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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 ^1H	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

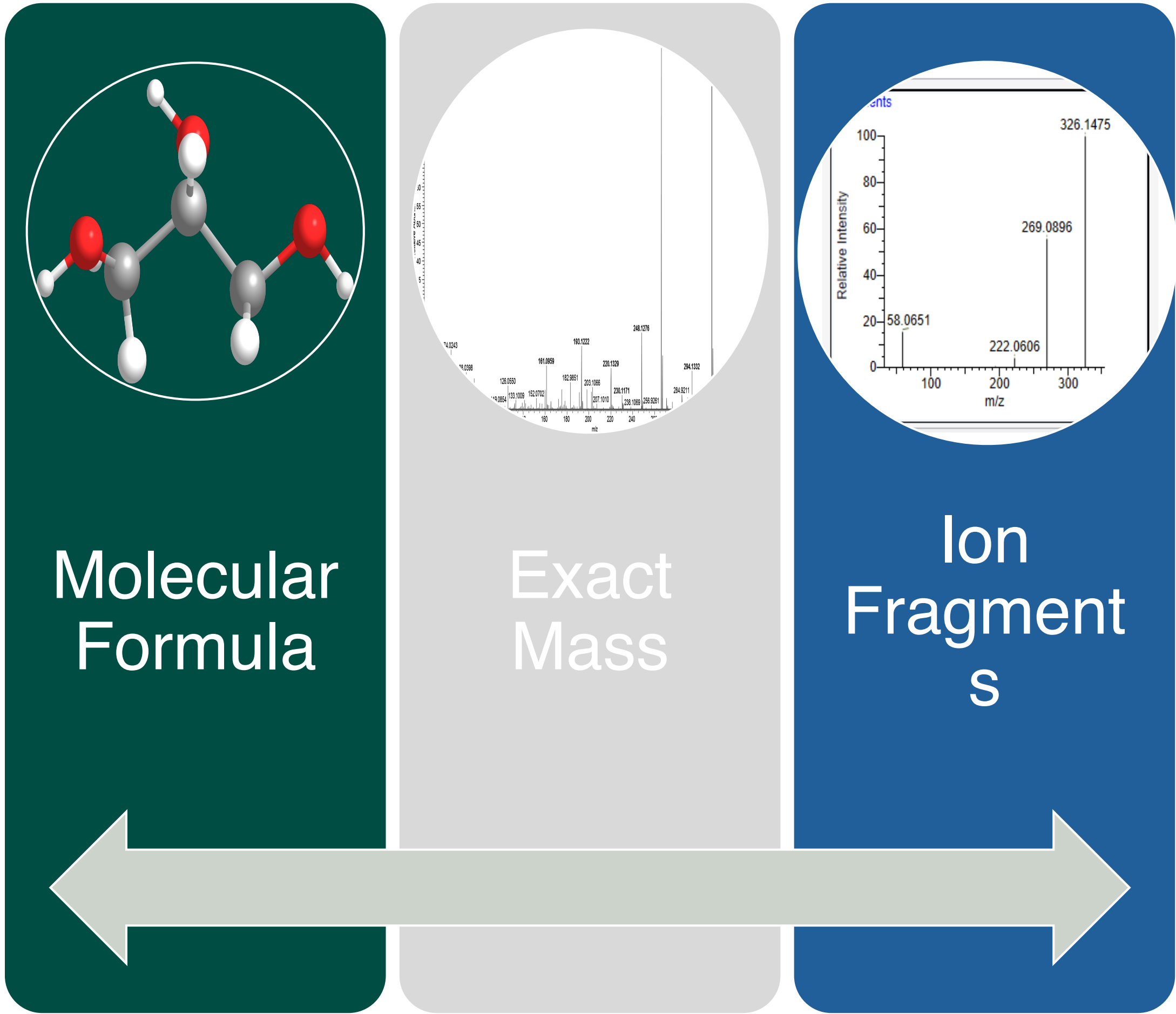
HRM S

- 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



2025_03_27_002_20250327103132_20250527101357_20250

Compound Database - HRMS Dev List

Compounds: 224

Compound Name	Peak Label	Peak Workflow	Associated Target Peak	Chemical Formula	MS Order	Precursor m/z	Product m/z	m/z
4-MAA (4-Methyl An	T1: 218.12879	TargetPeak		C12H15N3O	ms1	218.12879	0.000	218.12879
4-MAA (4-Methyl An	T1F1: 218.12879->56.4	Fragment	T1: 218.12879	C12H15N3O	ms2	218.12879	56.04948	217.12091
4-MAA (4-Methyl An	T1F2: 218.12879->97.1	Fragment	T1: 218.12879	C12H15N3O	ms2	218.12879	97.07602	217.12091
4-MAA (4-Methyl An	T1F3: 218.12879->187	Fragment	T1: 218.12879	C12H15N3O	ms2	218.12879	187.08659	218.12879
5-Hydroxyflunixin	T1: 313.07945	TargetPeak		C14H11F3N2O3	ms1	313.07945	0.000	313.07945
Acetaminophen (Par	T1: 152.07061	TargetPeak		C8H9NO2	ms1	0.000	0.000	152.07061
Acetyltiylosin	T1: 958.53699	TargetPeak		C48H79NO18	ms1	0.000	0.000	958.53699
Acetyltiylosin	T1F1: 1040.000->772.4	Fragment	T1: 958.53699	C48H79NO18	ms2	1040.000	772.44778	772.44778
Acetyltiylosin	T1F2: 1040.000->174.1	Fragment	T1: 958.53699	C48H79NO18	ms2	1040.000	174.11247	174.11247
Acetyltiylosin	T1F3: 1040.000->109.1	Fragment	T1: 958.53699	C48H79NO18	ms2	1040.000	109.06479	109.06479
Acyclovir	T1: 248.07541	TargetPeak		C8H11N5O3	ms1	0.000	0.000	248.07541
Alpha-Zearalanol (Ze	T1: 345.16725	TargetPeak			ms1	345.16725	0.000	345.16725
Alpha-Zearalanol	T1: 320.16238	TargetPeak			ms1	320.16238	0.000	320.16238
Amantadine	T1: 152.14338	TargetPeak		C10H17N	ms1	0.000	0.000	152.14338
Amoxicillin	T1: 366.11182	TargetPeak		C16H19N3O5S	ms1	0.000	0.000	366.11182
Amoxicillin	T1F1: 400.000->208.0	Fragment	T1: 366.11182	C16H19N3O5S	ms2	400.000	208.04249	208.04249
Amoxicillin	T1F2: 400.000->114.0	Fragment	T1: 366.11182	C16H19N3O5S	ms2	400.000	114.00071	114.00071
Amoxicillin Na	T1: 388.09376	TargetPeak		C16H19N3O5S	ms1	0.000	0.000	388.09376
Amoxicillin Na	T1F1: 388.09376->114	Fragment	T1: 388.09376	C16H19N3O5S	ms2	388.09376	114.00071	114.00071
Amoxicillin Na	T1F2: 388.09376->208	Fragment	T1: 388.09376	C16H19N3O5S	ms2	388.09376	208.04249	208.04249
Ampicillin	T1: 350.1169	TargetPeak		C16H19N3O4S	ms1	0.000	0.000	350.1169
Ampicillin	T1F1: 400.000->160.0	Fragment	T1: 350.1169	C16H19N3O4S	ms2	400.000	160.04248	160.04248
Ampicillin	T1F2: 400.000->174.0	Fragment	T1: 350.1169	C16H19N3O4S	ms2	400.000	174.05478	174.05478

Compound Details Pane

Compound Name

Ionization: None

Chemical Formula

CAS No

Compound Type: Analyte

Compound Groups

Neutral Mass: 0

Category

Internal Standard

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

- Resolution 70,000
- Scan range 120-1200 m/z
- AGC target 1e6

DIA Experiment

- Resolution 35,000
- (N)CE / stepped ce:15, 30, 60

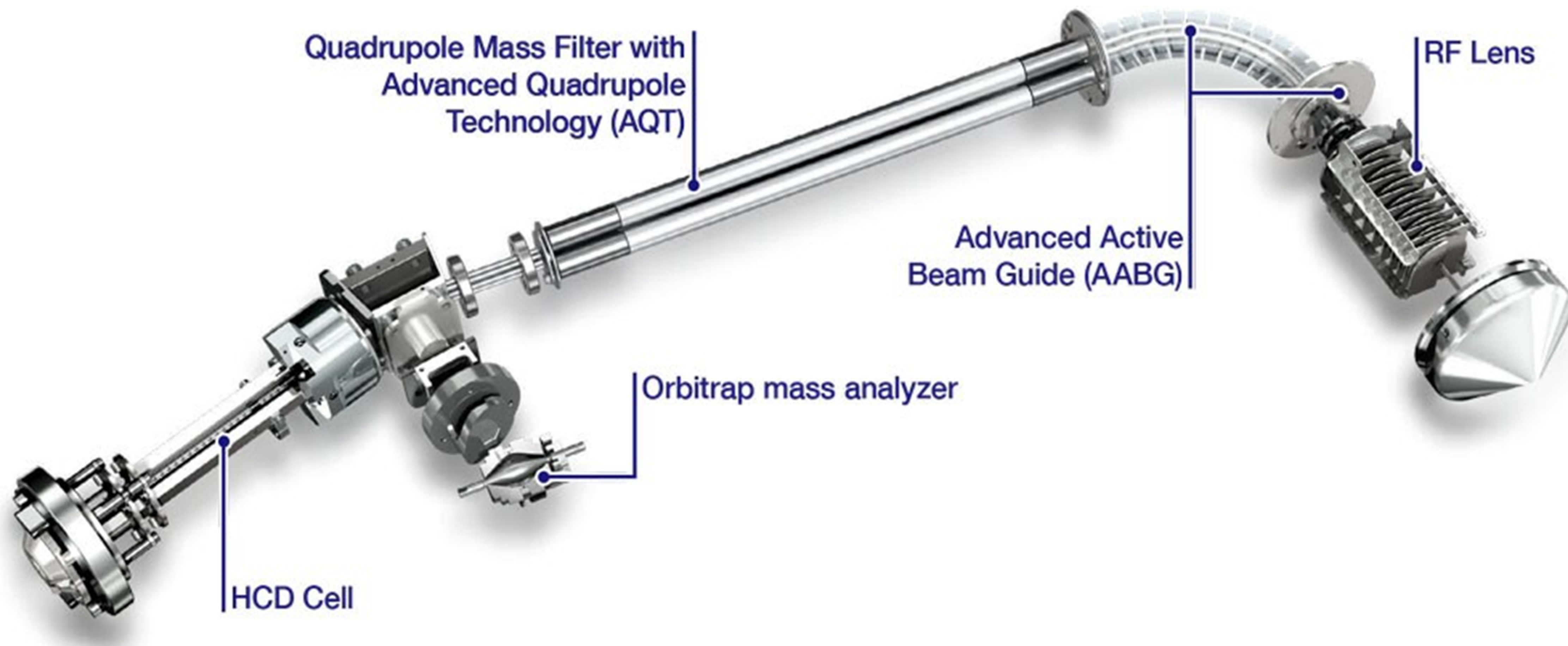
DIA Experiment

- Resolution 17,500
- (N)CE / stepped ce:15, 30, 60

T-SIM

- Resolution 70,000

Q-Exactive Orbitrap



Exploris 120 Master Method Parameters TM231

Positive Polarity

Full MS

- Resolution
60,000 milk
120,000 eggs
- Scan range
200-1200 m/z
- AGC target
standard

DIA Experiment

- Resolution
30,000
- CE: 15, 30, 60

DIA Experiment

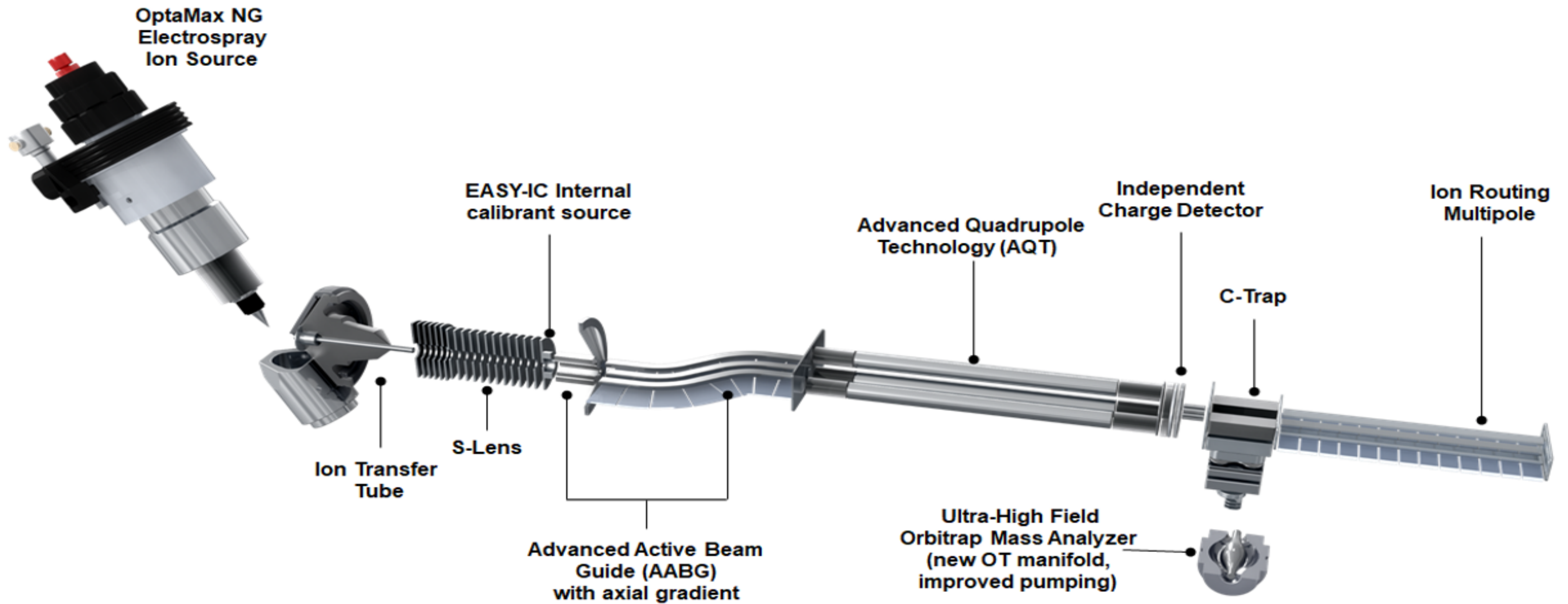
- Resolution
15,000
- CE:15, 30, 60

Negative Polarity

SIM

- Resolution
120,000

Exploris 120 Orbitrap



Key differences between Q-Exactive and Exploris systems

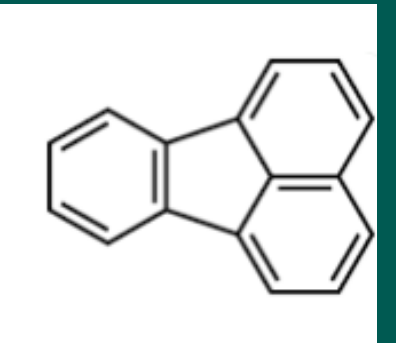


Q-Exactive resolution 17,500; 35,000, 70,000; & 140,000 (not used)

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 100 μ l internal standard, 500 μ l acetonitrile
- Vortex all tubes and allow to stand in the dark for 10mins.
- Add 130 μ l 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 0.22 μ m 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.

Qualitative Screening	Semi-Quantitative Screening	Quantitative Screening
CC β Selectivity Stability Ruggedness	CC β Selectivity Stability Ruggedness Precision *	CC β Selectivity Stability Ruggedness Precision Trueness Relative matrix effect

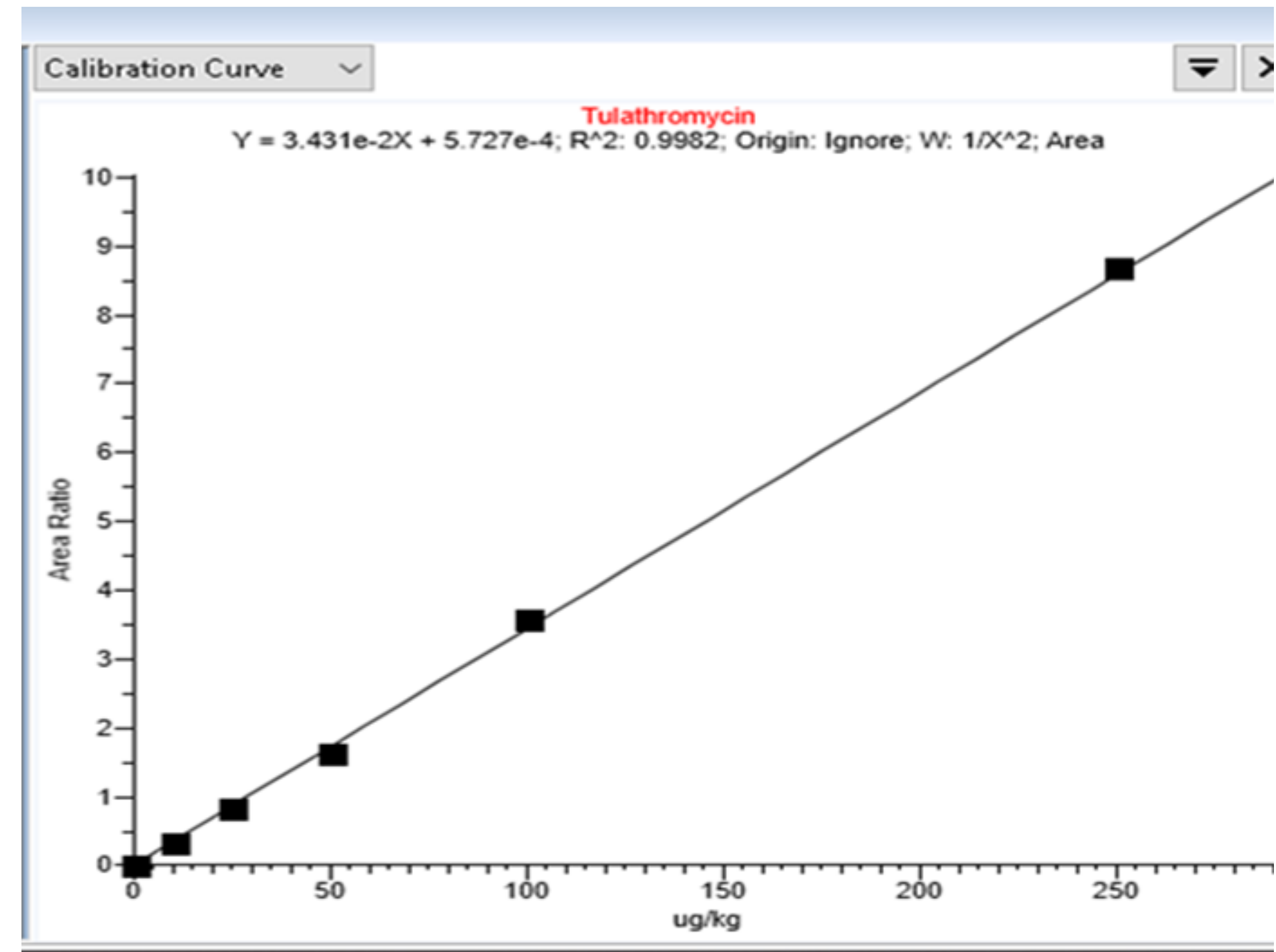
Several performance characteristics were determined simultaneously, and validation experiments were combined to minimise workload.

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

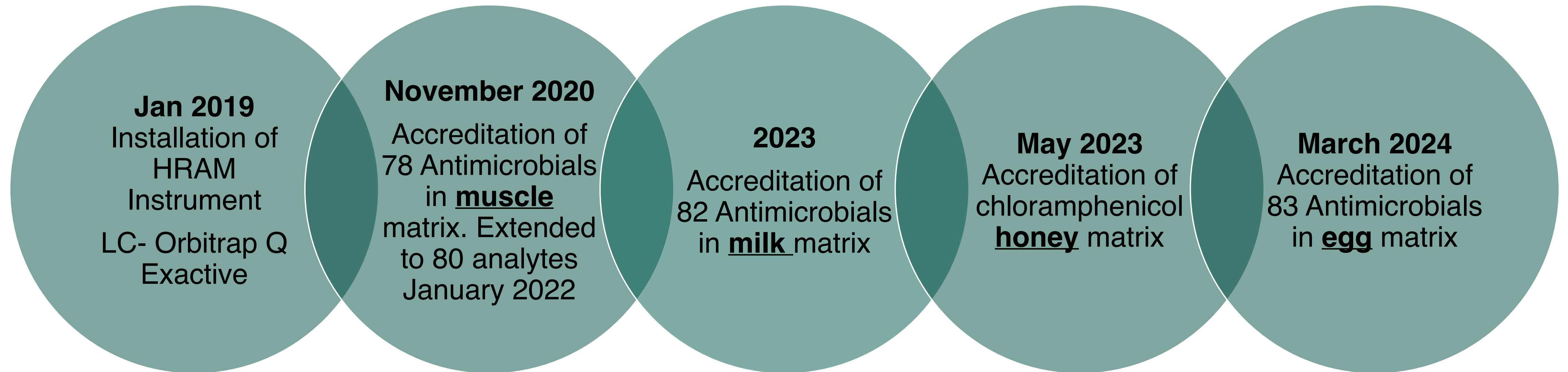
Quantitative Analysis Capability



- 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



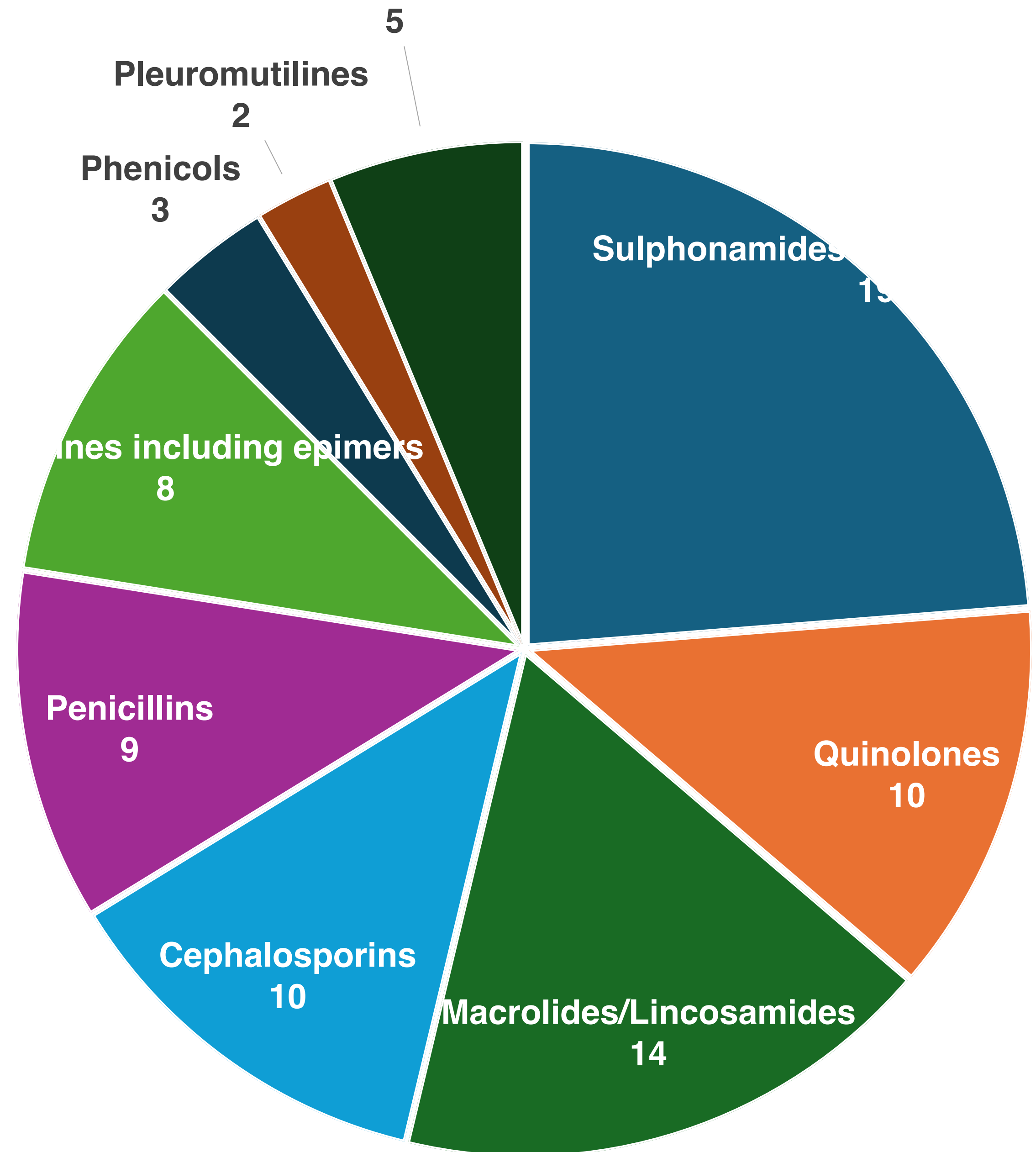
59 meet **Quantitative** criteria.



21 meet **Semi-quantitative** criteria.



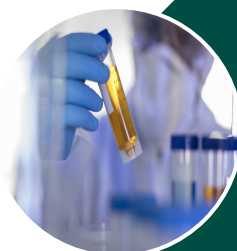
Accredited by the Irish National Accreditation Board in 2022.



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



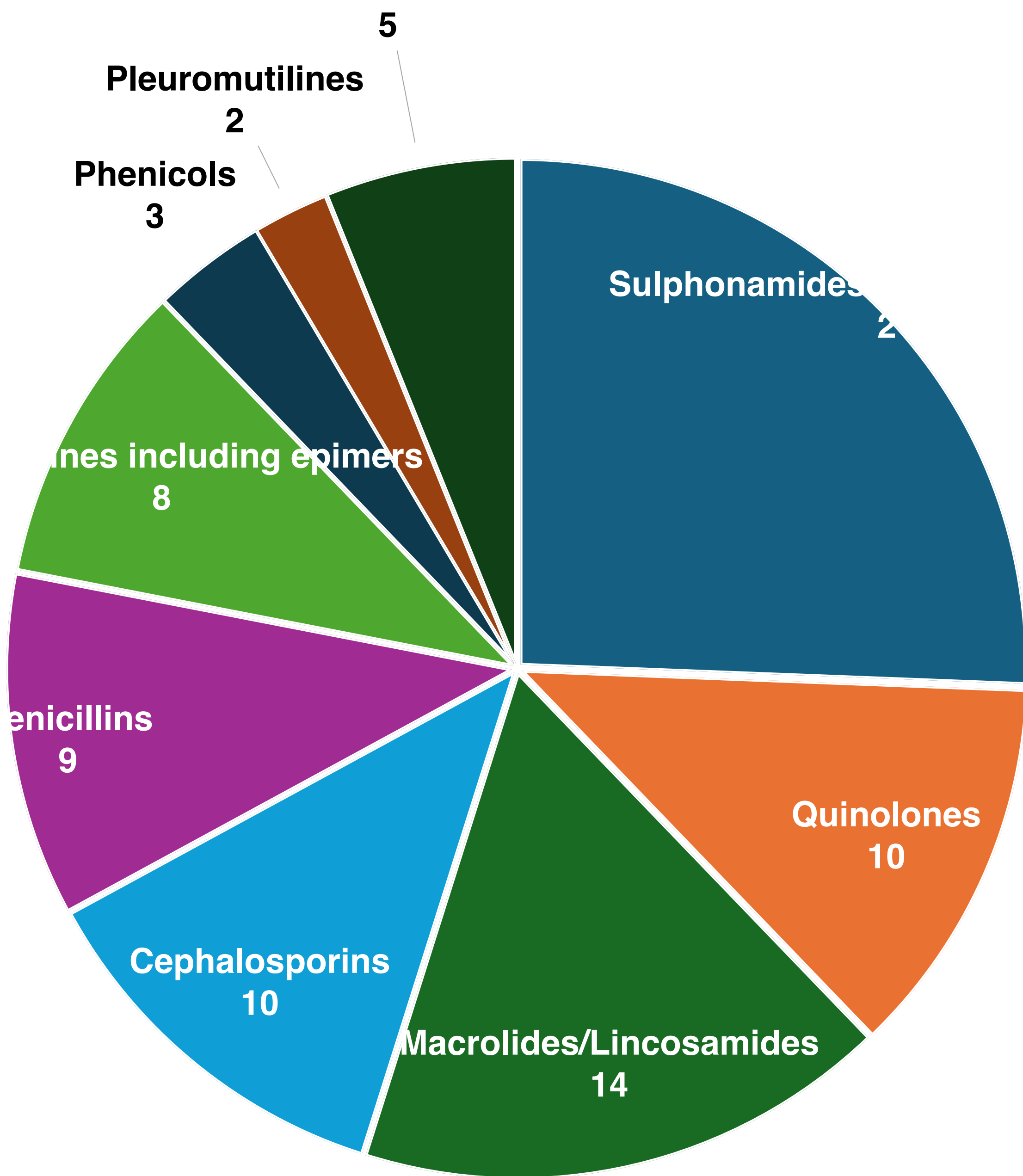
30 meet **Quantitative** criteria



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.



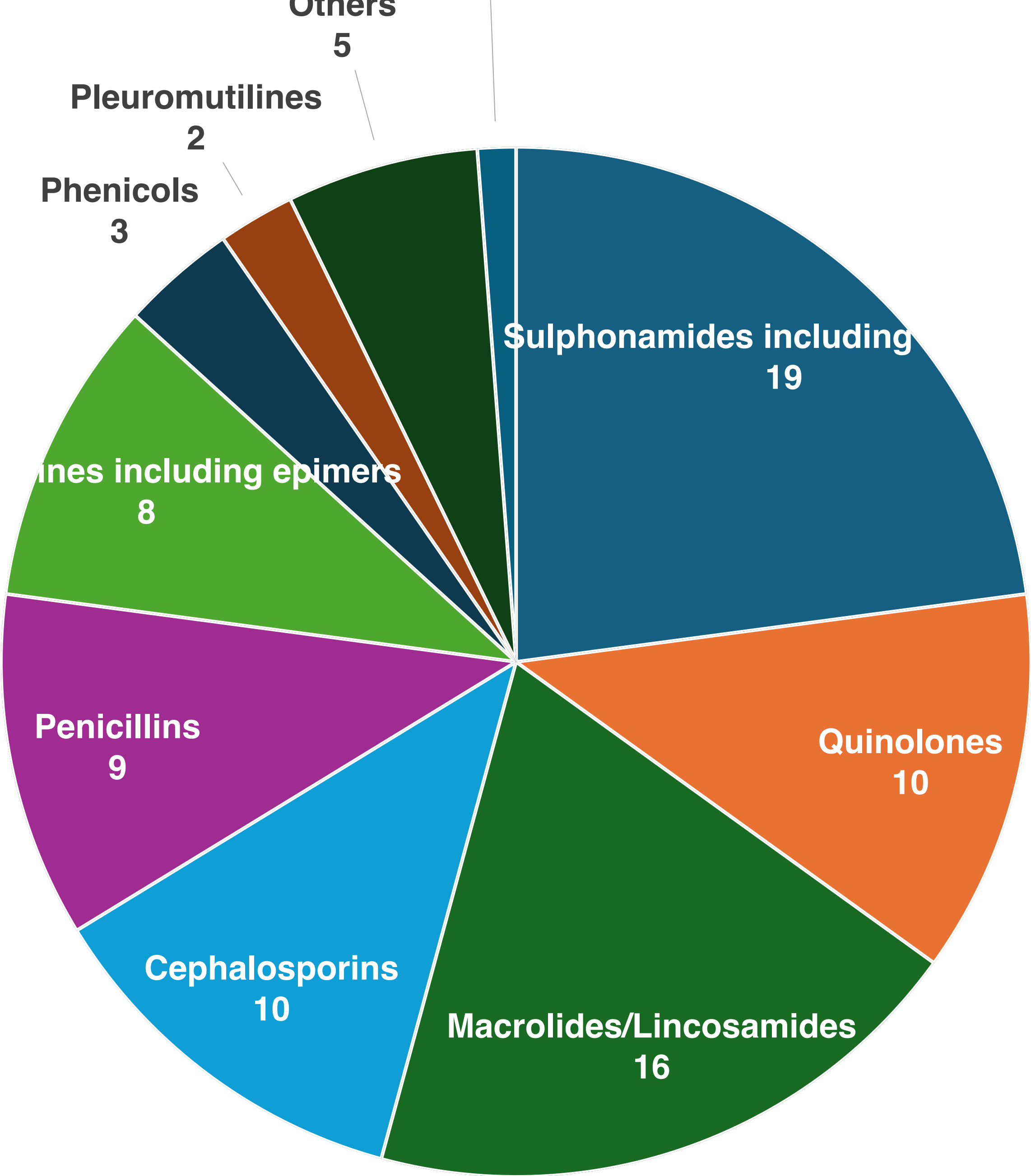
52 meet Quantitative criteria.



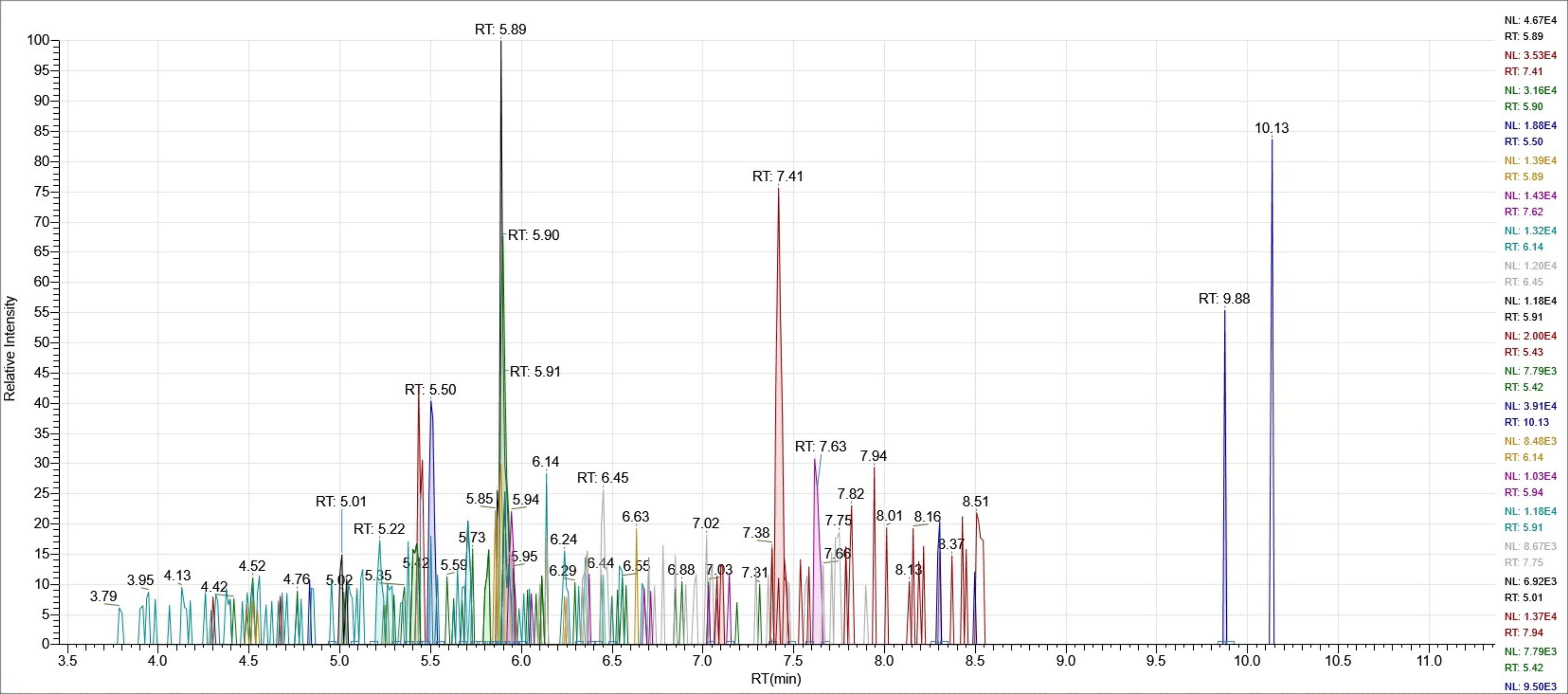
31 meet Semi-quantitative criteria.



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.

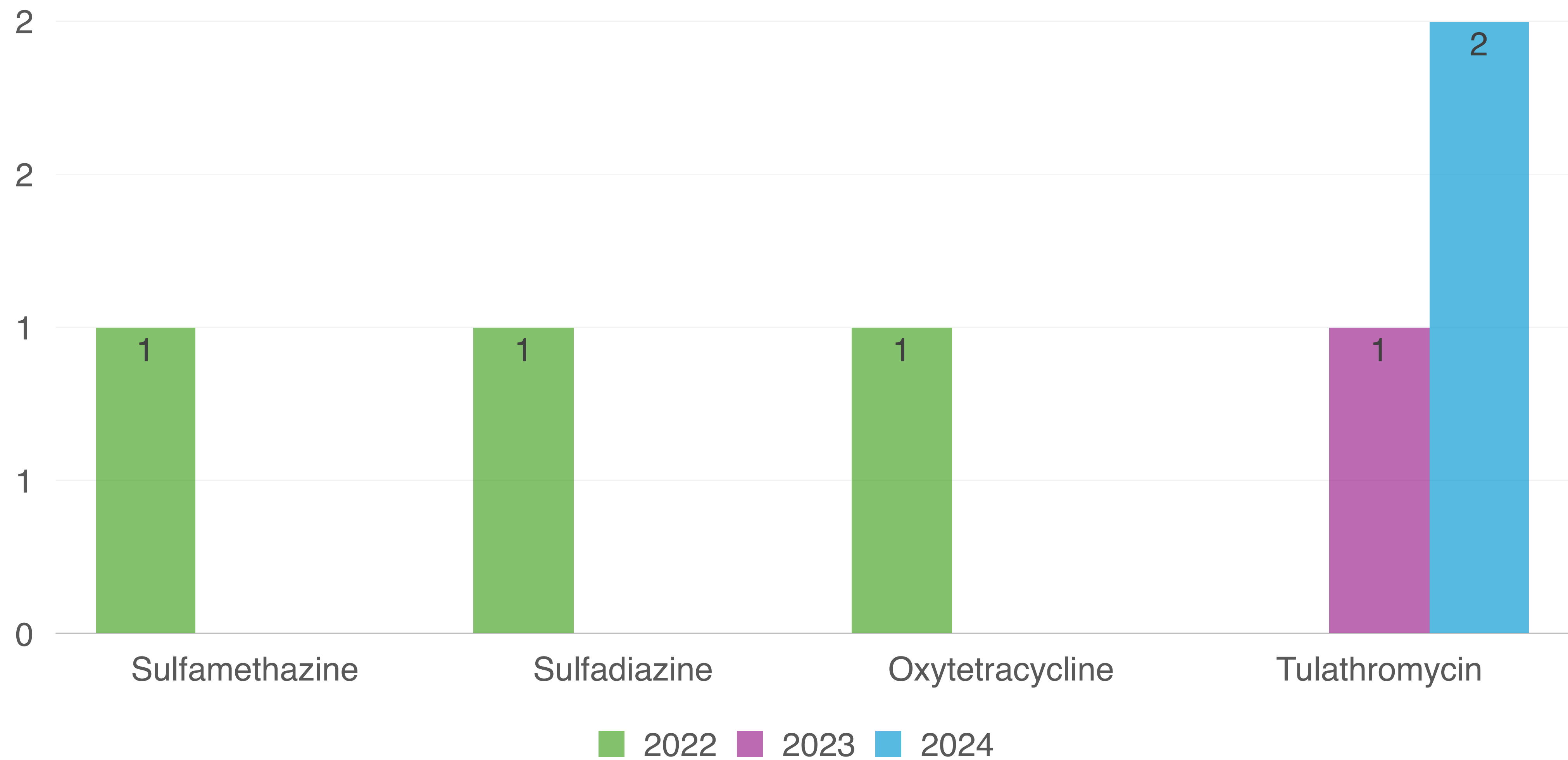
LC-HRMS analysis is used for all our antibiotic screening.

4 LC-Orbitrap Instruments are used in the lab.
2 Q-Exactive instruments and 2 Exploris 120 instruments

Positive Sample detection



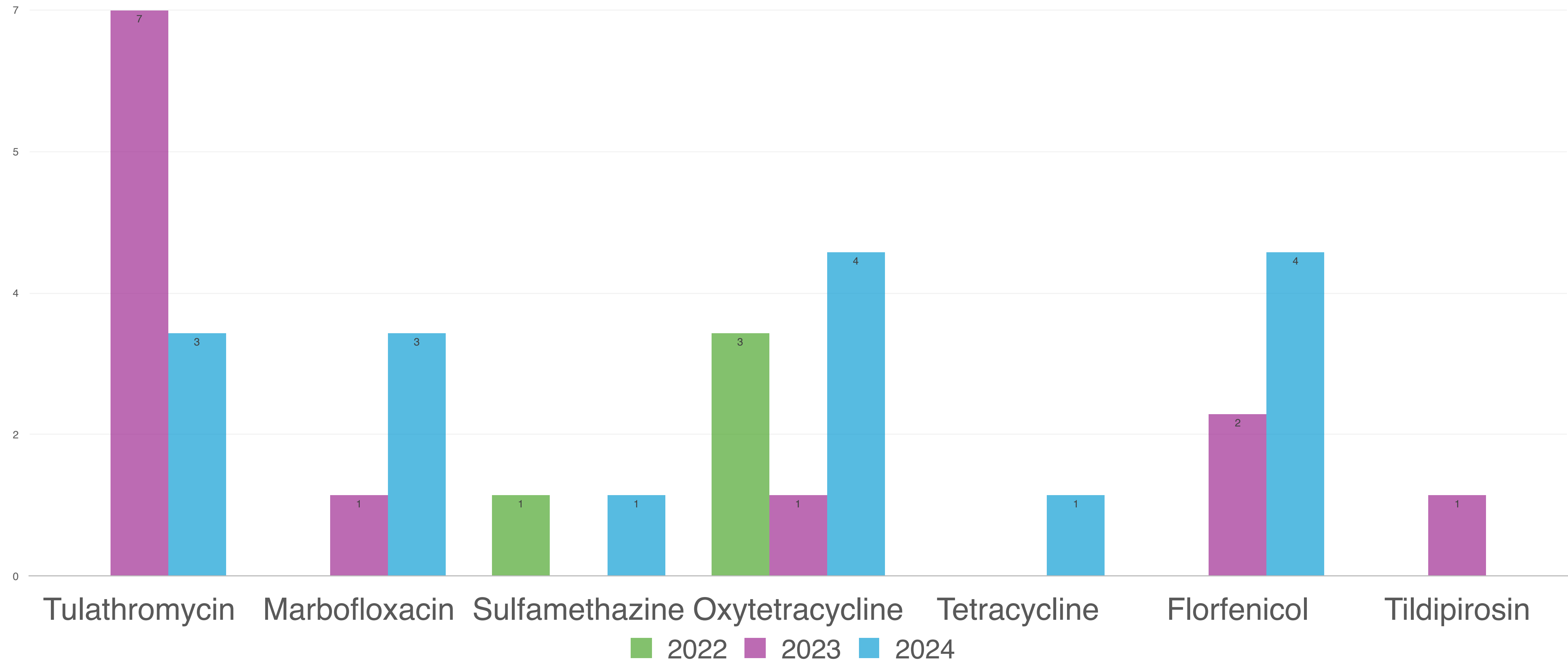
Target Muscle - Antibiotics Detected



Positive Sample detection



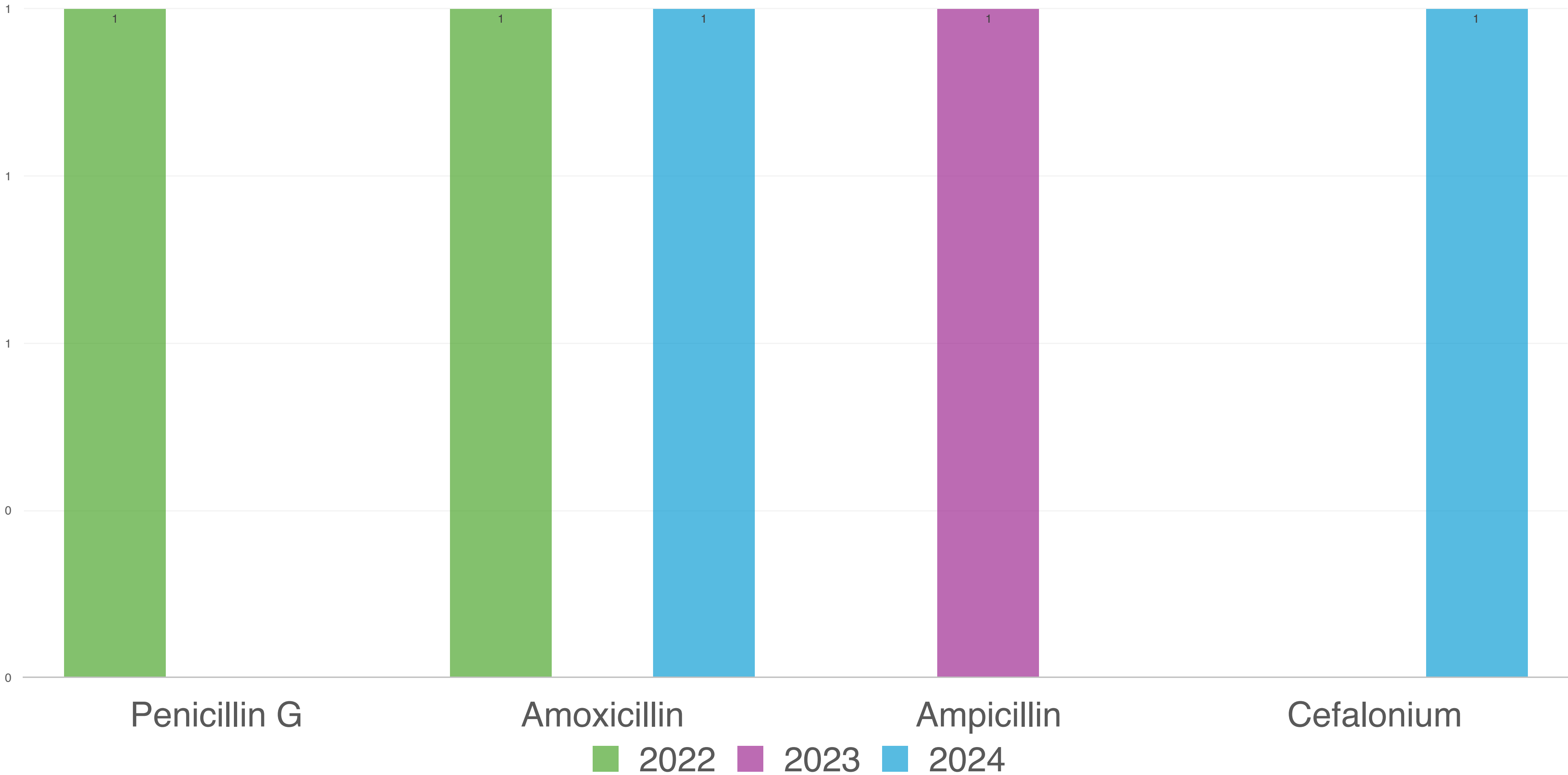
Suspect Muscle - Antibiotics detected



Positive Sample detection



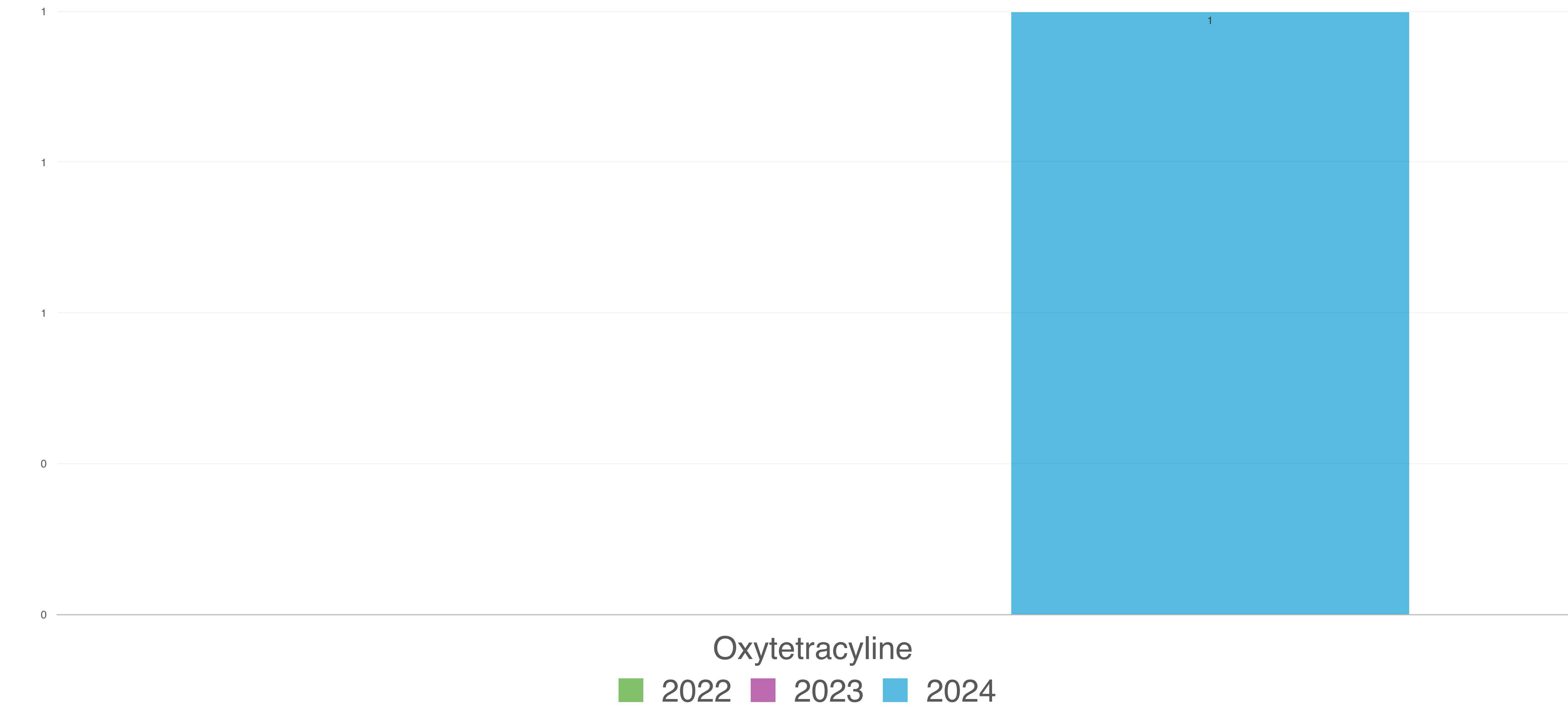
Milk - Antibiotics Detected



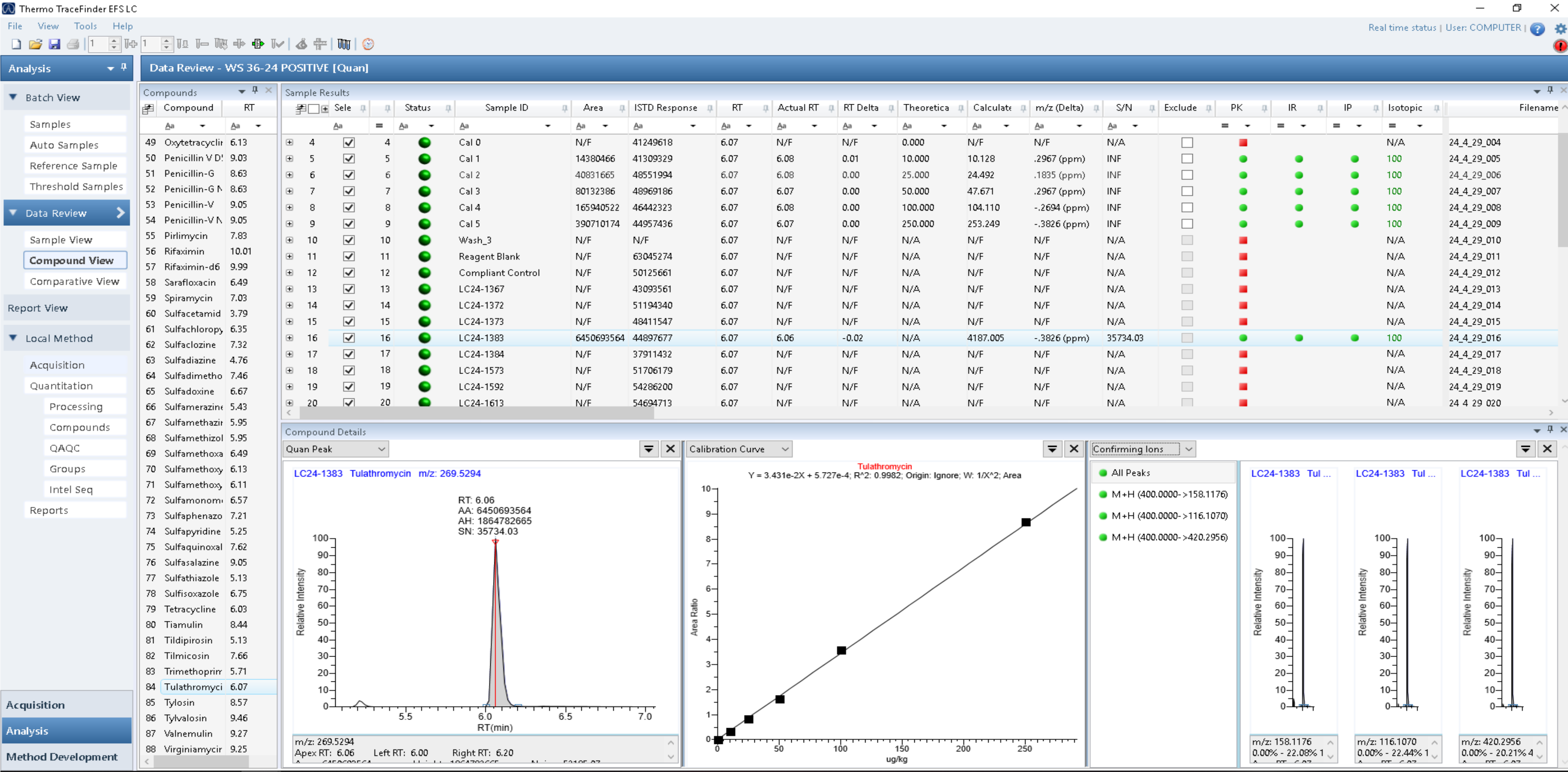
Positive Sample detection



Egg - Antibiotics Detected



Tulathromycin detected in bovine muscle sample



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		

<u>Vial Pos</u>	<u>Sample ID</u>	<u>File Name</u>	<u>Level</u>	<u>Sample Name</u>	<u>File Date</u>	<u>Comment</u>
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)
Sulfamethoxypyridazine-d3	6.03	6.02	0.01	.0769 (ppm)
Baicaloprim-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	Method:	WS 38-25 POSITIVE BATCH_TM231_Muscle_Positive Mode_001
Instrument:	ChromLC 142.0		TM231_Muscle_Positive Mode_001
User:	Thermo	Call File:	Calibration.cdx
Batch:	WS 38-25 POSITIVE BATCH		

<u>Vial Pos</u>	<u>Sample ID</u>	<u>File Name</u>	<u>Level</u>	<u>Sample Name</u>	<u>File Date</u>	<u>Comment</u>
B:06	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
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>CCBeta	TRUE
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Sample Conc: 507.833 ug/kg

Retention time:	8.57	Rt Difference	0.04
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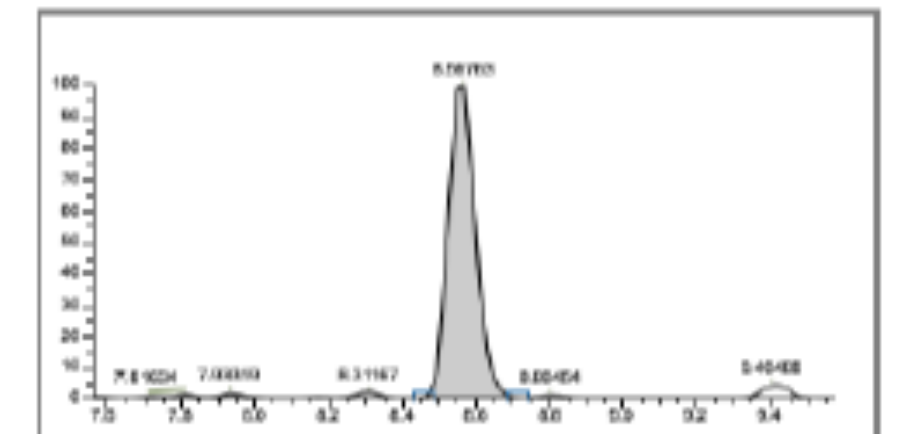
Area (Quan): 21068223

Height (Quan): 4083589

Qual ratio 1: Pass

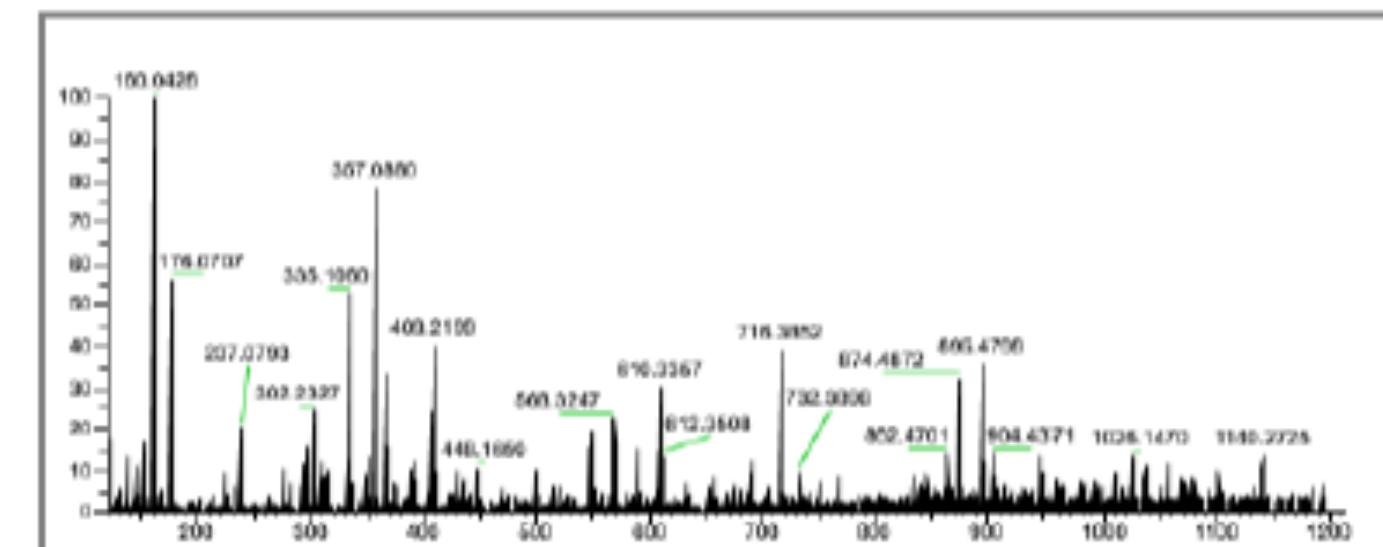
Qual ratio 2: Pass

Quan Peak: m/z335.1060 mz

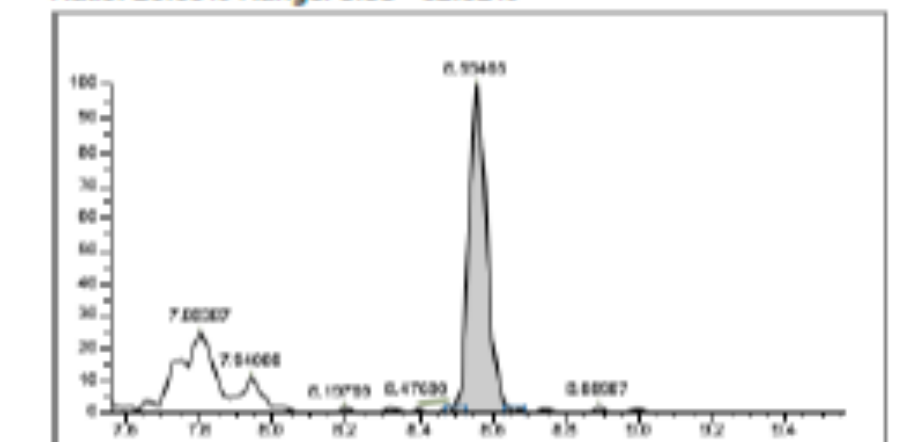
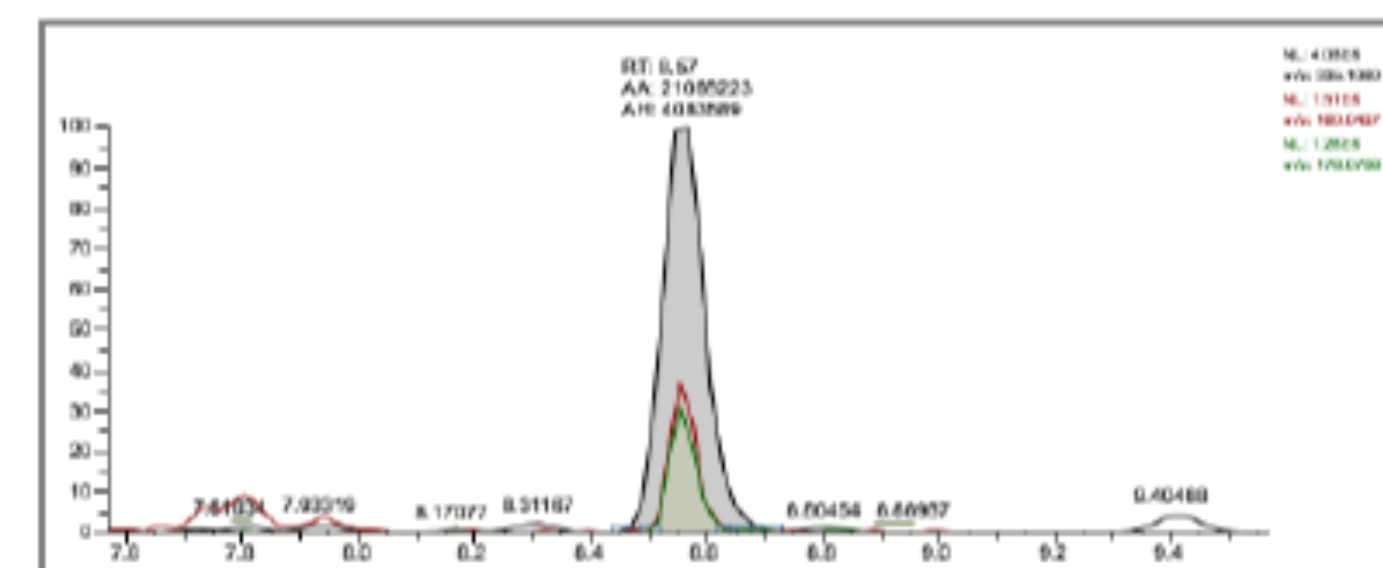


Qual Ion 1: 300.0000->160.0427 m/z
Ratio: 23.33% Range: 0.00 - 32.82%

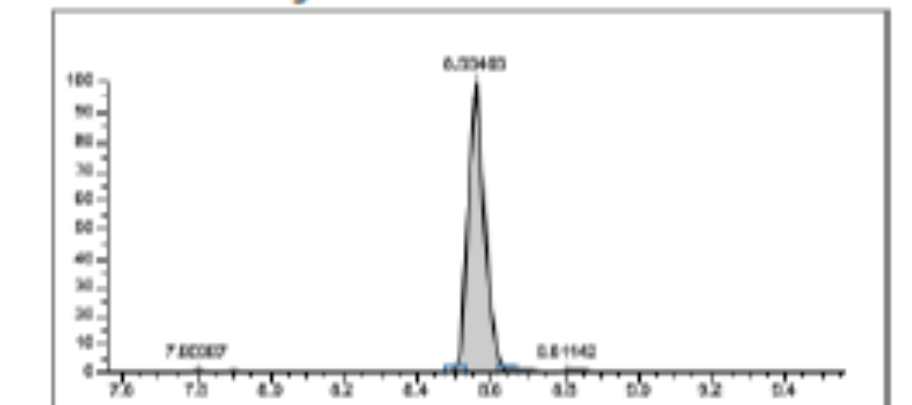
MS Spectra



Overlay



Qual Ion 2: 300.0000->176.0706 m/z
Ratio: 19.38% Range: 0.00 - 29.86%





Routine maintenance

Systems perform best when in continuous use.

Monthly Full calibration

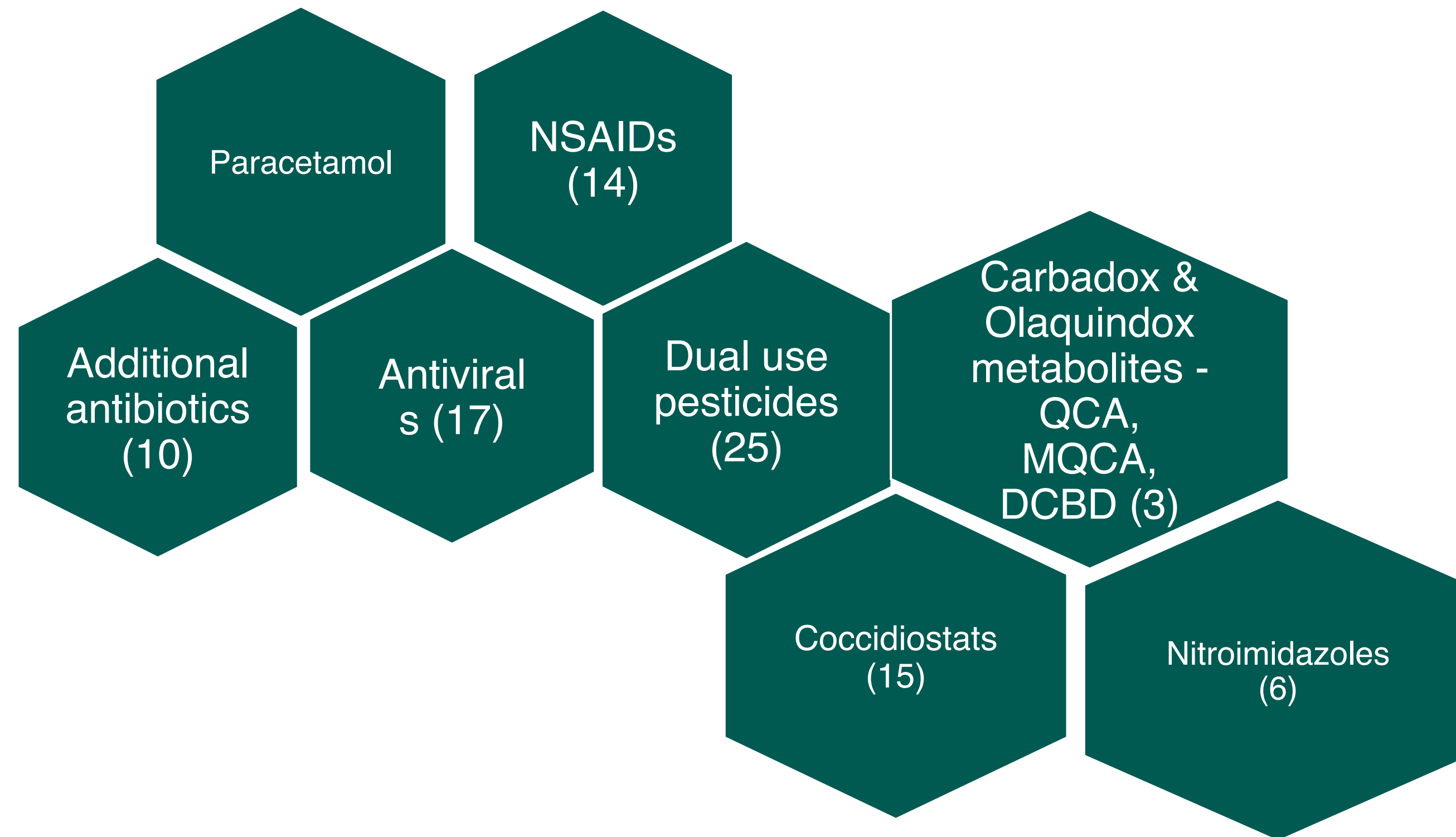
Mass calibration on day of use.

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 and 10% Acetone)

Excellent Platform for Further Expansion



Exploris 120 Muscle Development Method Positive

Time Range (min) 0-15 CLEAR

Full Scan

Intensity

Dynamic Exclusion

ddMS²

1 scans

Full Scan Properties

Orbitrap Resolution	60000
Scan Range (m/z)	100-1500
RF Lens (%)	70
AGC Target	Standard
Maximum Injection Time Mode	Custom
Maximum Injection Time (ms)	100
Microscans	1
Data Type	Profile
Polarity	Positive
Source Fragmentation	☐

Time Range (min) 0-15 CLEAR

Full Scan

Intensity

Dynamic Exclusion

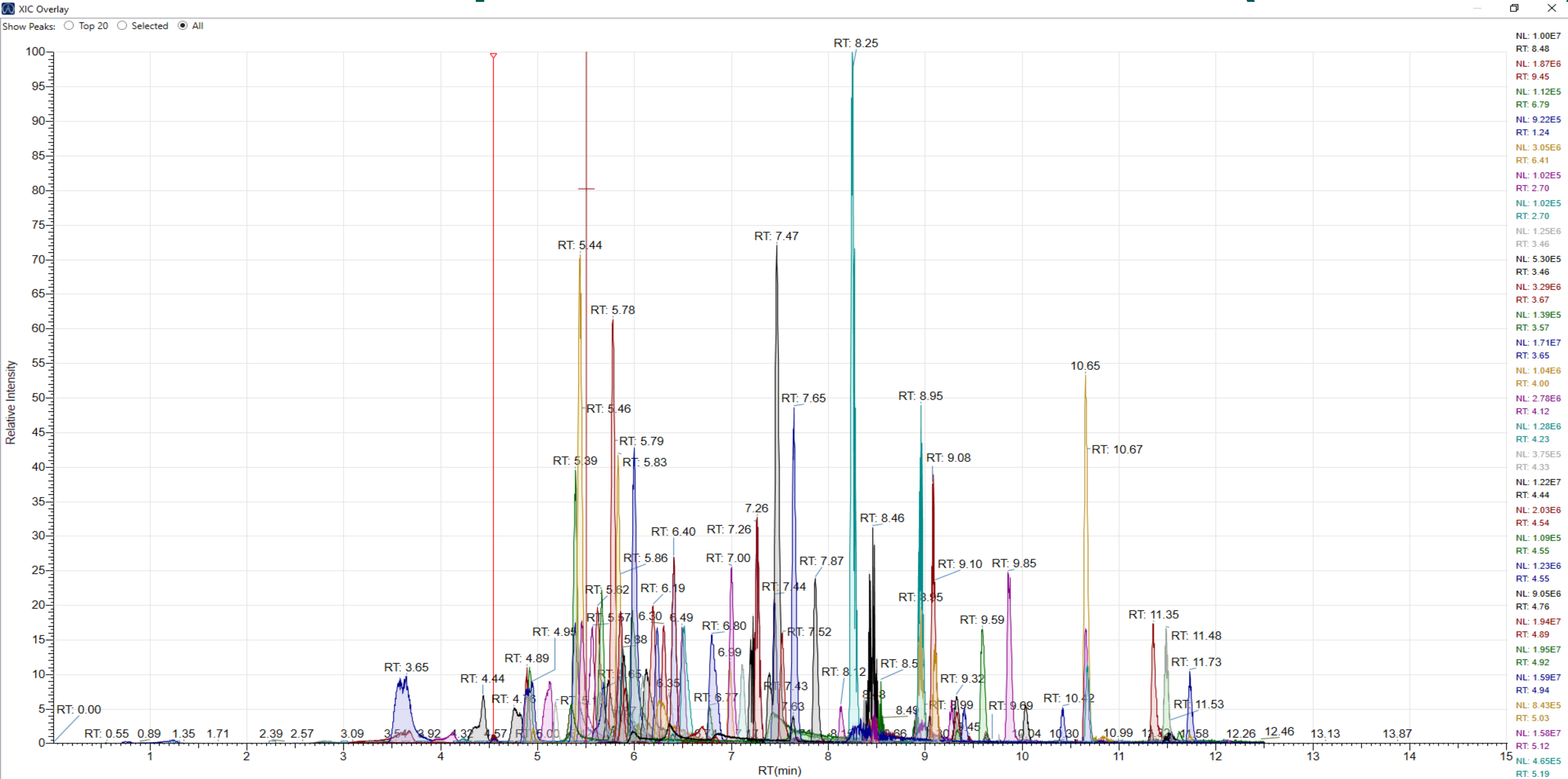
ddMS²

1 scans

Data-Dependent MS² Scan Properties

Isolation Window (m/z)	1.5
Isolation Offset	Off
Collision Energy Type	Normalized
HCD Collision Energies (%)	30,50,150
Orbitrap Resolution	15000
Scan Range Mode	Auto
AGC Target	Standard
Maximum Injection Time Mode	Custom
Maximum Injection Time (ms)	22
Microscans	1
Data Type	Profile

Muscle Development Method Positive XIC (Cal 2 Level)



Exploris 120 Muscle Development Method Negative

Time Range (min) 0-15 CLEAR

Full Scan
Intensity
Dynamic Exclusion
ddMS²

4 scans

Full Scan Properties

Orbitrap Resolution	120000
Scan Range (m/z)	100-1500
RF Lens (%)	70
AGC Target	Standard
Maximum Injection Time Mode	Custom
Maximum Injection Time (ms)	100
Microscans	5
Data Type	Profile
Polarity	Negative
Source Fragmentation	☐

Time Range (min) 0-15 CLEAR

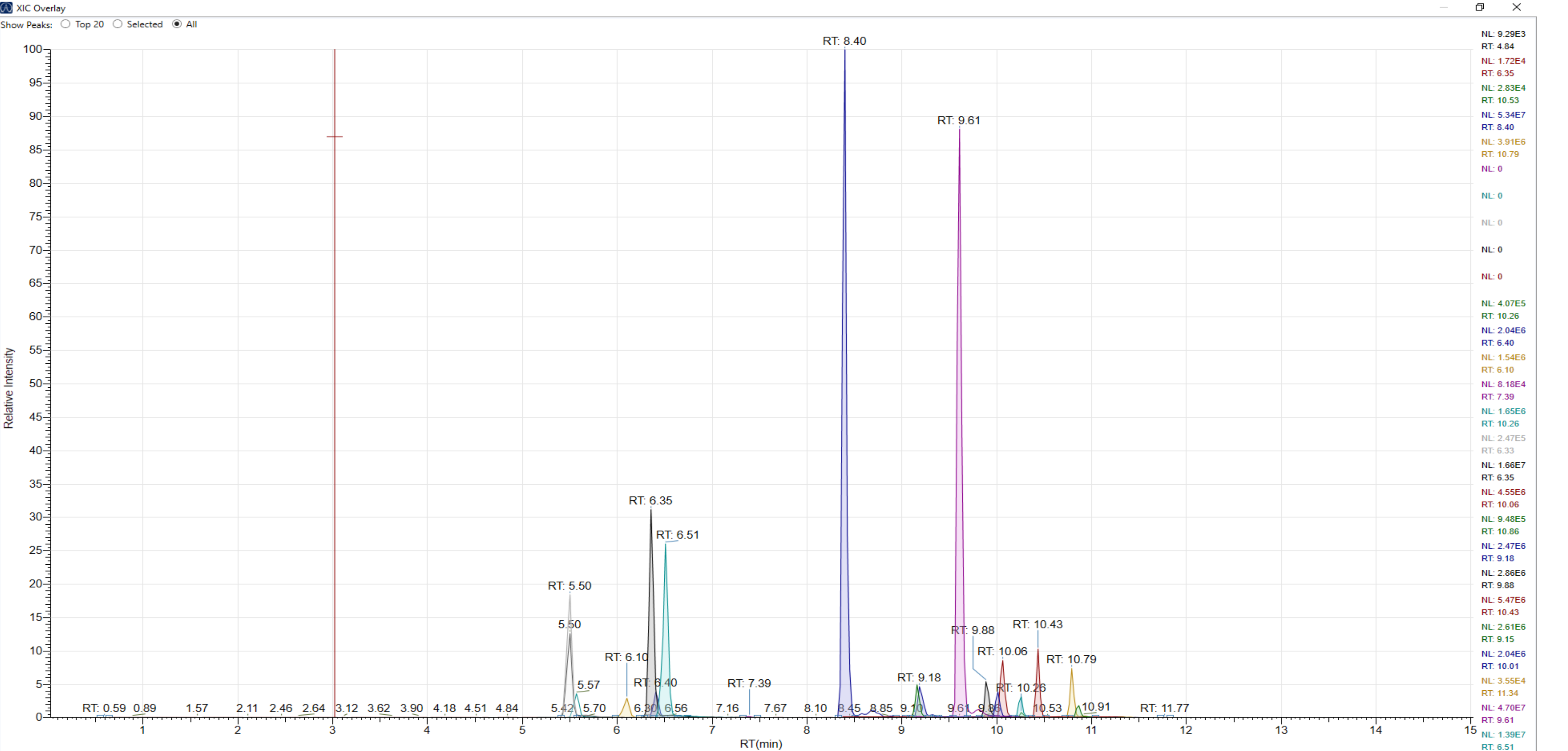
Full Scan
Intensity
Dynamic Exclusion
ddMS²

4 scans

Data-Dependent MS² Scan Properties

Isolation Window (m/z)	1.5
Isolation Offset	Off
Collision Energy Type	Normalized
HCD Collision Energies (%)	30,50,150
Orbitrap Resolution	15000
Scan Range Mode	Auto
AGC Target	Standard
Maximum Injection Time Mode	Custom
Maximum Injection Time (ms)	22
Microscans	1
Data Type	Profile

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

