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Transition to High-resolution Accurate Mass (HRAM) technology for Quantitative Screening of Antimicrobials in Food of animal origin

What is High Resolution Accurate Mass (HRAM) ?

Resolution is the ability to distinguish between peaks of similar mass. Higher resolution allows for more accurate mass measurements by providing better separation of peaks.

Accuracy describes how close a measured mass is to the true or expected mass. Accuracy is crucial for accurate identification of molecules.

Why move to HRMS analysis?

Multiple LC-QQQ screening methods based on family class

Microbiological based screening methods e.g. 6-plate

Multiple methods being used for residue screening of antibiotics, utilising a lot of laboratory resources. Single method with both quantitative and confirmation capability preferred.

Microbiological tests while fast had limited specificity and sensitivity.

Only identify class.

Quantitation not possible.

False positive and false negatives possible

All elements have a nominal and an exact mass.

Element	Nominal mass	Exact mass	Mass defect
Carbon ^{12}C	12	12.000000	0.0000000
Hydrogen ^{1}H	1	1.0078250	0.0078250
Nitrogen ^{14}N	14	14.0030740	0.0030740
Oxygen ^{16}O	16	15.9949146	- 0.0050854
Chlorine ^{35}Cl	35	34.9688527	- 0.0311473
Fluorine ^{19}F	19	18.9984032	- 0.0015968
Sulfur ^{32}S	32	31.9720707	- 0.0279293

LRMS Versus HRMS

LRMS

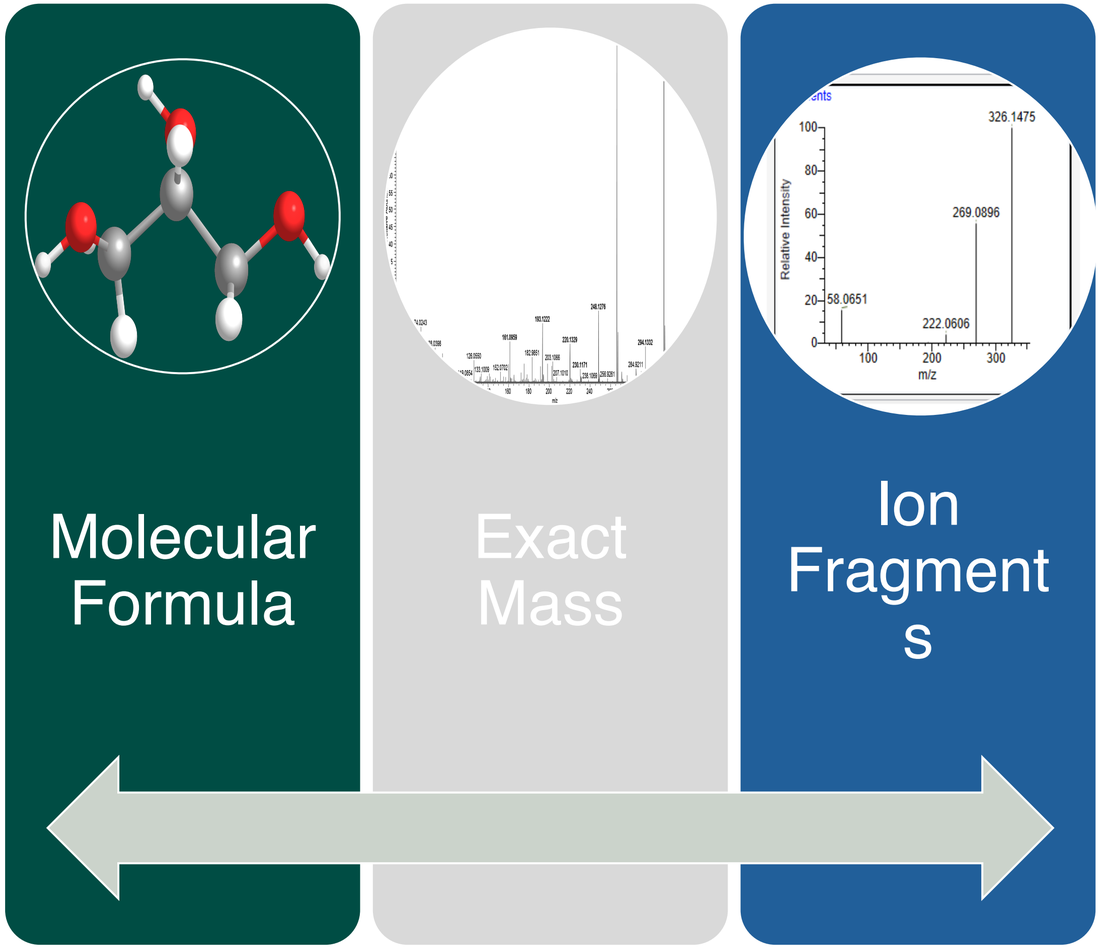
- Nominal mass
- Low resolution <10,000
- Time consuming to tune individual compounds
- Only scans for what you ask

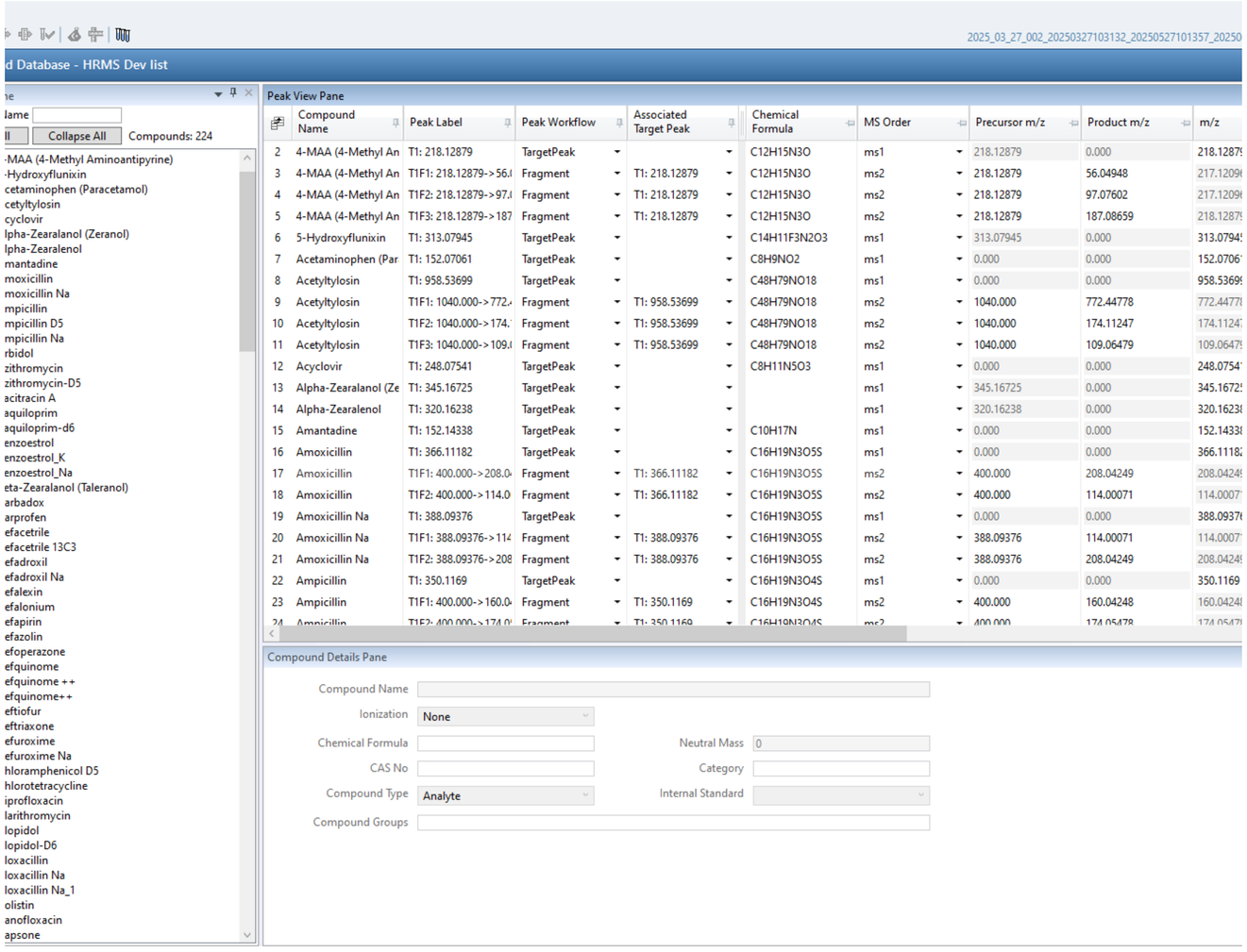
HRMS

- Exact Mass
- High resolution up to 140,000
- Easy set up - no need to tune individual compounds
- Full scan and retrospective analysis

Development of a single HRMS Screening method - TM231

Step 1: Preparation of compound database and instrument Methods





Thermo Vanquish LC System Parameters TM231

Thermo Accucore VDX Column 2.1mm x 100mm 2.6um

Mobile Phase A: 0.1% Formic acid in Water

Mobile Phase B: Methanol

Gradient: t0 1%B, t2 1% B, t10 90%B, t10.1 98%B, t12 98%B

Flowrate: 0.3ml/min

Run time: 14mins

Injection Volume: 5ul (Positive Mode), 3ul (Negative Mode)

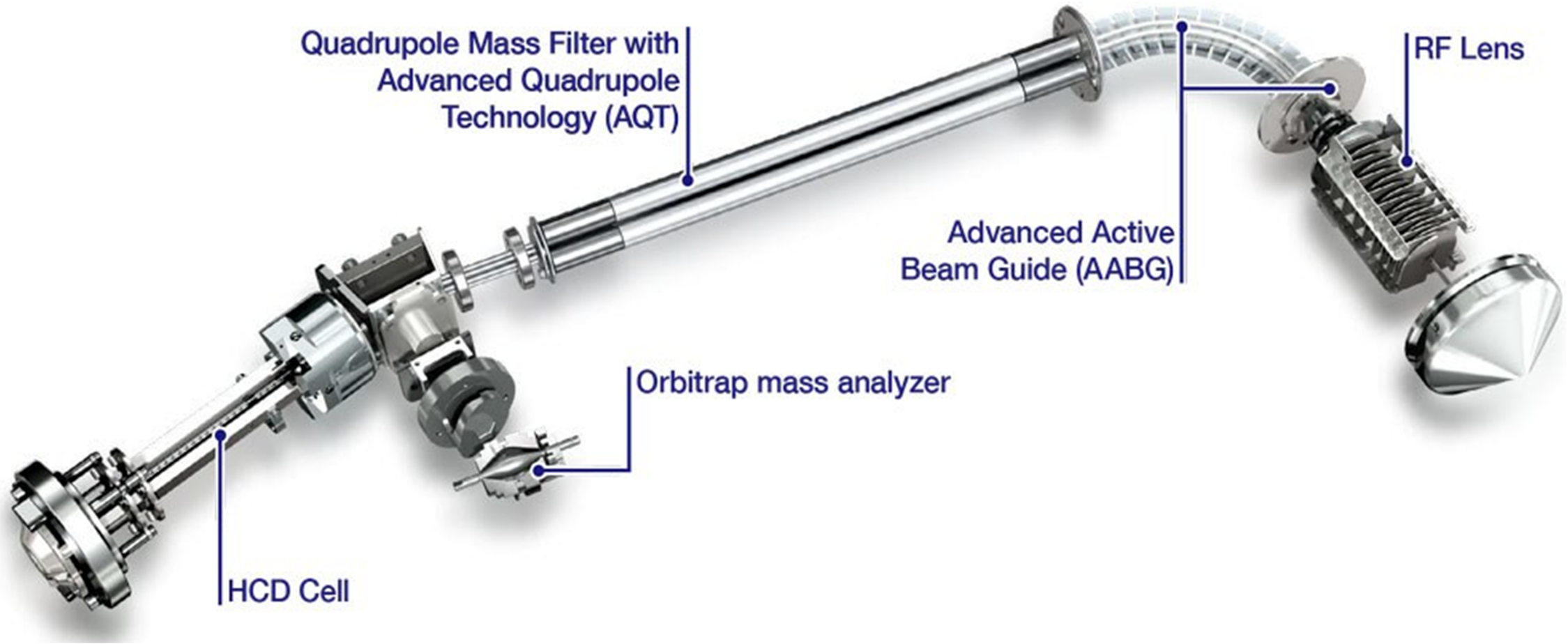
Q-Exactive Master Method parameters TM231

Positive Polarity

Negative Polarity

Full Scan MS	**DIA** Experiment	**DIA** Experiment	T-SIM
• Resolution 70,000 • Scan range 120-1200 m/z • AGC target 1e6	• Resolution 35,000 • (N)CE / stepped ce:15, 30, 60	• Resolution 17,500 • (N)CE / stepped ce:15, 30, 60	• Resolution 70,000

Q-Exactive Orbitrap



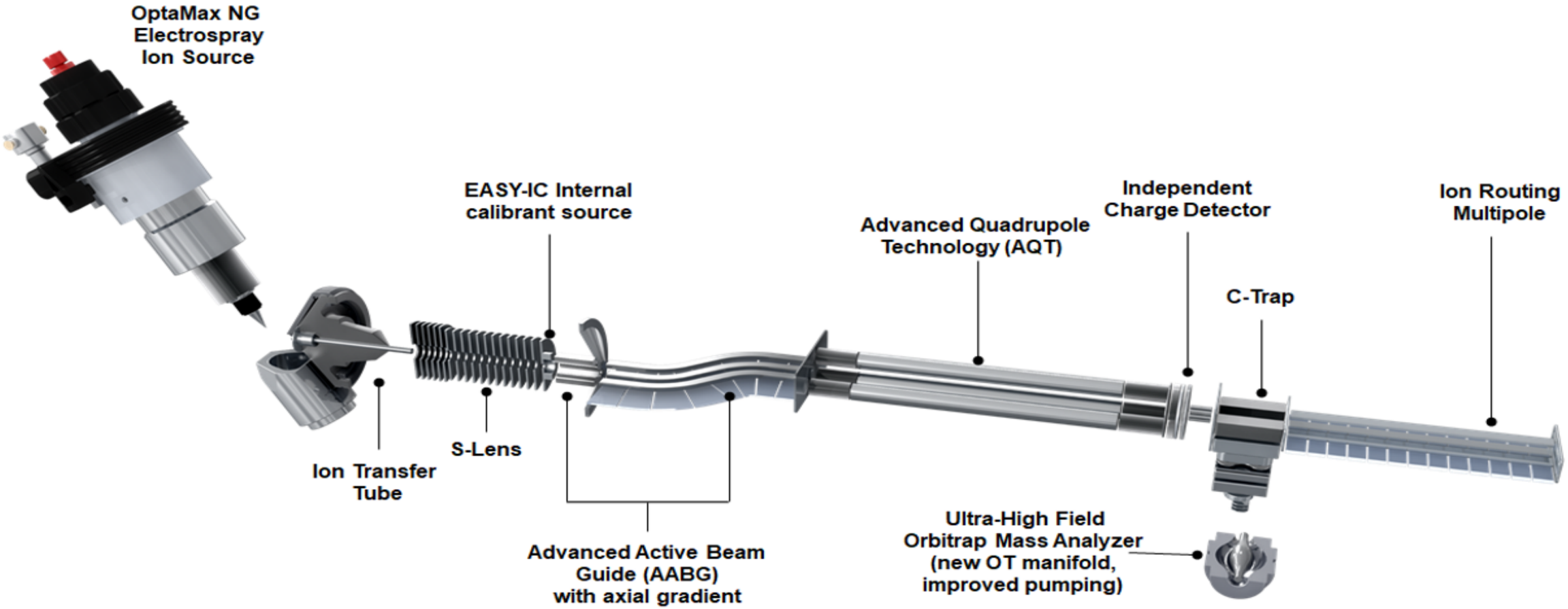
Exploris 120 Master Method Parameters TM231

Positive Polarity

Negative Polarity

Full MS	**DIA** Experiment	**DIA** Experiment	SIM
• Resolution 60,000 milk 120,000 eggs • Scan range 200-1200 m/z • AGC target standard	• Resolution 30,000 • CE: 15, 30, 60	• Resolution 15,000 • CE:15, 30, 60	• Resolution 120,000

Exploris 120 Orbitrap

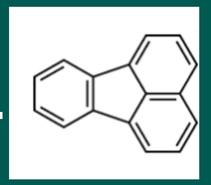


Key differences between Q-Exactive and Exploris systems

Q-Exactive resolution 17,500; 35,000, 70,000; & 140,000 (not used)
Exploris resolution 15,000; 30,000; 60,000 & 120,000

Exploris scan rate of 22Hz compared to Q-Exactive at 12 Hz.

Exploris has Easy-IC automatic Internal calibration (Flouranthene).



Different tuning solutions used for each instrument.

Updated Tune Software on Exploris.

Step 2: Sample Preparation

Weigh 2g Matrix into a centrifuge tube.

- Add 100µl internal standard, 500µl acetonitrile
- Vortex all tubes and allow to stand in the dark for 10mins.
- Add130µl 0.1M Na2EDTA and a ceramic mixer.

Extraction 1

- 4mls ACN/Water (80/20)
- Shake for 10mins
- Centrifuge 5mins @ 4000RPM
- Decant extract to glass evaporation tube containing 200ul of DMSO

Extraction 2

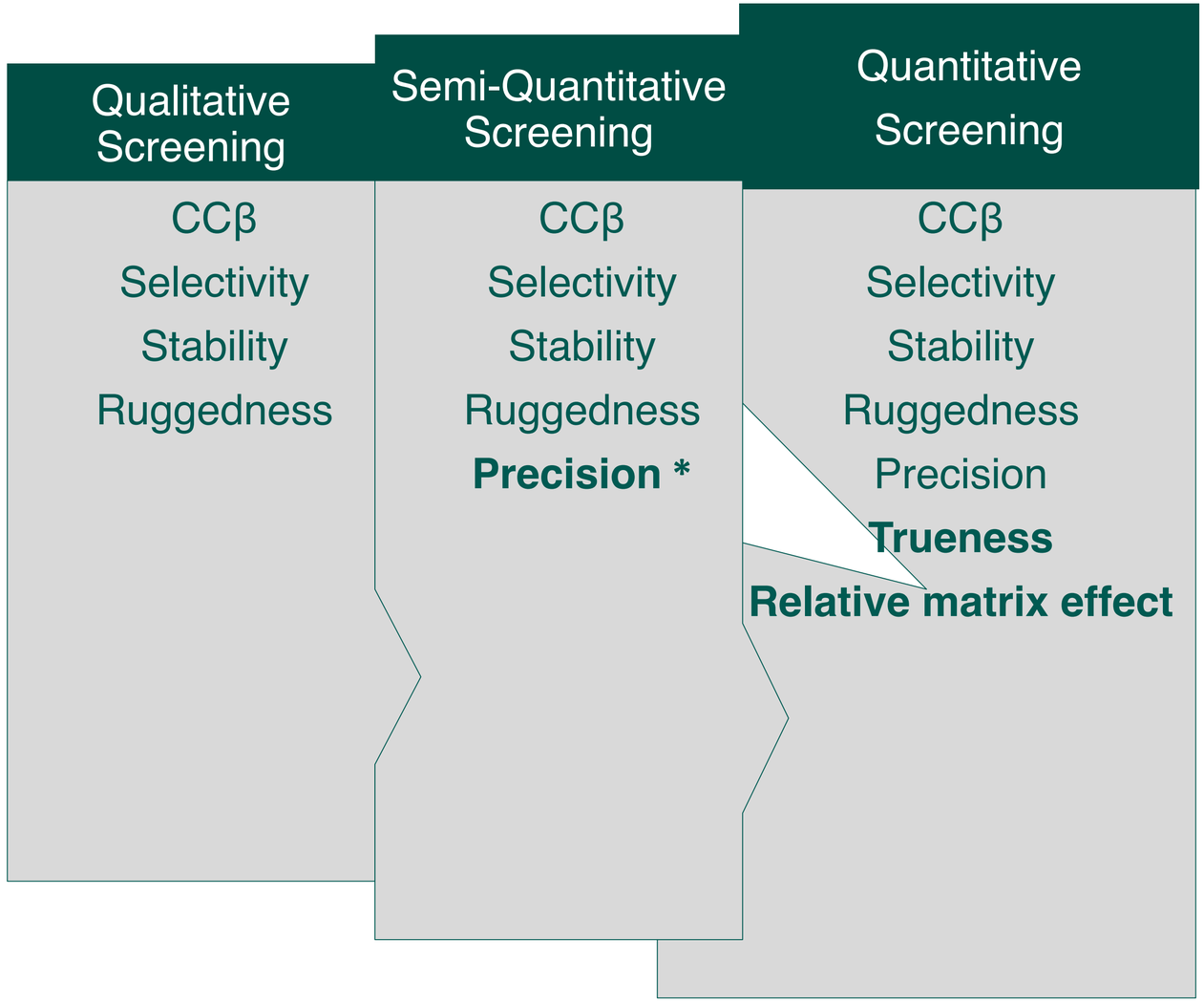
- 4mls ACN
- Shake for 10mins
- Centrifuge 5mins @ 4000RPM
- Add extract 2 to extract 1
- *Additional QuEChERS extraction for Milk samples*

Evaporate under Nitrogen to 200ul mark with Turbovap at 50°C

- Reconstitute with 800ul 80/20 (Ammonium acetate/ACN)
- Centrifuge 5mins @18000RPM and filter with 0.22um PVDF filter into LC vial

Step 3: Validation – Design of Experiments

Chapter 2 of Annex 1 to Commission Implementing Regulation (EU) 2021/808 describes the performance characteristics to be determined for the validation of analytical methods for the determination of pharmacologically active substances in food of animal origin.

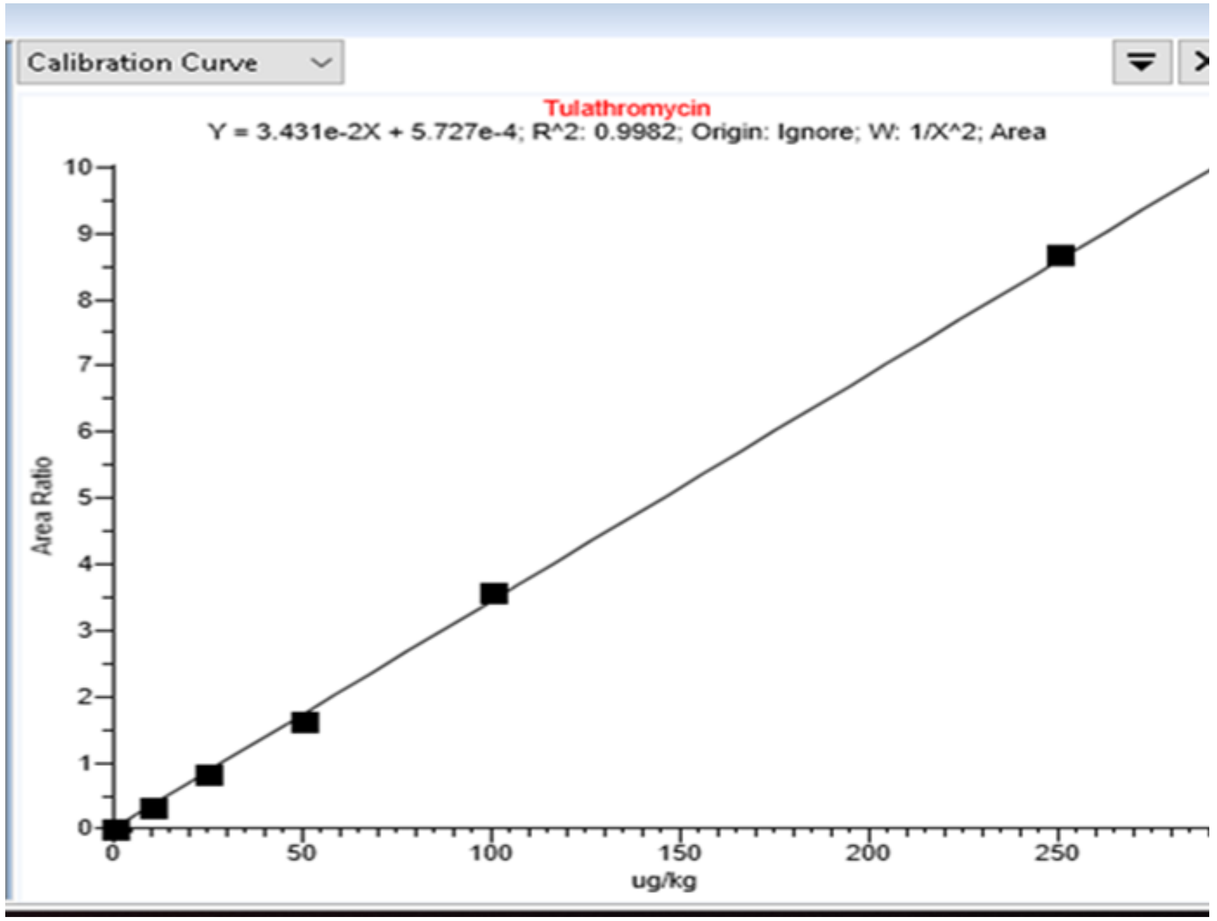


Several performance characteristics were determined simultaneously, and validation experiments were combined to minimise workload.

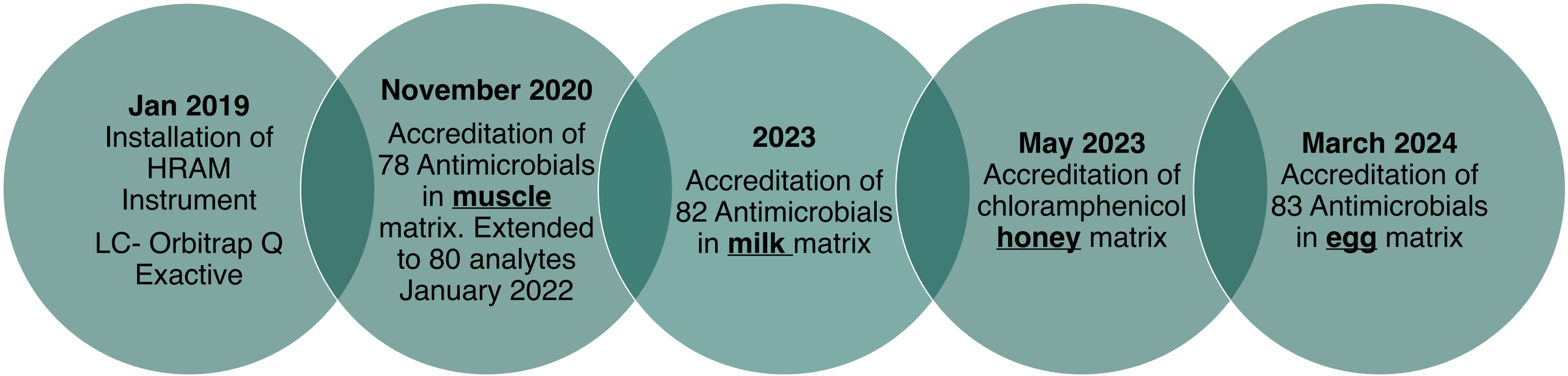
Validation Series	Muscle Samples	Milk Samples	Honey Samples	Egg Samples
Relative Matrix Effects	24	20	6	21
Specificity	21	21	21	21
Ruggedness	8 aliquots of one sample	8 aliquots of one sample	8 aliquots of one sample	8 aliquots of one sample
Series 1 Precision	7 5 fortification levels	7 5 fortification levels	7 4 fortification levels	7 3 fortification levels
Series 2 Precision	7 5 fortification levels	7 5 fortification levels	7 4 fortification levels	7 3 fortification levels
Series 3 Precision	7 5 fortification levels	7 5 fortification levels	7 4 fortification levels	7 3 fortification levels

Quantitative Analysis Capability

- Minimum 5-point calibration curve included for all substances
- Detection of authorised Antibiotics at 0.5*MRL or lower
- Detection of prohibited substances Chloramphenicol & Dapsone at 0.1*RPA/ MMPR



Validation/Accreditation Timeline



TM231 Muscle Validation

Bovine, Ovine, Porcine, Poultry, Cervine and Equine muscle included in validation.

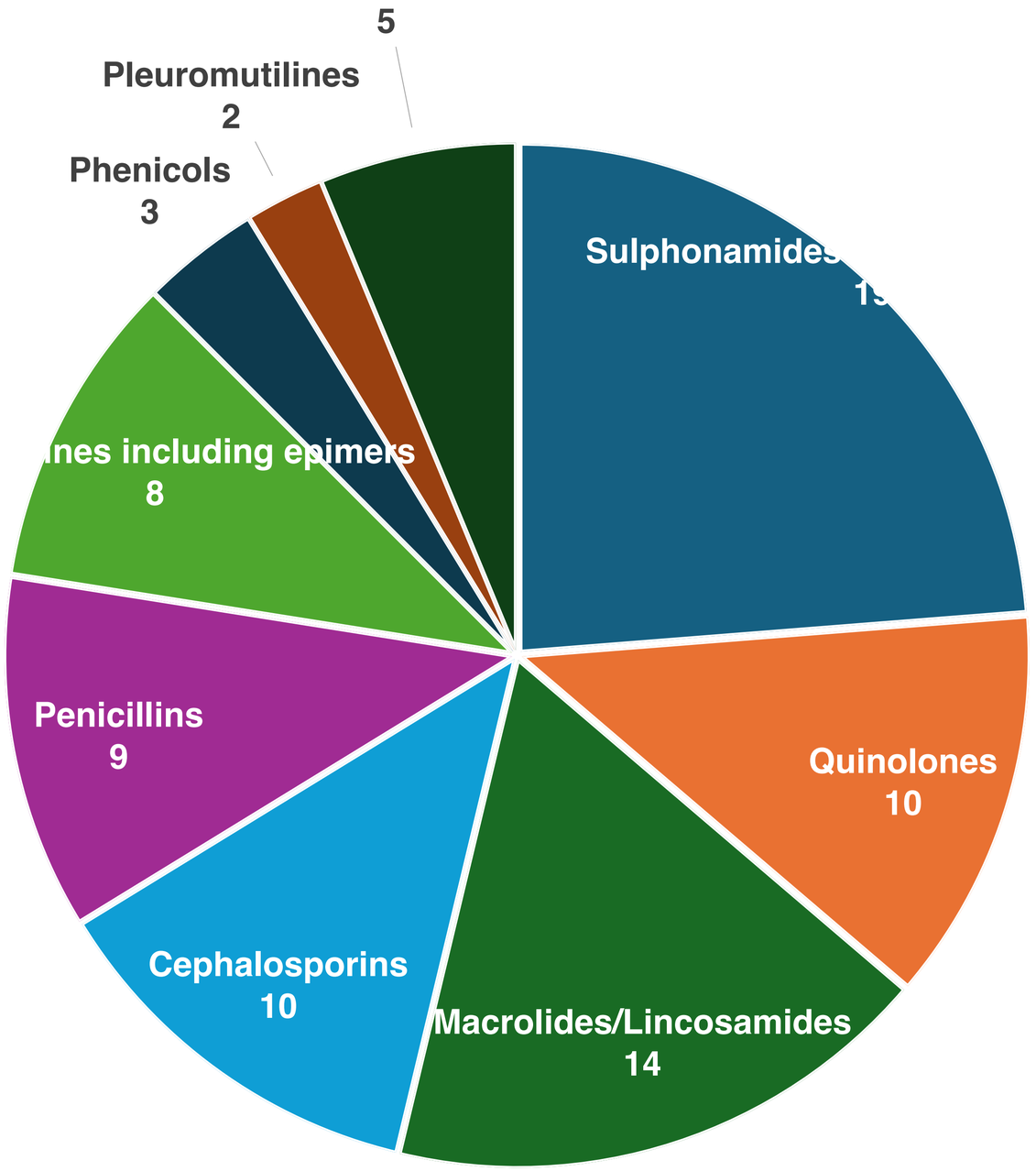
Method **validated** according to Commission Implementing Regulation (EU) 2021/808.

80 compounds from **10 family classes** passed validation

59 meet **Quantitative** criteria.

21 meet **Semi-quantitative** criteria.

Accredited by the Irish National Accreditation Board in 2022.



TM231 Milk Validation

Bovine, Ovine, Caprine Milk included

QuEChERS extract was used as an additional sample clean-up. This improved responses for low responding analytes.

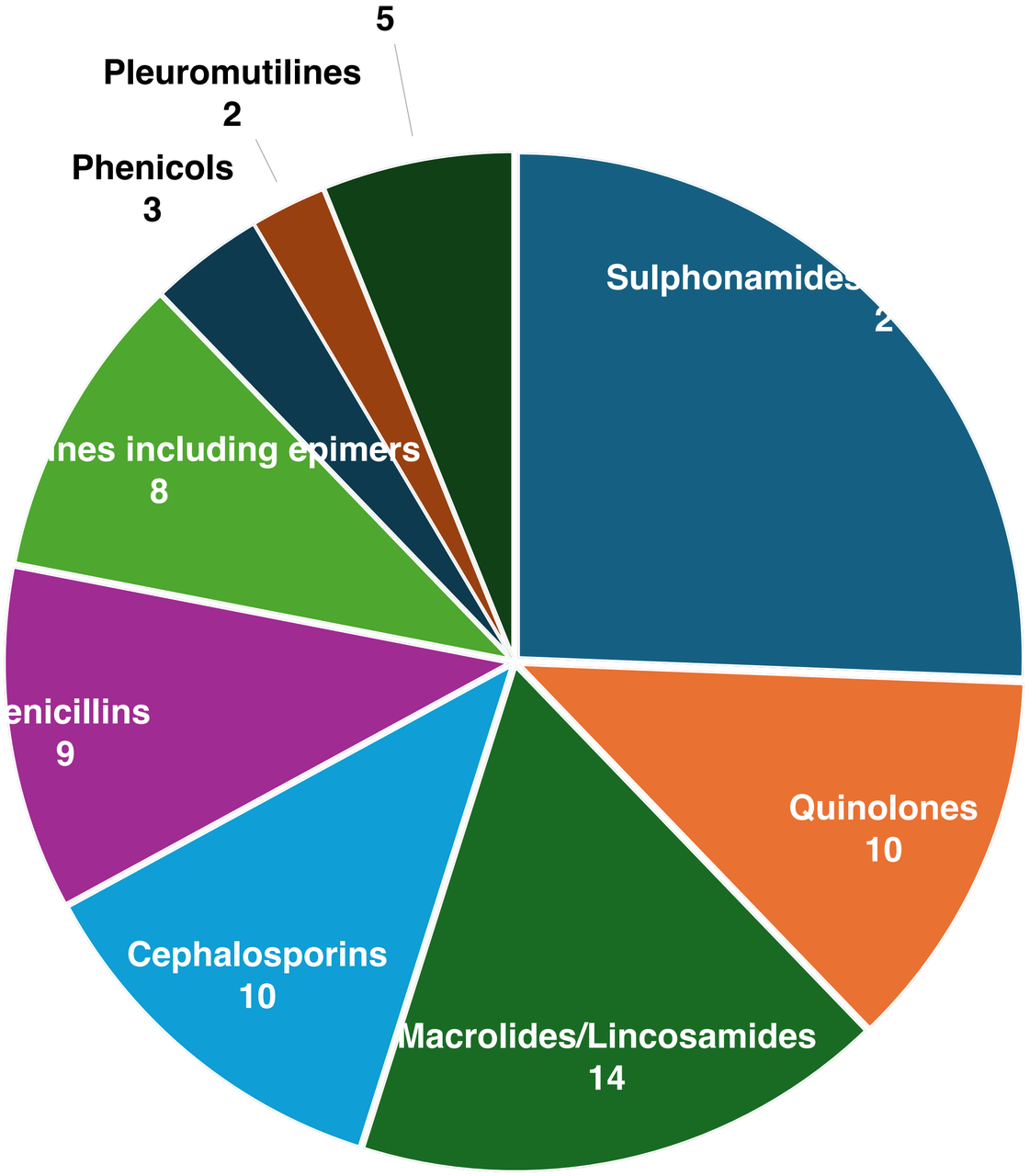
Method **validated** as a screening method according to Commission Implementing Regulation (EU) 2021/808.

82 compounds from **10 family classes** passed validation

30 meet **Quantitative** criteria

52 meet **Semi-quantitative** criteria

Accredited by the Irish National Accreditation Board in 2023



TM231 Egg Validation

Poultry, Quail eggs included

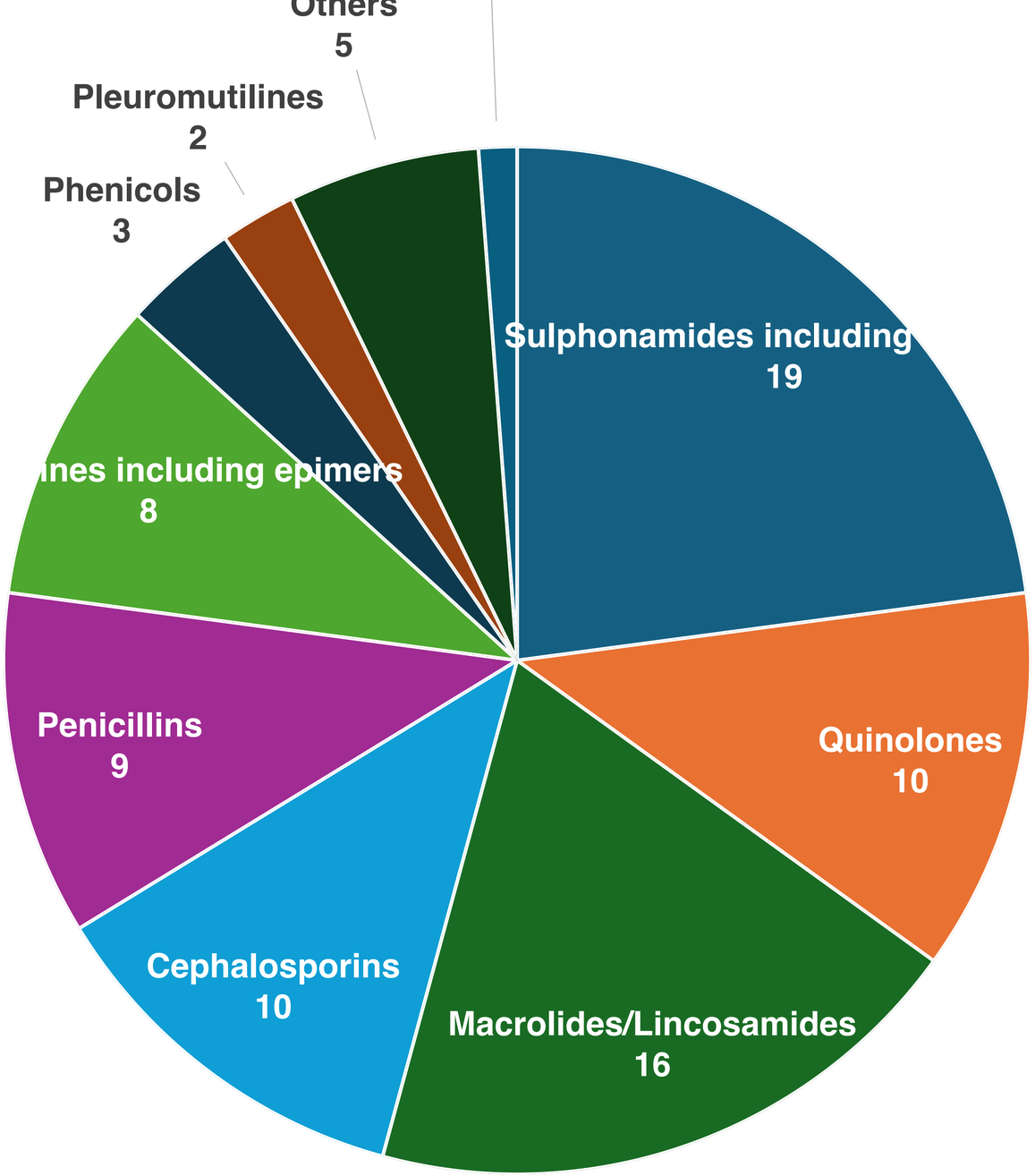
Method **validated** as a screening method according to Commission Implementing Regulation (EU) 2021/808

83 Compounds from **11 family classes** passed validation.

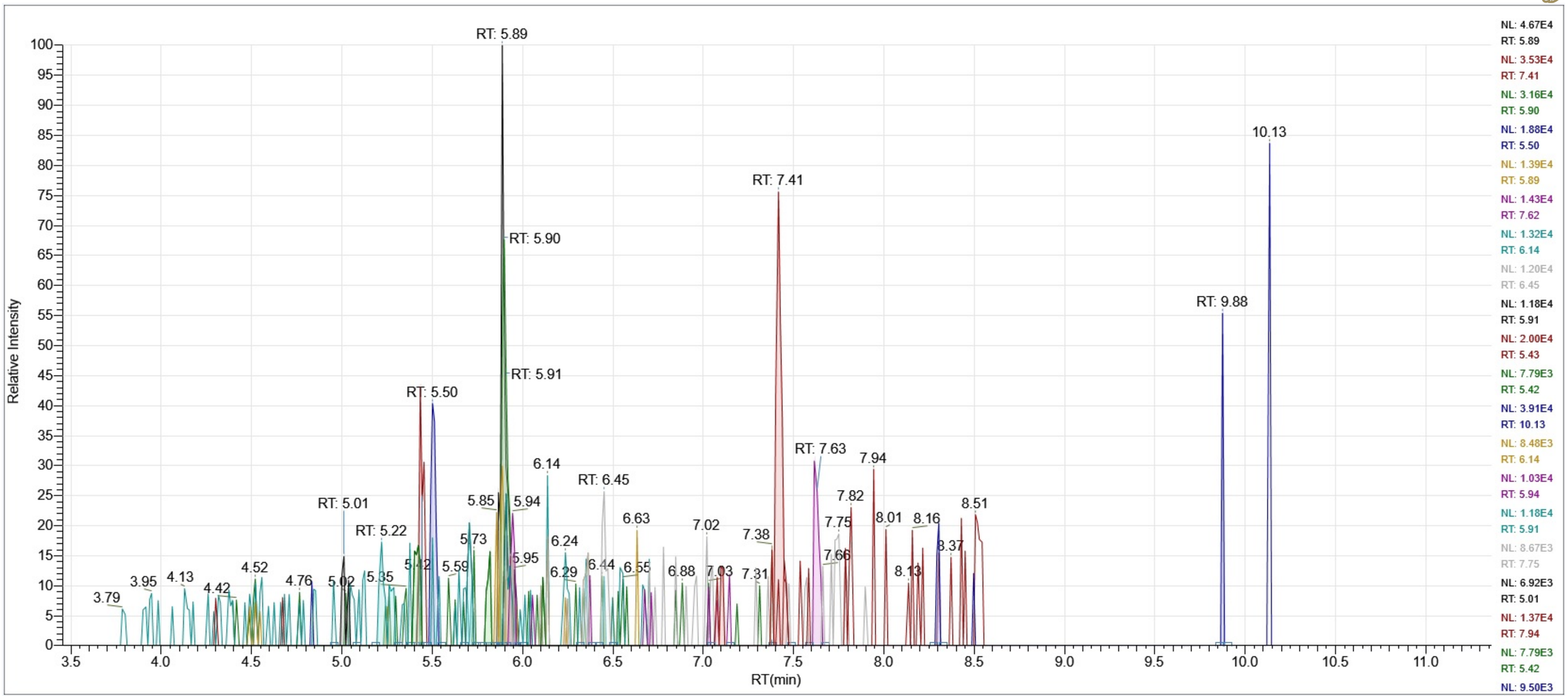
52 meet **Quantitative** criteria.

31 meet **Semi-quantitative** criteria.

Accredited by the Irish National Accreditation Board in 2024.



Positive Mode XIC of analytes (Egg Matrix)
Cal 3



Routine use of method in Official Controls

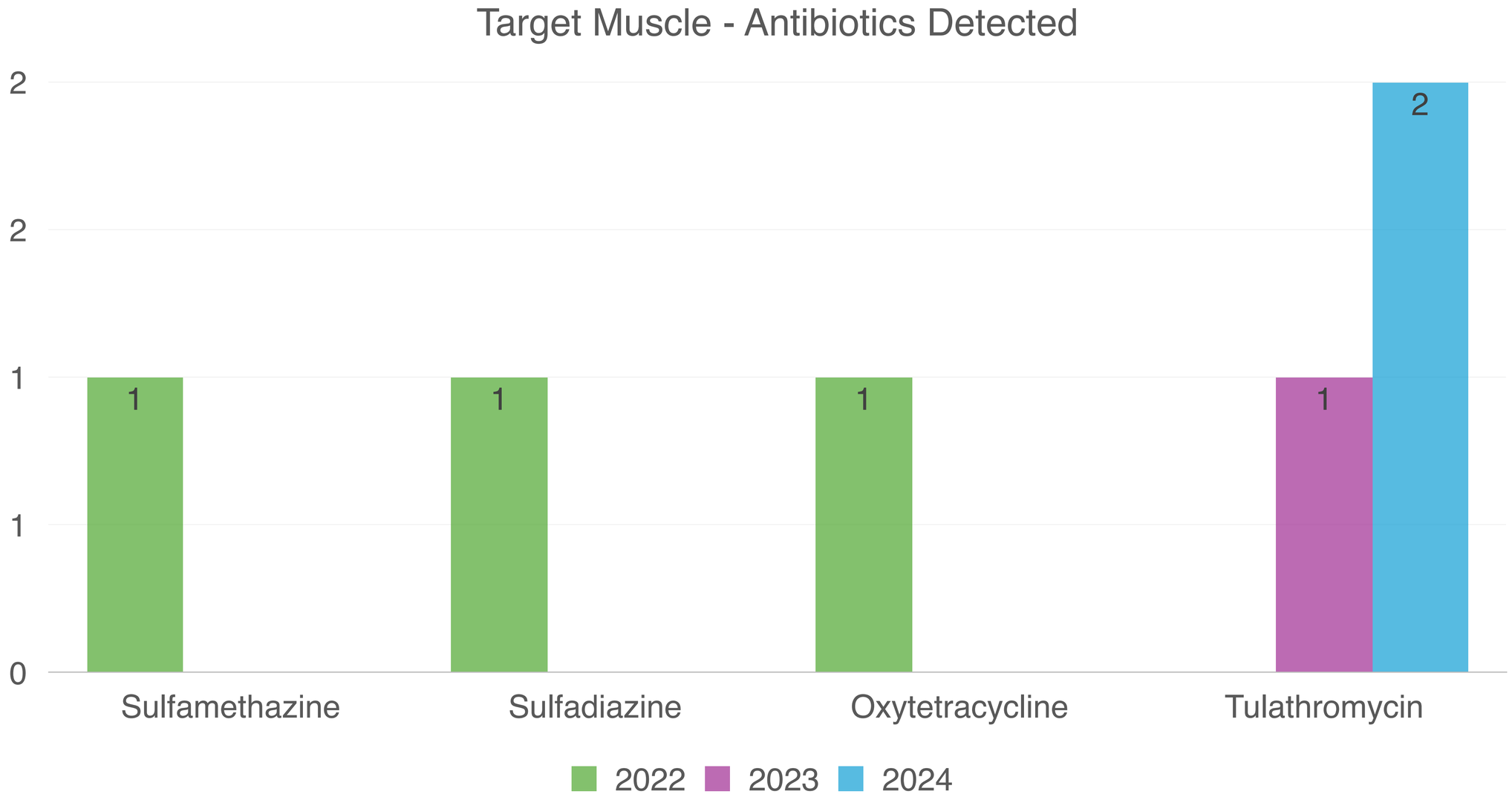
TM231 has been in routine use since 2022.

LC-HRMS analysis is used for all our antibiotic screening.

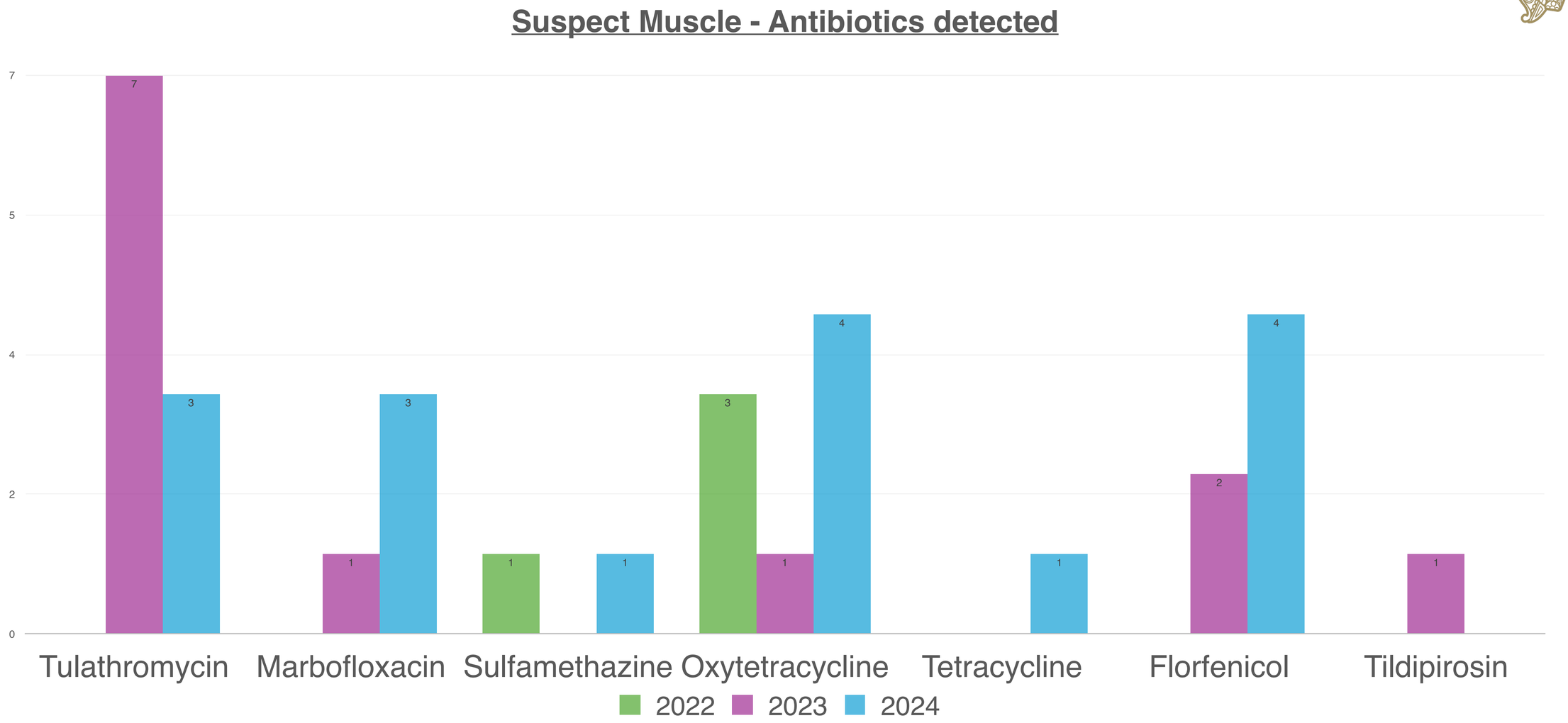
4 LC-Orbitrap Instruments are used in the lab.

2 Q-Exactive instruments and 2 Exploris 120 instruments

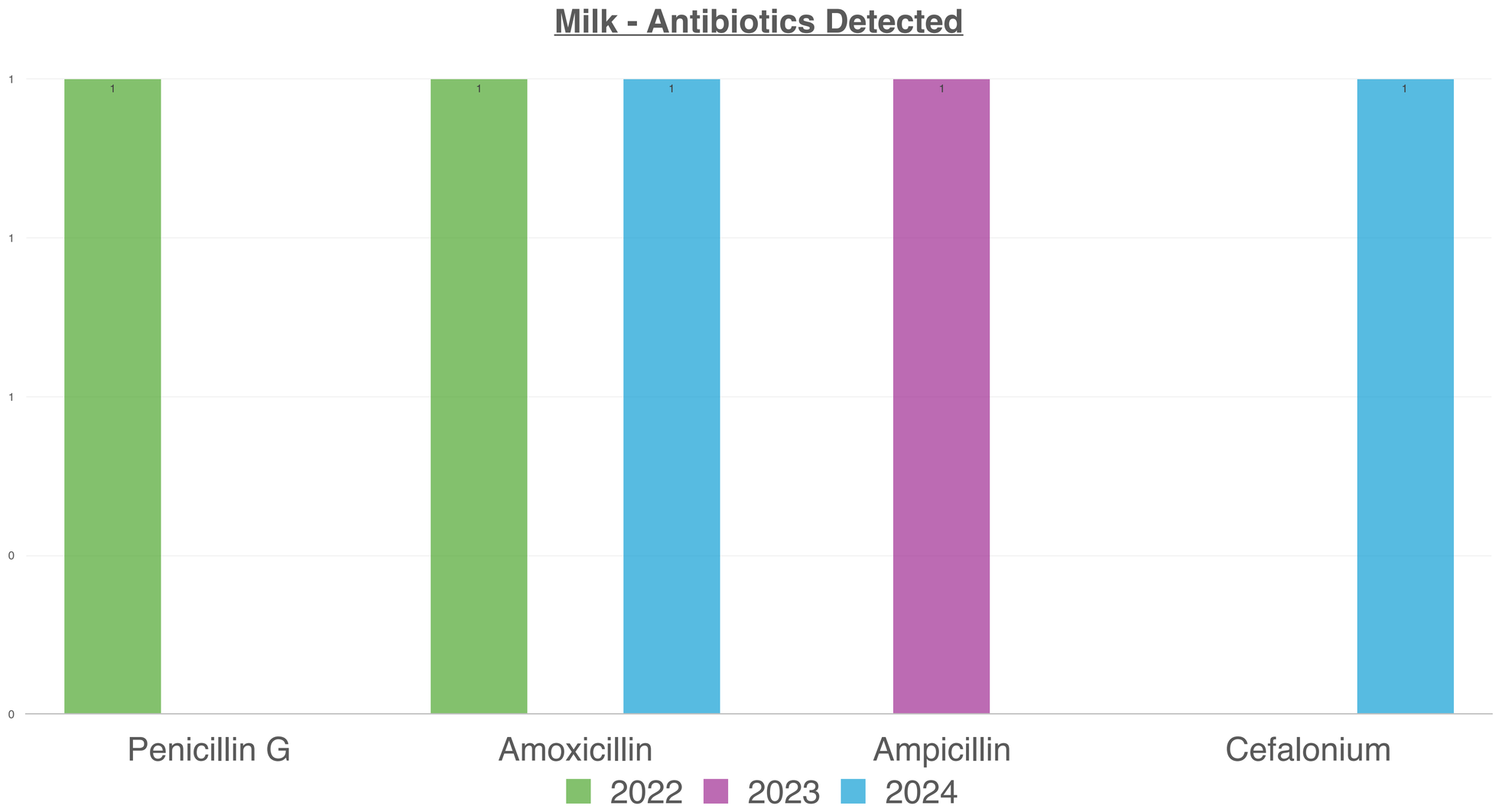
Positive Sample detection



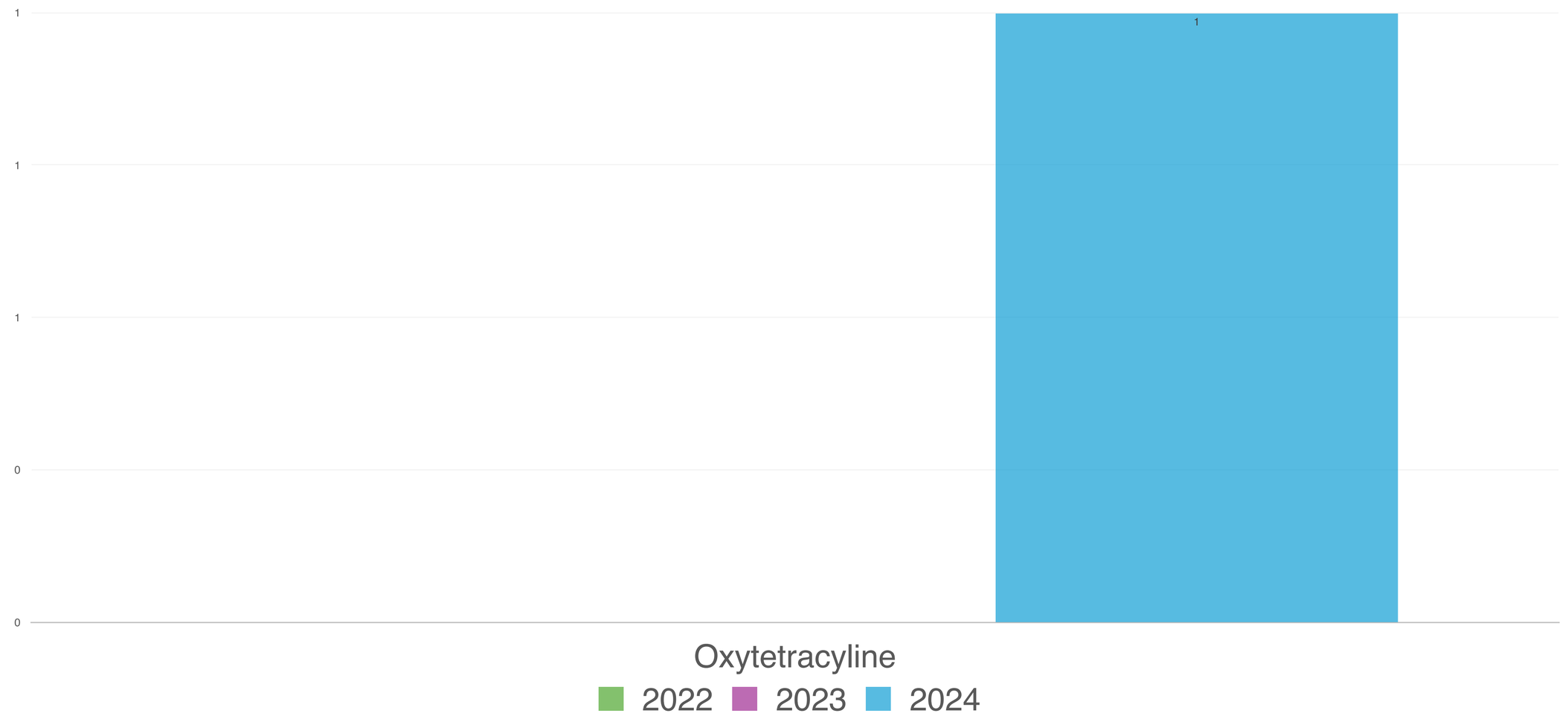
Positive Sample detection



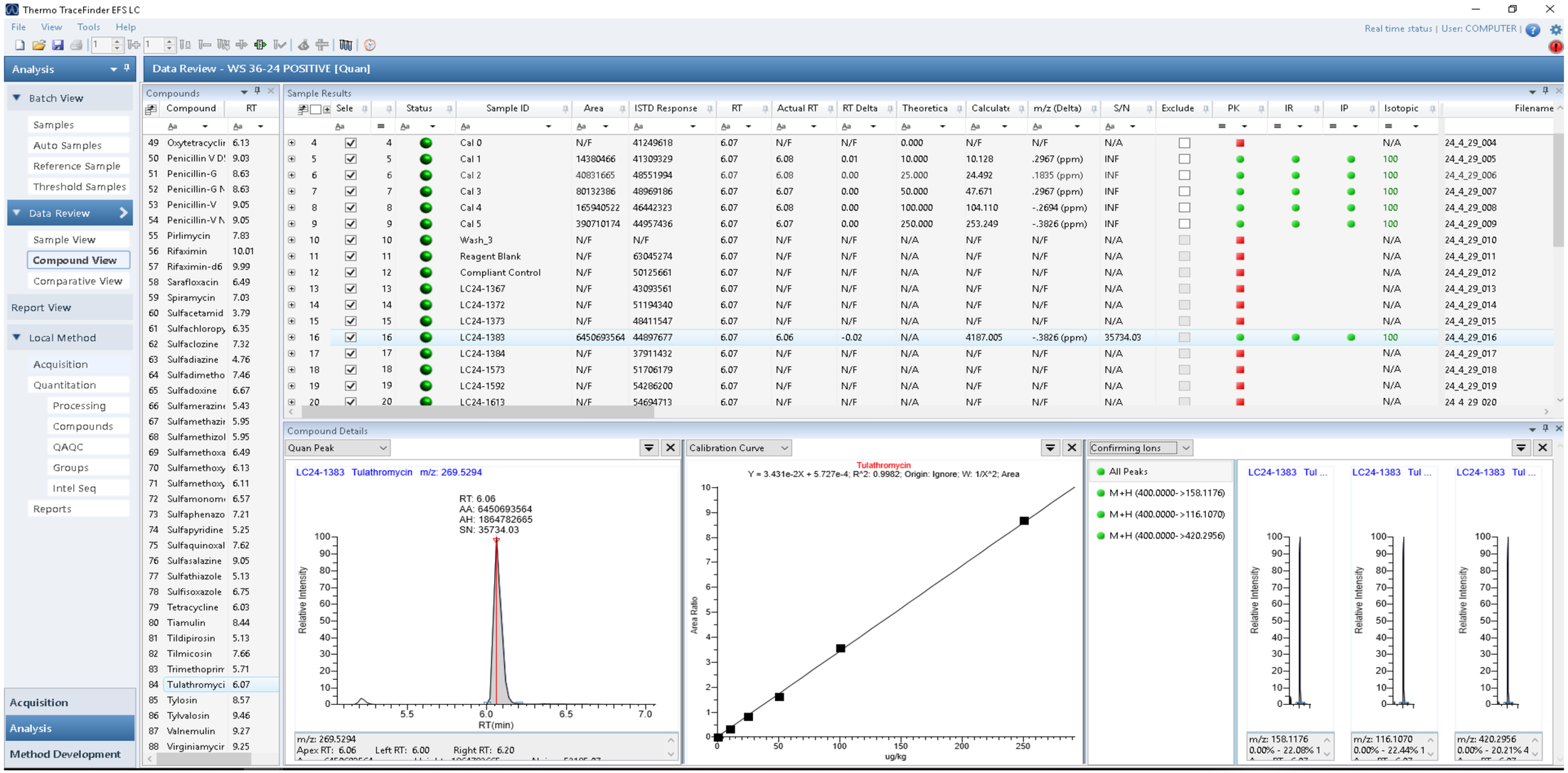
Positive Sample detection



Positive Sample detection



Tulathromycin detected in bovine muscle sample



Customised Reports

Screening Report TM 231

Lab Name: Veterinary Residues

Instrument: ChromLC 142.0 Method Name: WS 38-25 POSITIVE BATCH_TM231_Muscle_Positive Mode_001

Master Method: TM231_Muscle_Positive Mode_001

Batch: WS 38-25 POSITIVE BATCH

Vial Pos	Sample ID	File Name	Level	Sample Name	File Date	Comment
B:D3	LC25-1837 (PT)	2025_05_21_033	N/A	LC25-1837 (PT)	5/21/2025 10:53:53 PM	

Internal Standards

Compound Name	Retention Time	Expected RT	RT Delta	m/z Delta
Ampicillin D5	6.34	6.33	0.00	1.8174 (ppm)
Azithromycin	7.14	7.14	0.00	2.1110 (ppm)
Penicillin V D5	8.98	8.97	0.02	-2.3314 (ppm)
Oxolinic Acid D5	7.47	7.47	0.00	-.7755 (ppm)
Rifaximin-d6	9.92	9.91	0.01	-1.2604 (ppm)
Sulfamethoxypyrodazine-d3	6.03	6.02	0.01	.0769 (ppm)
Baquiloprim-d6	3.23	3.44	-0.21	-.1963 (ppm)

The following compounds were found in the sample

Compound Name	Retention Time	Expected RT	RT Delta	Calculated Amount (µg/Kg)	Sum Compounds	>CCβ	m/z Delta
Flumequine	8.47	8.47	0.00	144.16		TRUE	-.4568 (ppm)
Baquiloprim	3.74	3.88	-0.15	0.54		FALSE	1.0872 (ppm)
Tilmicosin	7.56	7.55	0.02	0.49		FALSE	.8728 (ppm)
Valnemulin	9.17	9.14	0.02	0.14		FALSE	-1.0187 (ppm)
Difloxacin	6.26	6.25	0.01	0.12		FALSE	3.6058 (ppm)
Erythromycin	8.38	8.51	-0.13	0.09		FALSE	4.6344 (ppm)
Enrofloxacin	6.05	6.04	0.01	0.01		FALSE	-.5543 (ppm)

TM231 Positive Screening Report

Lab Name: Veterinary Residues
Instrument: ChromLC 142.0
User: Thermo
Batch: WS 38-25 POSITIVE BATCH

Method: WS 38-25 POSITIVE BATCH_TM231_Muscle_Positive Mode_001
TM231_Muscle_Positive Mode_001
Cali File: Calibration.calx

Vial Pos	Sample ID	File Name	Level	Sample Name	File Date	Comment
B:D6	LC25-1848 (PT)	2025_05_21_036	N/A	LC25-1848 (PT)	5/21/2025 11:45:17 PM	

Compound Name: Penicillin-G M/Z Delta 0.145

>CCBeta TRUE

Sample Conc: 507.833 ug/kg

Retention time: 8.57 Rt Difference 0.04

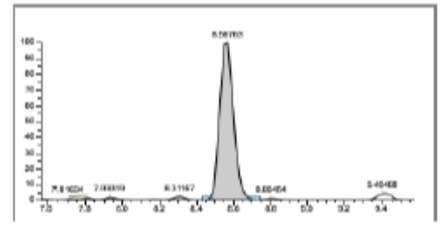
Area (Quan): 21068223

Height (Quan): 4083589

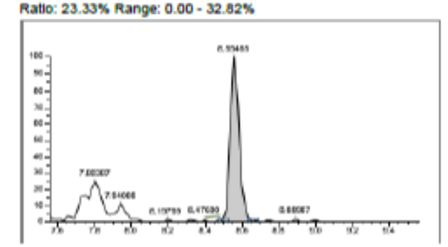
Qual ratio 1: Pass

Qual ratio 2: Pass

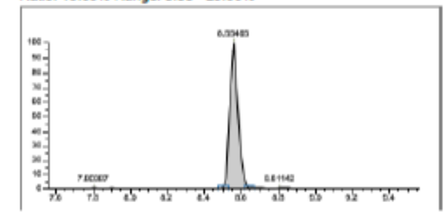
Quan Peak: m/z335.1060 mz



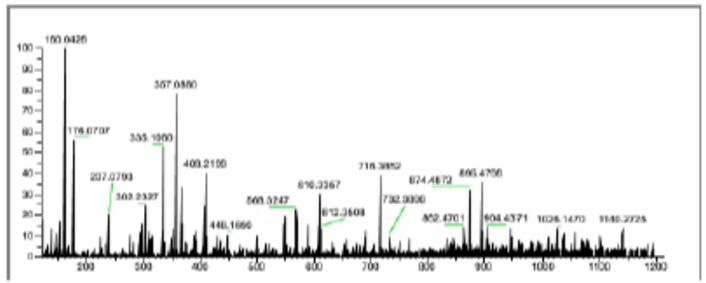
Qual Ion 1: 300.0000->160.0427 mz
Ratio: 23.33% Range: 0.00 - 32.82%



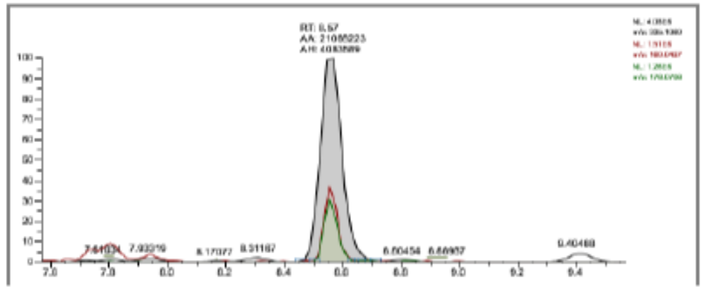
Qual Ion 2: 300.0000->176.0706 mz
Ratio: 19.36% Range: 0.00 - 29.86%



MS Spectra



Overlay



Routine maintenance

Systems perform best when in continuous use.

Monthly Full calibration

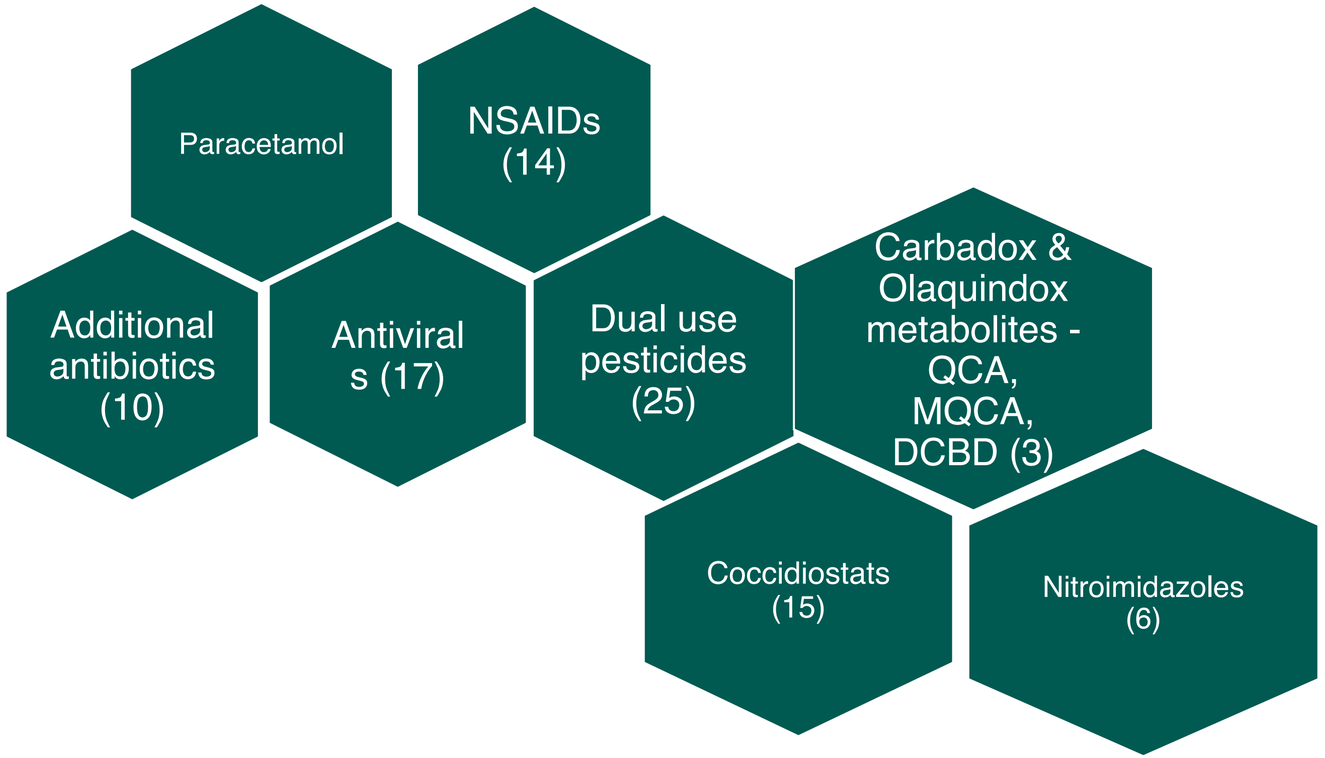
Mass calibration on day of use.

Ion transfer tube and Ion sweep cone cleaned weekly

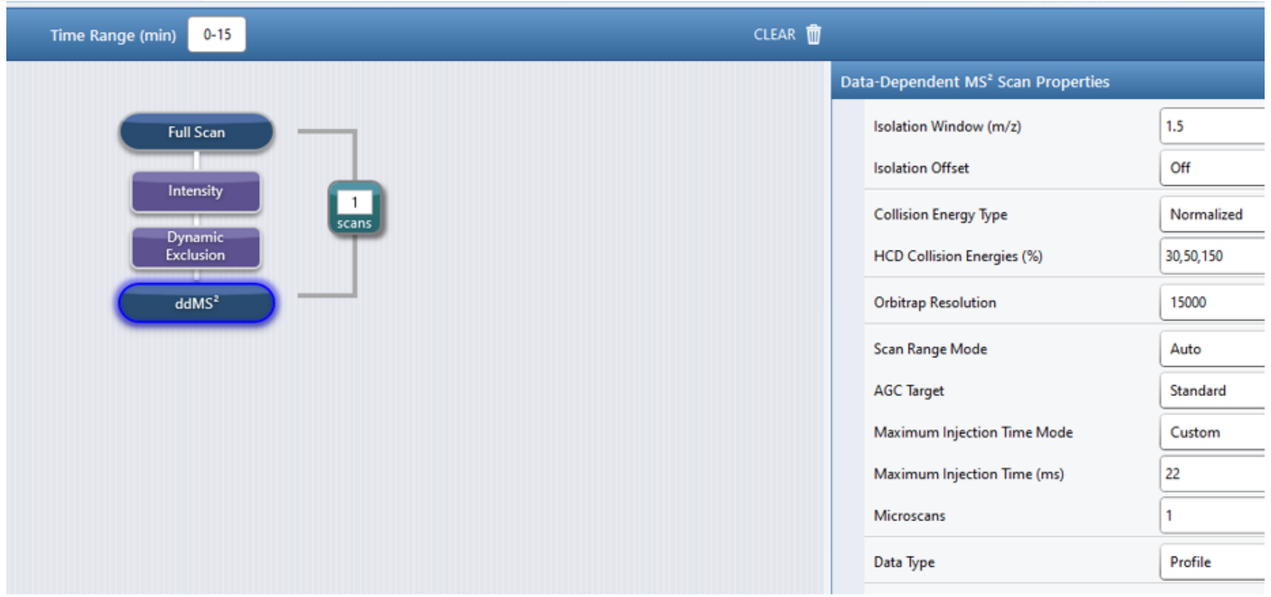
S-Lense cleaned quarterly

Poor peak resolution and loss of sensitivity caused by hydrophobic contamination. Column cleaned with “Chromacare” solution (45% IPA, 45% ACN and10% Acetone)

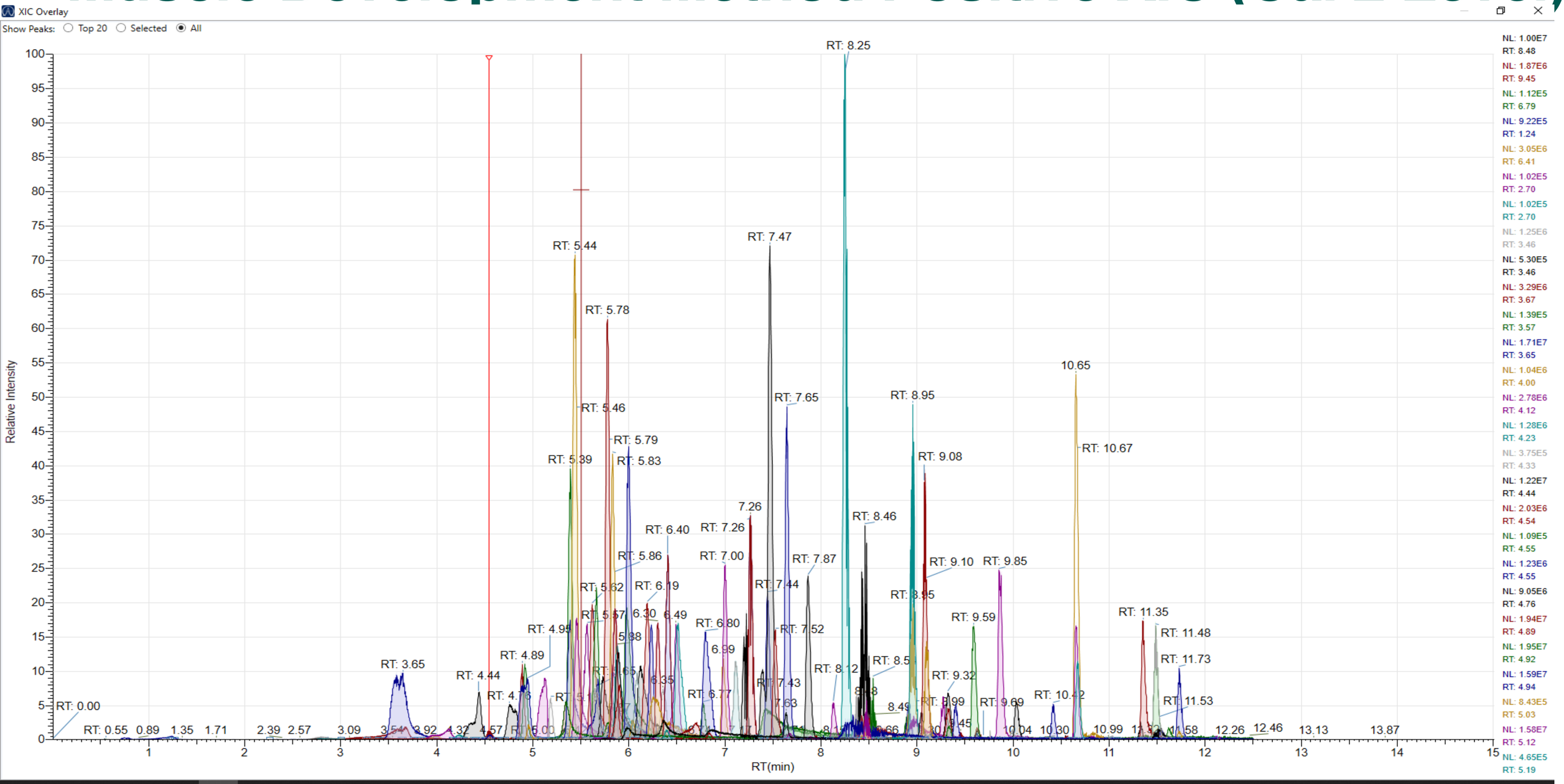
Excellent Platform for Further Expansion



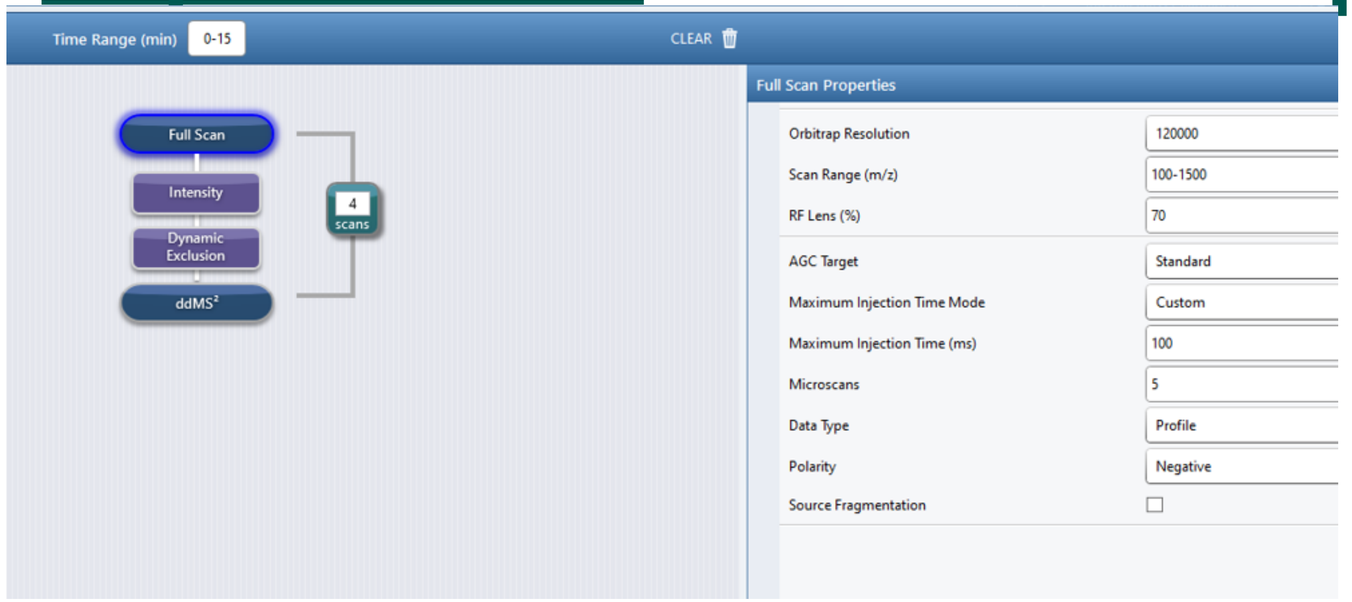
Exploris 120 Muscle Development Method Positive



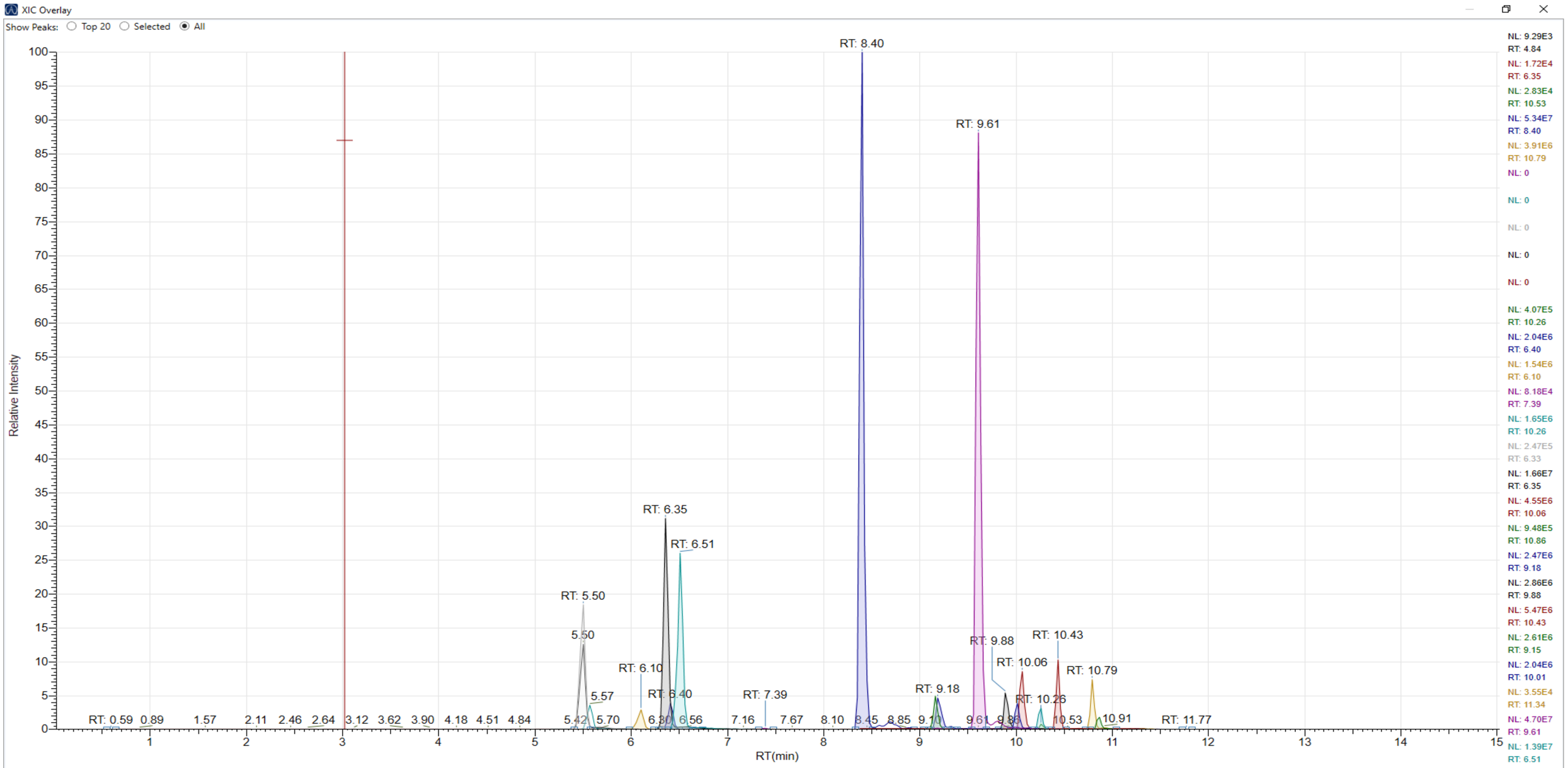
Muscle Development Method Positive XIC (Cal 2 Level)



Exploris 120 Muscle Development Method Negative



Muscle Development Method Negative XIC (Cal 2 Level)



Limitations

- Legislation has not kept up to pace with use of HRMS techniques.
- Guidelines are heavily based on QQQ methods.
- No criteria for Isotopic pattern and Library matches
- Revision of HRMS guidelines are underway, this will hopefully allow additional identification and confirmatory criteria specific to HRMS.
- Difficult to obtain reference standards and to keep solutions within their expiry dates.

Conclusions

- Thank you for your attention.
- Any questions?
- Email. Ian.Kelleher@agriculture.gov.ie