 Vannes, France

[2] Univ Rennes, Ecole Nationale Supérieure de Chimie de Rennes, CNRS, ISCR (Rennes Institute of Chemical Sciences), UMR 6226, F-35000 Rennes, France

Introduction and goals

Consumer health is at risk when veterinary drugs residues are found in **animal-based food matrices** like milk, eggs, and muscle. More and more **multi-residue approaches** using **liquid chromatography** coupled to **high-resolution mass spectrometry** (**LC-HRMS**) are being developed to check that residue levels do not exceed the legal limitations imposed by Europe for banned and unbanned substances. High-resolution techniques have the advantage of producing ever-more-complete data thanks to Data Independent Analysis method with high selectivity and sensitivity. Therefore, these methods have the capacity to carry out **retrospective analysis** for the research of new substances. The French reference laboratory for food control (ANSES Fougères) has expressed its intention to implement this kind of analysis method for the **national surveillance plans** for monitoring veterinary drugs from 2025.

Method development and optimization → Validation according to the new regulation **(EU) 2021/808** → Implementation for **national surveillance plans**

Materials and methods

Instrumentation :

- **Liquid chromatography :** ELUTE UHPLC (BRUKER)
- **Mass spectrometer :** Q-TOF Impact II (BRUKER)
- **Ion source :** VIP-HESI (BRUKER)

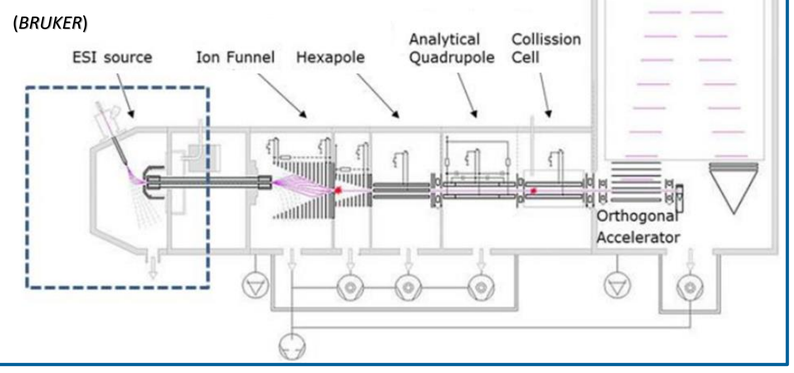
Acquisition method :

- **F**ull **S**can (**FS**): mass range m/z **30 - 1200**
- **B**road**B**and **C**ollision-**I**nduced **D**issociation (**bbCID**) : non-targeted fragmentation on m/z range **30 - 1200**

Acquisition speed of 3 Hz with sequential analysis of a FS spectrum and a bbCID spectrum.
Collision energy : 24 eV and 36 eV

Time-of-flight (TOF) mass spectrometer scheme :

(*BRUKER*)



Molecules and databases

Antibiotics : Penicillins, Cephalosporins, Cyclins, Sulfamides, Quinolones, Macrolides, ...

Antiparasitics : Anthelminthics, Avermectins, Organophosphates, Pyrethroids, Benzoylureas, Nitroimidazoles, ...

Sedatives

Hormonal drugs

Alkaloids

Anticoccidials

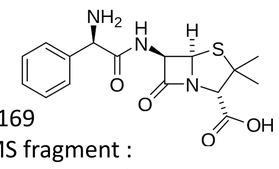
Non-steroidal anti-inflammatory drugs (NSAID)

Total : **257 targeted substances**

Two databases :

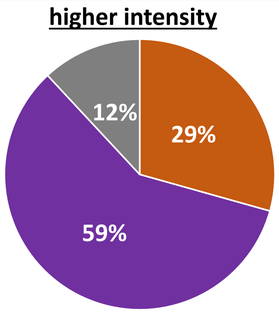
- Positive ionisation (**235**)
- Negative ionisation (**22**)

Example Ampicillin :
$C_{16}H_{19}N_3O_4S$
R_t = 4.71 min
m/z $[M+H]^+$ = 350.1169
Characteristic MS/MS fragment :
C_7H_7N (m/z $[M+H]^+$ = 106.0651)

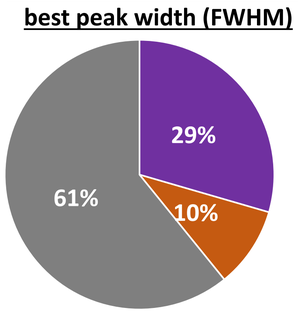


Chromatographic conditions

Proportion of molecules with higher intensity

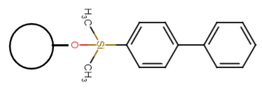


Proportion of molecule with best peak width (FWHM)



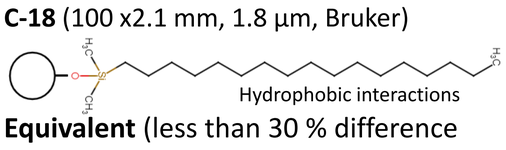
Chromatographic LC column

- **Biphenyl** (100 x 2.1 mm, 1.8 µm, RESTEK) ☑ Best results



π-π interactions

- **C-18** (100 x2.1 mm, 1.8 µm, Bruker)

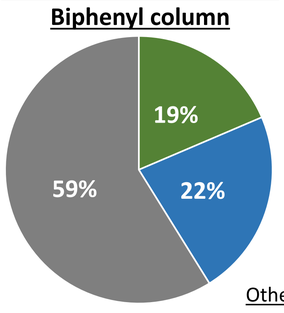


Hydrophobic interactions

- **Equivalent** (less than 30 % difference between columns)

Mobile phase conditions (positive mode)

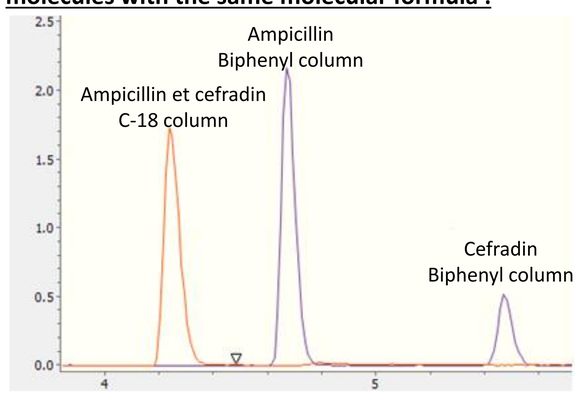
Sensitivity comparison between two mobile phases on the Biphenyl column



- H_2O/CH_3OH, 0.1 % Formic acid and 0.5 mM ammonium formate → - $[M + NH_4]^+$ Adducts, + Penicillins, + NSAID, + Benzimidazoles ☑ Best families compromise
- H_2O/CH_3OH, 0.01 % Formic acid and 5 mM ammonium formate → + $[M + NH_4]^+$ Adducts, + Cyclins, + Anticoccidials, + Alkaloids
- Equivalent (less than 30 % difference between both conditions)

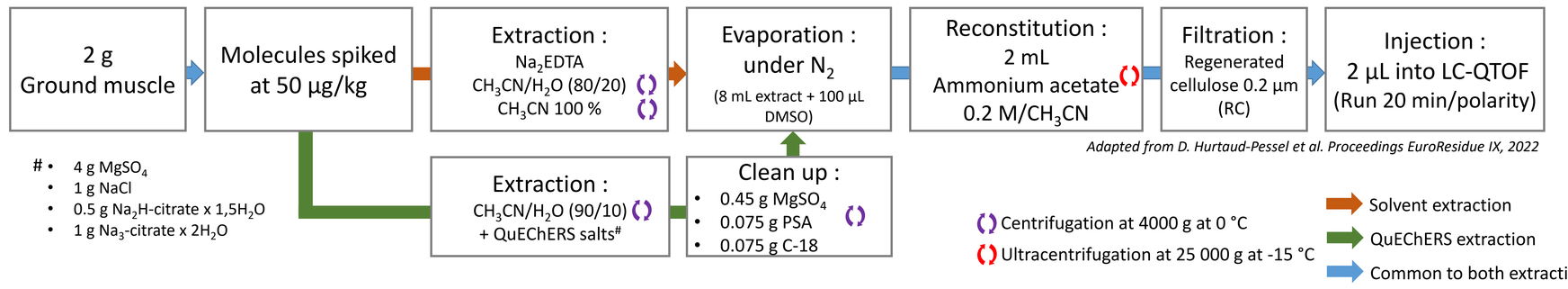
Other tested condition : H_2O/CH_3OH 0.1 % Formic acid → - - $[M + NH_4]^+$ Adducts, + + Penicillins

Example of chromatographic separation of two molecules with the same molecular formula :



Extraction conditions

Extraction strategies comparison



Adapted from D. Hurtaud-Pessel et al. Proceedings EuroResidue IX, 2022

QuEChERS: poor absolute recovery for penicillins, cephalosporins and cyclins

Solvent extraction: absolute recovery between 19% (Ticarcillin) and 111% (DMF-dimethylformamidin)

Method validation

(EU) 2021/808 : expected criteria for screening and confirmation methods

Method	Screening		Confirmation	
	Qualitative	Quantitative	Qualitative	Quantitative
Identification points			X	X
CCα			X	X
CCβ	X	X		
Trueness		X		X
Precision		X		X
Selectivity/specificity	X	X	X	X

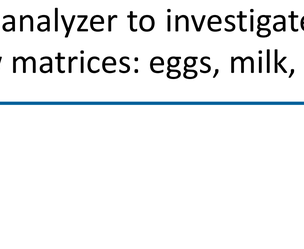
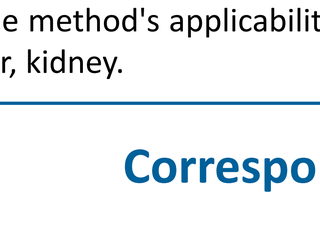
Validation plan

			Calibration range						Validation samples				
Day	Matrix	Concentration (µg/kg)	0	1	10	50	100	250	0	1	10	50	100
1	Poultry muscle	Number of extracts	1	1	1	1	1	1	7	7	7	7	7
2	Pork muscle	Number of extracts	1	1	1	1	1	1	7	7	7	7	7
3	Bovine muscle	Number of extracts	1	1	1	1	1	1	7	7	7	7	7
								Total	21	21	21	21	21

Validation results

Proportion of molecules at concentration level :

- 1 µg/kg
- 10 µg/kg
- 50 µg/kg
- 100 µg/kg
- Not determinated

	Qualitative	Quantitative	Conclusion
Screening			At the 10 µg/kg reference value (below the half mean MRL*) : • 84 % are qualitatively screened • 62 % are quantitavely screened 5 % still remain unscreenable at any concentration *MRL : Maximum Residue Limit
Confirmation			At the 10 µg/kg reference value : • 69 % are qualitatively confirmed • 53 % are quantitavely confirmed 11 % are not confirmed at any concentration

Conclusions and perspectives

This work has resulted in a rapid and sensitive method for screening and confirming of veterinary drug residues in muscle. The results showed that the method performed well for both qualitative and quantitative screening with 62 % of substances quantified at a concentration level of 10 µg/kg, whose 40 % at 1 µg/kg. Moreover, the qualitative and quantitative confirmation results showed that 69 % and 53 % of molecules reached 10 µg/kg level, respectively. Furthermore, these results will be compared to those produced by the ANSES laboratory with an Orbitrap analyzer to investigate the method's applicability on multiple high-resolution mass spectrometry technologies. Subsequently, further validations will be carried out on new matrices: eggs, milk, liver, kidney.

Acknowledgment

We thanks BRUKER and A. Verdu for the opportunity to outline our results.

Corresponding author's email

eloi.marilleau@inovalys.fr