FDA U.S. FOOD & DRUG ADMINISTRATION
OFFICE OF REGULATORY AFFAIRS

Applications of High Resolution MS to Veterinary Drug Residue Analysis in Aquaculture and Animal Feed

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Laboratory Methods and Services Committee
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Aquaculture

Growing industry

By 2030 over 50% of fish for human consumption will be supplied by aquaculture

Global industry

38% fish produced globally was exported in 2010

China and Southeast Asia major producers

Varied types of species

tilapia, shrimp, salmon, catfish, frog legs, eel

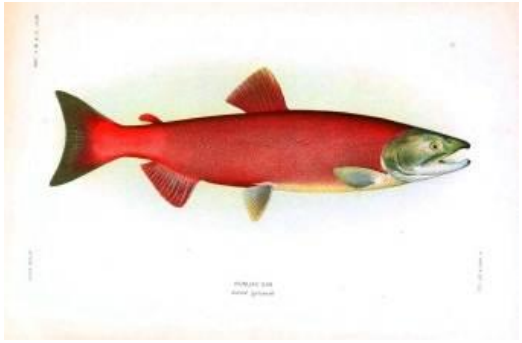
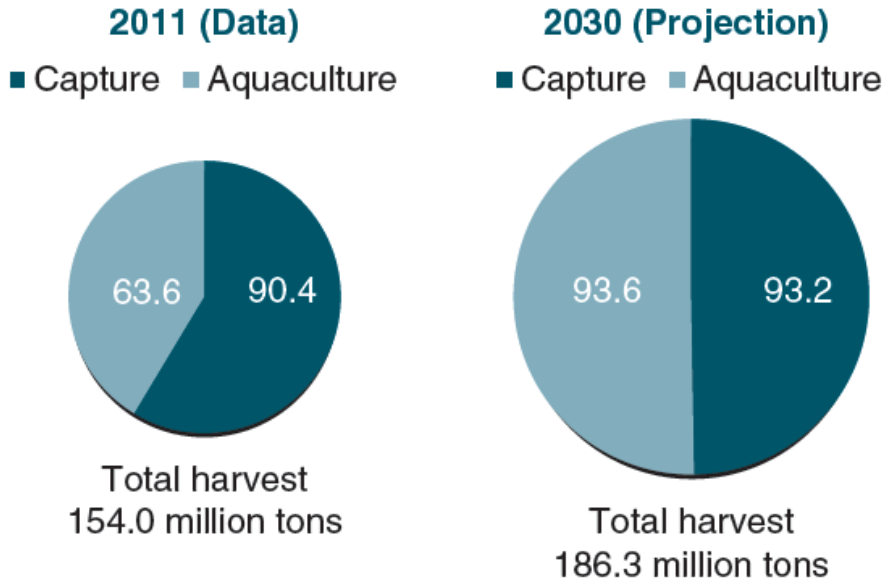


FIGURE 3.2: Volume and Share of Capture and Aquaculture Production in Global Harvest



Sources: FishStat and IMPACT model projections.

World Bank Report, *Fish to 2030: Prospects for Fisheries and Aquaculture*. 2013

Veterinary drug residues in aquaculture

Use:

To prevent spread of infection in dense populations

Approval:

- Very few drugs approved for aquaculture use in the US
- More approved in the EU and Japan
- Many more drugs potentially used in other countries

Potential human health effects:

- Acute and Chronic Effects
  - Chloramphenicol – aplastic anemia
  - Triphenylmethane dyes - carcinogenic
- **Antimicrobial Resistance**

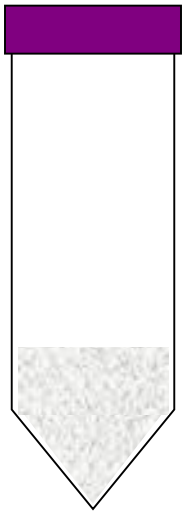
High Resolution MS: potential advantages for residue analysis

- Full scan data collection with accurate mass allows screening for virtually unlimited number of compounds.
- Don’t preselect analytes to monitor, so target and nontarget analytes are detected.
- Data can be evaluated retrospectively.
- Fragment ions can be obtained for further characterization of analyte.

Objectives for method to screen for drugs in aquaculture

- Develop analytical screening method for veterinary drug residues in fish using HRMS.
- Initially optimize and validate method for 70 test compounds most likely to be used in aquaculture.
- Use HRMS capability with vet drug database to screen samples for hundreds of additional compounds.

Extraction procedure

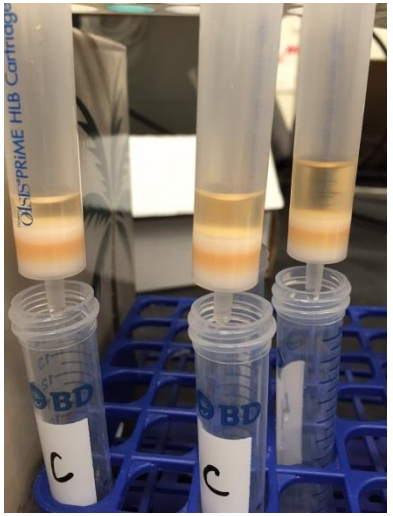


Acidic acetonitrile (ACN) extraction

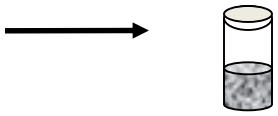
2 g tissue
Add 8 mL ACN with 0.2% p-toluene sulfonic acid and 2% glacial acetic acid
Centrifuge

OASIS HLB PRiME SPE (200 mg)

Pass 3 mL of extract through SPE
Evaporate to near dryness
(Save portion of eluent to analyze directly for nonpolar compounds)

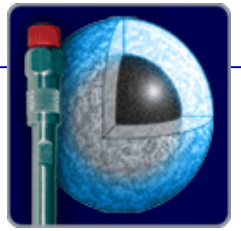


Reconstitute in 400 µL 10% ACN in water
Centrifuge
Aliquot portion to LC vial



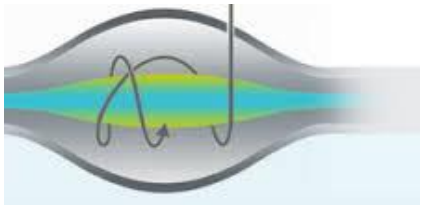
Data acquisition

LC: Thermo Ultimate 3000 LC system with C18 fused-core reversed-phase column. Mobile phase gradient 0.1 % formic acid and acetonitrile (ACN)



MS: Thermo Q-Exactive Orbitrap High Resolution MS with a heated electrospray source (using both classic QE and QE-HF)

Two types of acquisition programs were evaluated:



Nontargeted: collect product ion data for all precursor ions simultaneously *All Ion Fragmentation (AIF)* **or** sequentially by isolating segments of precursor ions *Data Independent Analysis (DIA)*

Targeted: isolate and collect product ion data only if targeted precursor ion on a list has abundance above threshold *Data Dependent MS^2 ($DDMS^2$)* **or** always when analyte is eluting *Parallel Reaction Monitoring (PRM)* using inclusion lists

Data analysis

1) Initial NonTargeted Data Acquisition with AIF or DIA

a) Targeted Data Analysis: Limit testing and identification of test compounds

- Use "TraceFinder Quant" to analyze 70-100 test compounds
- Match 5 ppm window (MS^1), 0.5 min retention time, one fragment ion (10 ppm)
- Compare to matrix-extracted standard fortified with test compounds at TTL

b) Semi-targeted Data Analysis: Expand screening for more drug residues

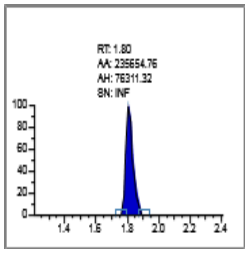
- Use "TraceFinder Screening" to search against larger analyte database (N > 450)
- Use 3 ppm window with higher signal criteria to limit detections
- Compare RT and fragment ions if known

2) Additional Targeted Data Acquisition

Data Analysis of Product Ion Spectra

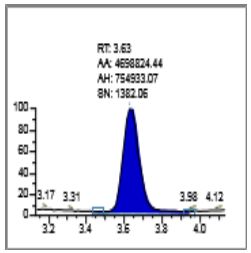
- Examine product ion spectra for analytes on inclusion list found in sample
- Use "TraceFinder "Quant" and "Screening" to compare residues to database
- Follow up with manual evaluation of spectral data and compare to known spectra

Example MS^1 data spiked sample



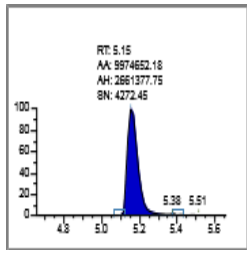
Amoxicillin

Quan Peak:	366.11182 m/z
Peak Area:	235655
RT:	1.80 min (1.80)
Amount:	95 ng/g



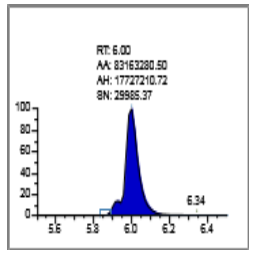
Sulfathiazole

Quan Peak:	256.02089 m/z
Peak Area:	4698824
RT:	3.63 min (3.60)
Amount:	9.5 ng/g



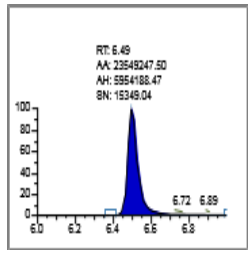
Sarafloxacin

Quan Peak:	386.13107 m/z
Peak Area:	9974652
RT:	5.15 min (5.40)
Amount:	4.6 ng/g



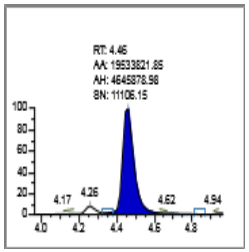
Tilmicosin

Quan Peak:	435.29030 m/z
Peak Area:	83163280
RT:	6.00 min (6.09)
Amount:	44 ng/g



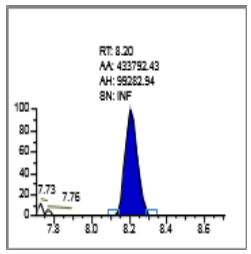
Oxolinic acid

Quan Peak:	262.07100 m/z
Peak Area:	23549248
RT:	6.49 min (6.60)
Amount:	9.5 ng/g



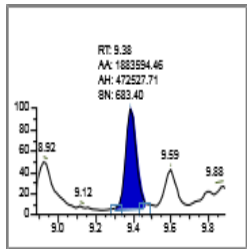
Oxytetracycline

Quan Peak:	461.15546 m/z
Peak Area:	19533822
RT:	4.46 min (4.70)
Amount:	93 ng/g



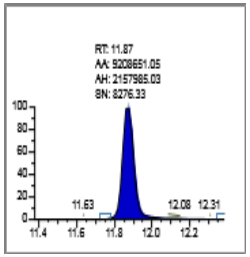
Leucomalachite green

Quan Peak:	331.21688 m/z
Peak Area:	433792
RT:	8.20 min (8.24)
Amount:	0.78 ng/g



Methyl testosterone

Quan Peak:	303.23186 m/z
Peak Area:	1883594
RT:	9.38 min (9.40)
Amount:	0.37 ng/g



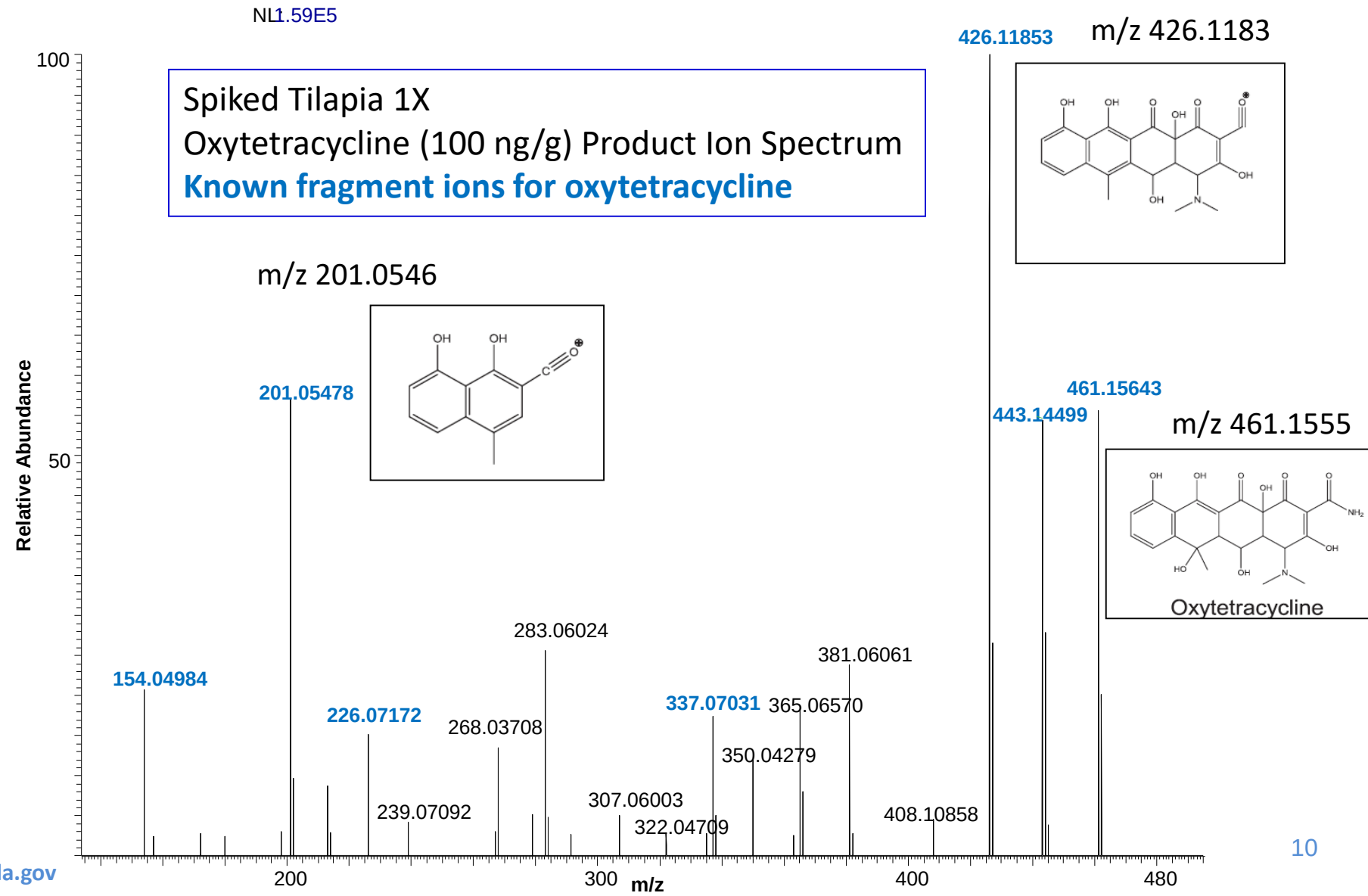
Ivermectin B1a

Quan Peak:	897.49708 m/z
Peak Area:	9208651
RT:	11.87 min (12.00)
Amount:	130 ng/g

Tilapia spiked with 70 compounds at target testing level.

MS^1 data shown.
Also collected MS^2 data and evaluated time and isotopic match.

MS^2 data for spiked sample



Validation of method

Fortified samples:

- 70 validation compounds (60 positive ion; 10 negative ion) in 5 species, 2-3 sources for each species of fish
- Fortified at target testing level (1X) to determine threshold for limits test (**Semi-quantitative screen with MS identification**)
- Also fortified at 2X, 0.5X, and 0.1X to determine minimum detection levels and lowest confirmation levels
- Determined false positive and false negative rates; approximate recoveries compared to solvent standards

Based on

- *FDA OFVM Guidelines for Validation of Chemical Methods v2*
- *Acceptance Criteria for Confirmation of Identity of Chemical Residues using Exact Mass Data for the FDA FVM Program*

J Ag. Food Chem. (2017)

Comparison of data acquisition

Residues confirmed at 1X target testing level

Confirmed = MH^+ (5 ppm), one fragment (10 ppm), RT match

Nontargeted

- > 90% validation compounds confirmed at 1X with AIF
- Most confirmed with AIF at much lower levels (0.1-0.5X of target testing level)
- Recently compared different DIA methods to AIF with similar results

Targeted

- ~ 70% of validation compounds depending on matrix with $DDMS^2$
- Compounds with low target testing levels (dyes) or low method recovery(β-lactams) don't meet threshold to trigger $DDMS^2$
- Some confirmed at higher levels
- Recently compared PRM (limited # of compounds) to $DDMS^2$ w/ better results

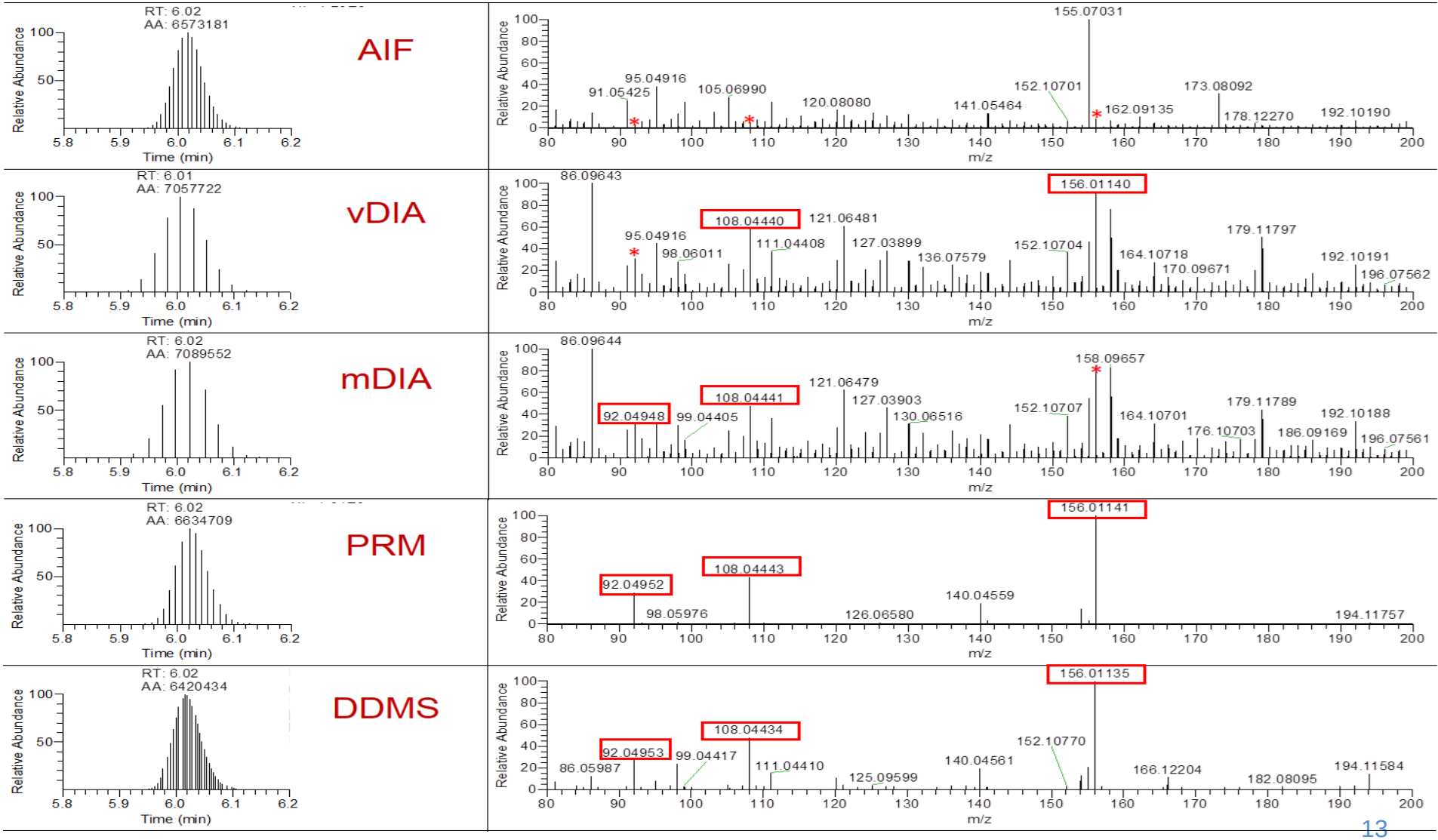
Continue to improve method by exploring different data acquisition methods

Comparison of scan types

Sulfadoxine 10 ng/g in spiked eel

EIC of MS1 (m/z 311.0809)

MS2 Spectra



Submitted to Rapid Commun. MS (2019)

Application of HRMS screen

- Analyze incurred aquaculture samples obtained from CVM.
  - Analyzed dosed salmon, trout, catfish
  - Detected and characterized metabolites in addition to parent compounds
- Applied method to violative regulatory samples
- Include additional analytes in method (beyond veterinary drugs)

Application of method: incurred fish

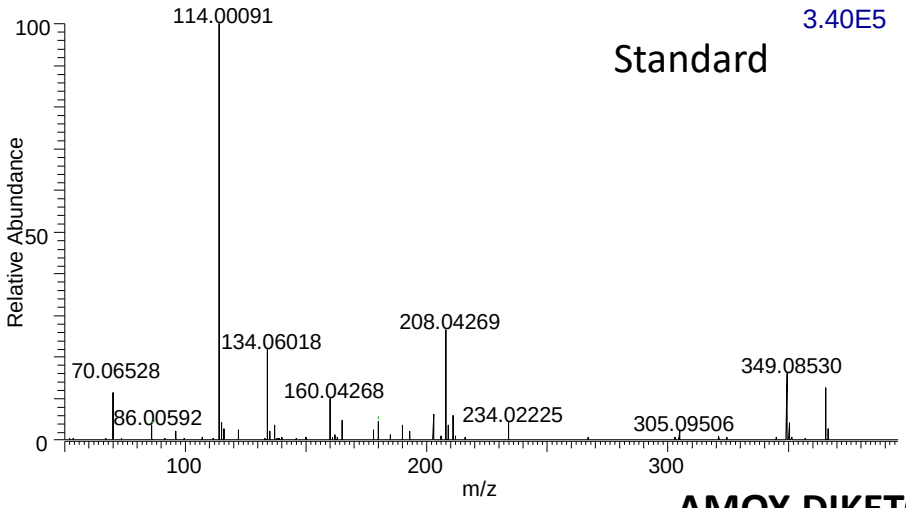
Fish	Dosed with	Test Compounds found by HRMS Screen (ng/g)*	Other compounds found by HRMS
Tilapia	Sulfadiazine	Sulfadiazine (220)	N^4 acetyl sulfadiazine, Ethoxyquin Dimer
Catfish	Enrofloxacin	Enrofloxacin (600) Ciprofloxacin (30)	Desethylene enrofloxacin
Salmon	Difloxacin	Difloxacin (102) *Sarafloxacin (1)*	
Salmon	Doramectin	*Doramectin (23)*	
Salmon	Malachite green, Brilliant green, Crystal violet	Malachite green (2) Leucomalachite green (0.8) Brilliant Green (4)	
Trout	Ampicillin	Ampicillin (125)	
Trout	Amoxicillin	Amoxicillin (90)	Amoxicillin diketone

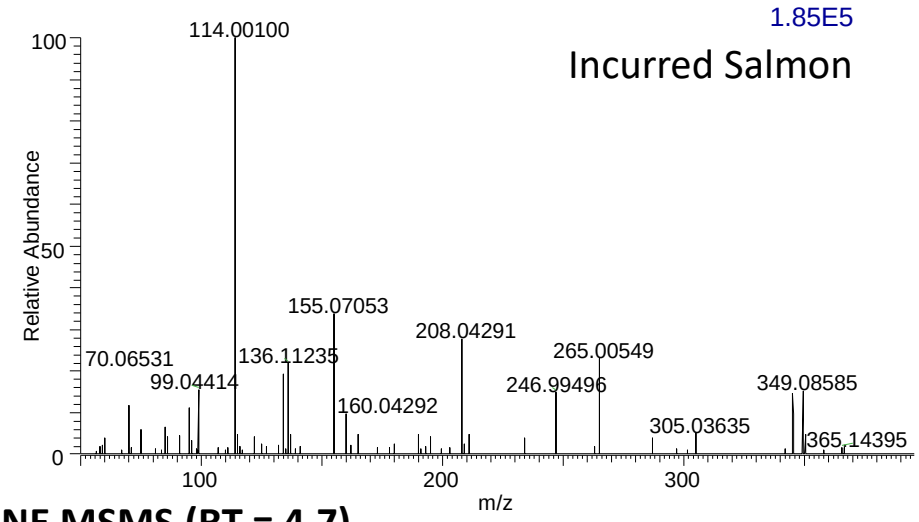
**The concentration of test compounds found by HRMS screen compared well to values obtained by QqQ methods (when available)*

Analytical Bioanalytical Chem. (2018)

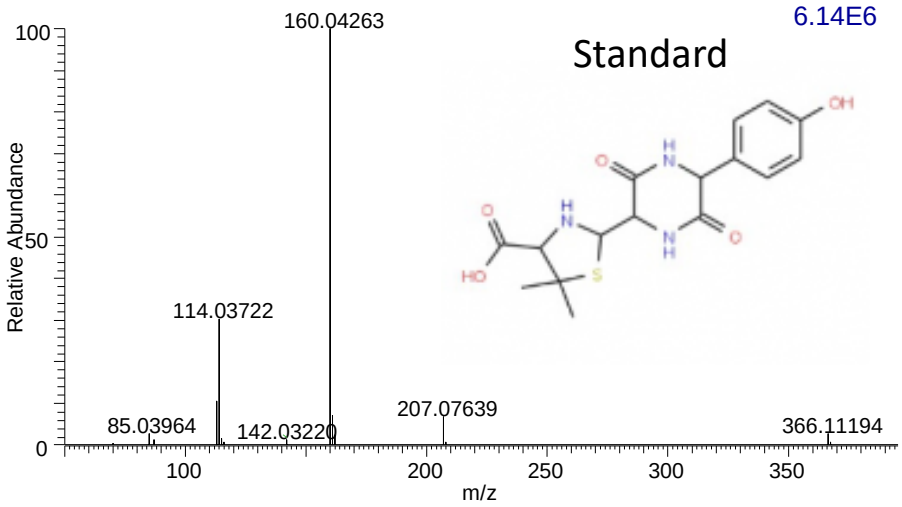
Amoxicillin incurred fish

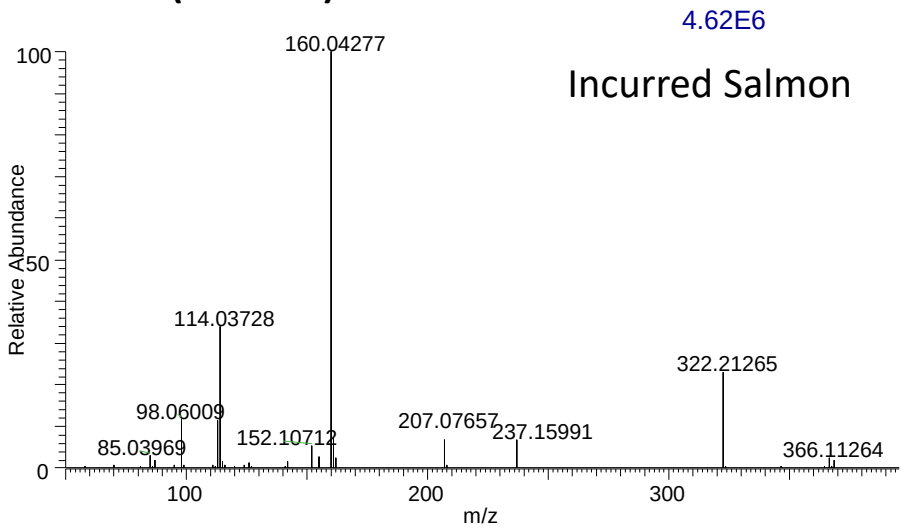
AMOX MSMS (RT = 1.9)





AMOX DIKETONE MSMS (RT = 4.7)





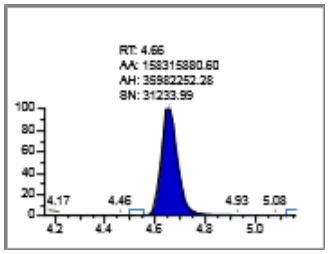
Application: Imported eel sample

- Farm raised eels are susceptible to the use of chemotherapeutics because they are raised in confined spaces (tanks or barrels)
- Multiple veterinary drug residues have been found in imported eel samples using targeted LC-MS/MS method (triple quadrupole)
- **Can we use HRMS screening method to determine what other residues or chemical contaminants might we be missing?**

Image: Chang W. Lee/The New York Times (7/3/2007)

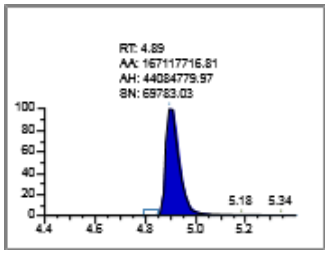
Application: Imported eel sample

Presumptive positive for test compounds



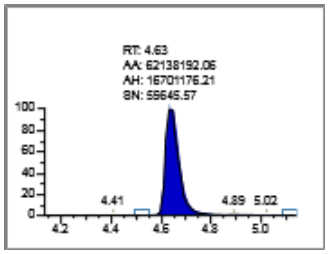
Sulfamethazine

Quan Peak: 279.09102 m/z
RT: 4.66 min
Amount: 85 ng/g



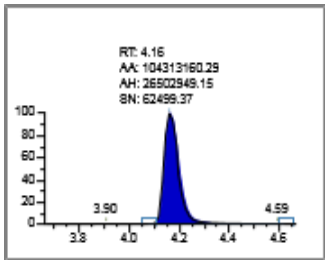
Enrofloxacin

Quan Peak: 360.17180 m/z
RT: 4.89 min
Amount: 58 ng/g



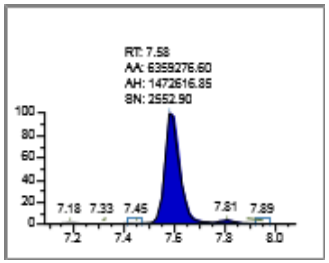
Ciprofloxacin

Quan Peak: 332.14050 m/z
RT: 4.63 min
Amount: 44 ng/g



Trimethoprim

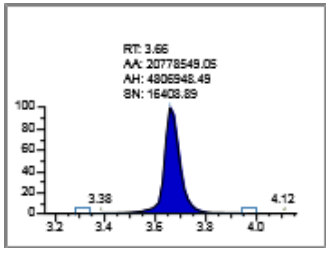
Quan Peak: 291.14517 m/z
RT: 4.16 min
Amount: 22 ng/g



Ethoxyquin

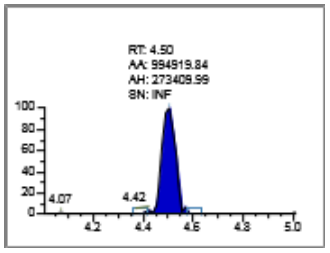
Quan Peak: 218.15394 m/z
RT: 7.58 min
Amount: 87 ng/g

Other test compounds found (< 50% TTL)



Lincomycin

Quan Peak: 407.22103 m/z
RT: 3.66 min
Amount: 11 ng/g

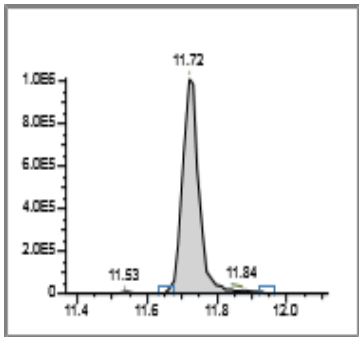


Oxytetracycline

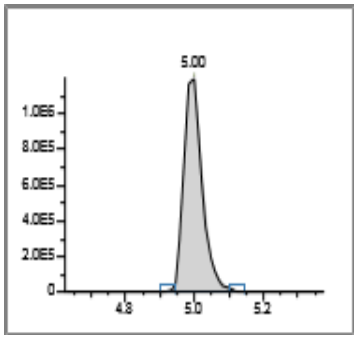
Quan Peak: 461.15546 m/z
RT: 4.50 min
Amount: 1.8 ng/g

Data from eel sample

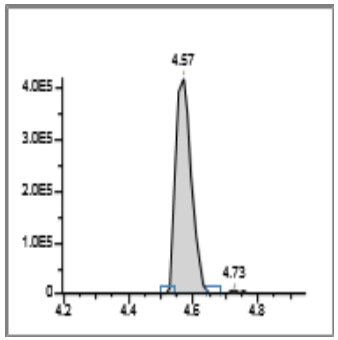
From screening larger database compounds (N ~450):



Ethoxyquin Dimer
AA: 3341515
RT: 11.72 min
m/z: 433.285 (433.285)
D m/z (ppm): 0.05

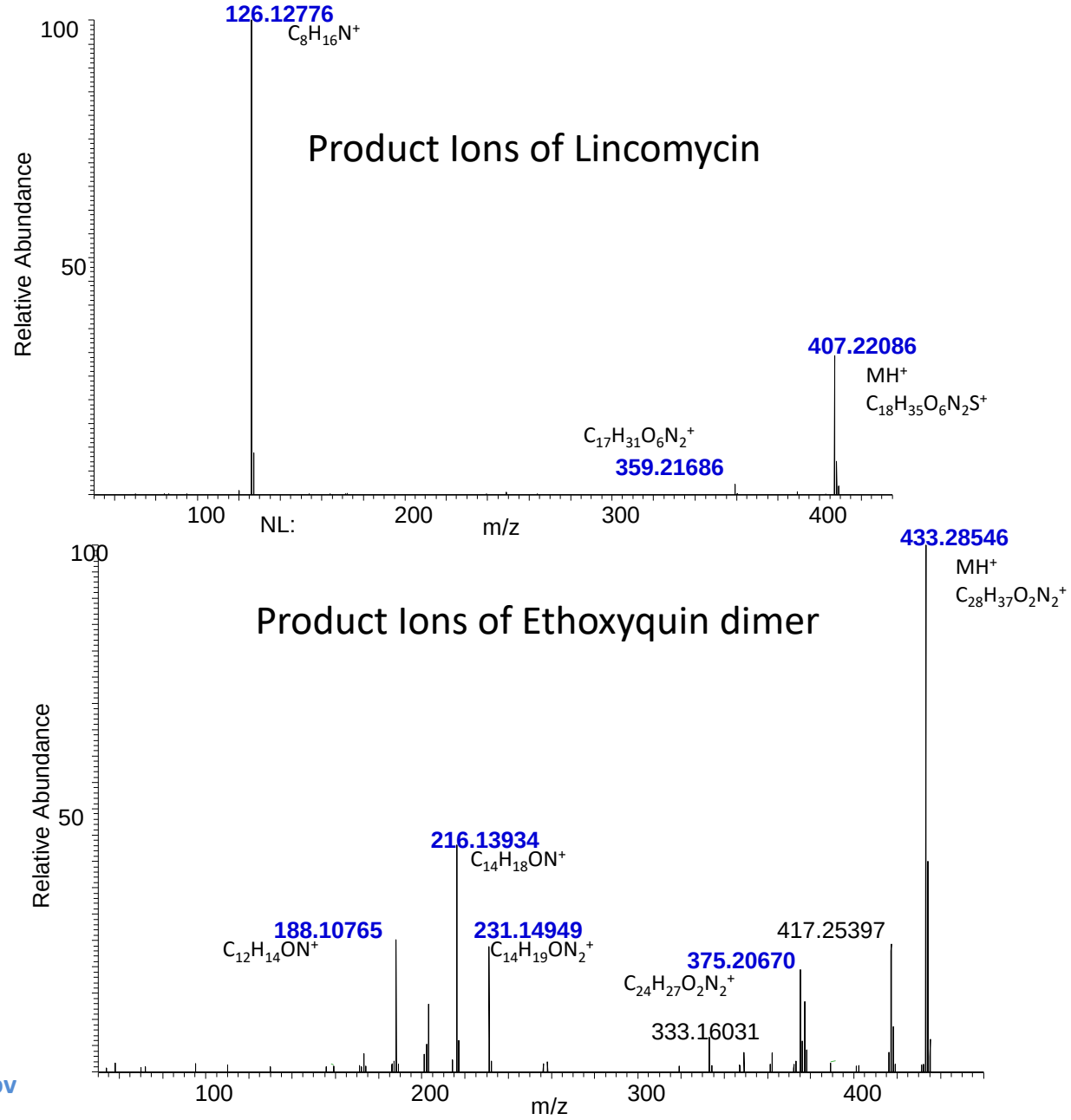


N4-acetyl-sulfamethazine
AA: 4372013
RT: 5 min
m/z: 321.1013 (321.1016)
D m/z (ppm): -0.85



Desethylene Enrofloxacin
AA: 1424542
RT: 4.57 min
m/z: 334.157 (334.1562)
D m/z (ppm): 2.4

Targeted MS2 data from eel

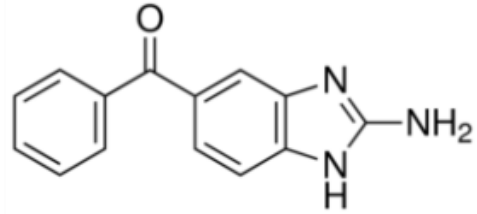


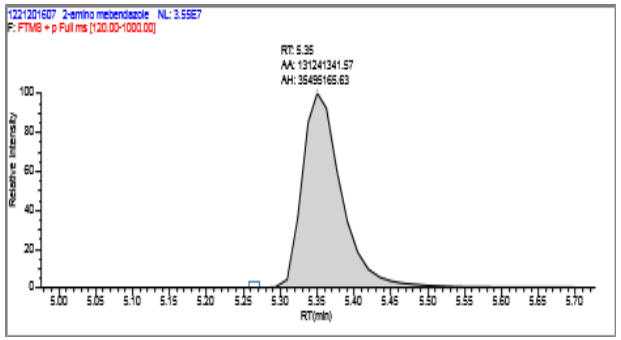
Retrospective data analysis of sample

- Other potential hits included **2-amino mebendazole (+)** but we did not initially have retention time or known fragment ions for this compound.

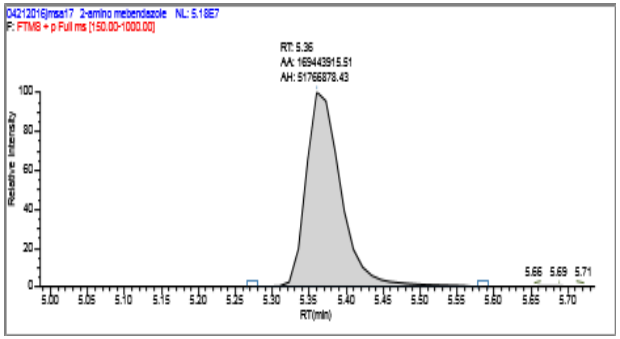
- After obtaining and analyzing standards of 2-amino mebendazole we reevaluated the data from eel samples. 2-amino mebendazole was confirmed (time and fragment ions match)

2-amino mebendazole





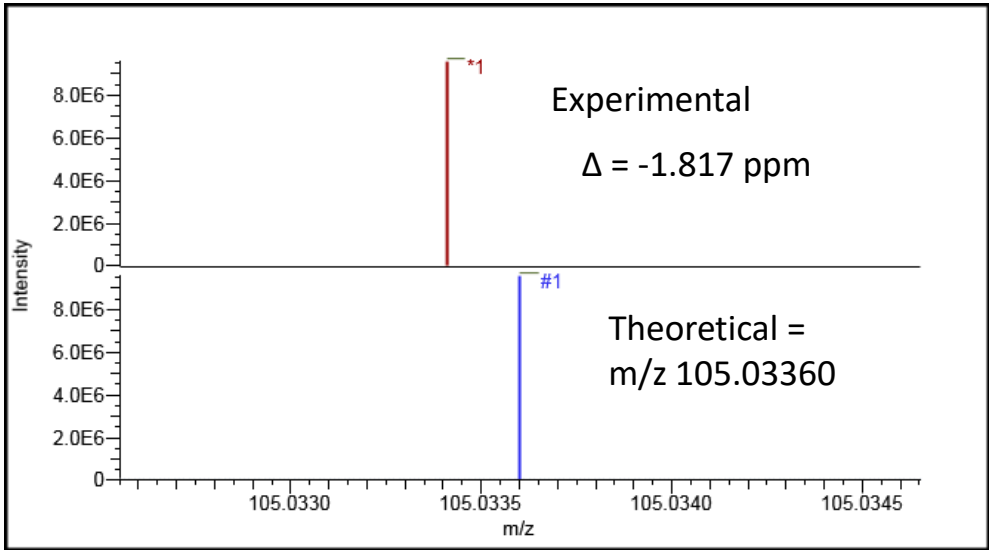
100 ng/mL std



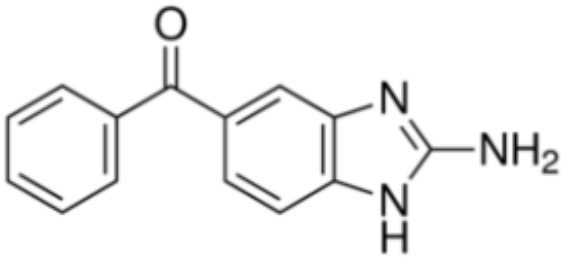
Eel #1

2-amino mebendazole in eel

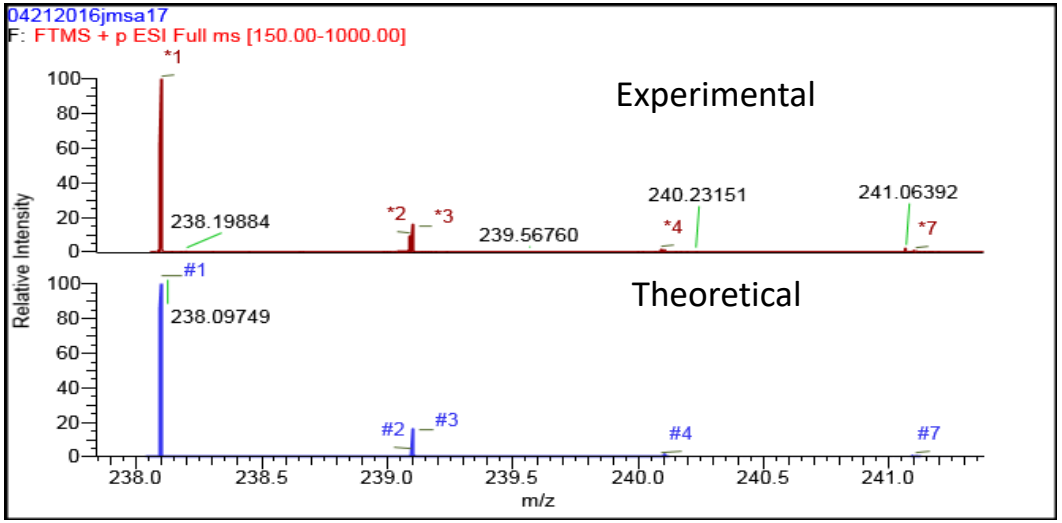
PRODUCT IONS



AIF Data



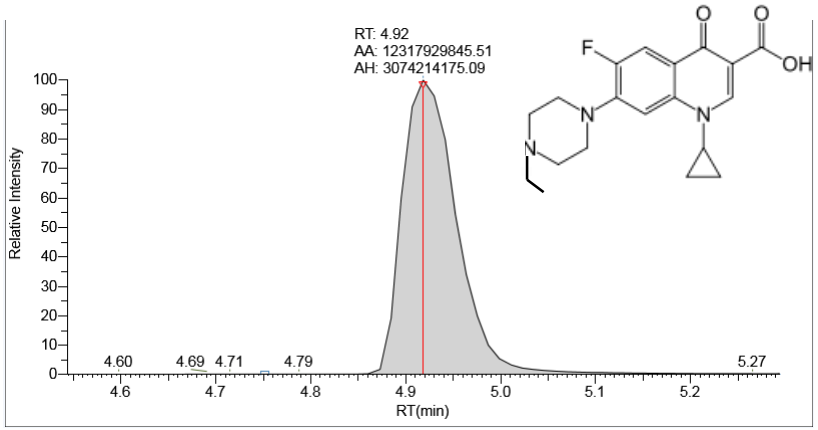
ISOTOPE IONS



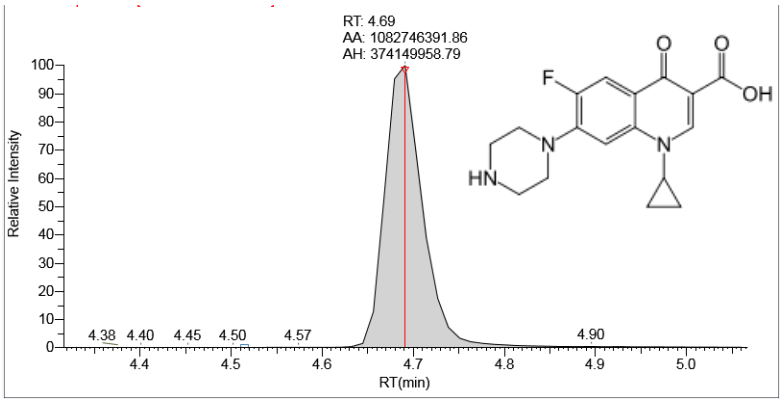
Previous Work : Residue study of mebendazole and its metabolites in eel after bath treatment, *Drug Metab Disp*. **1997**

2-amino mebendazole has since been added to routine FDA QqQ regulatory method

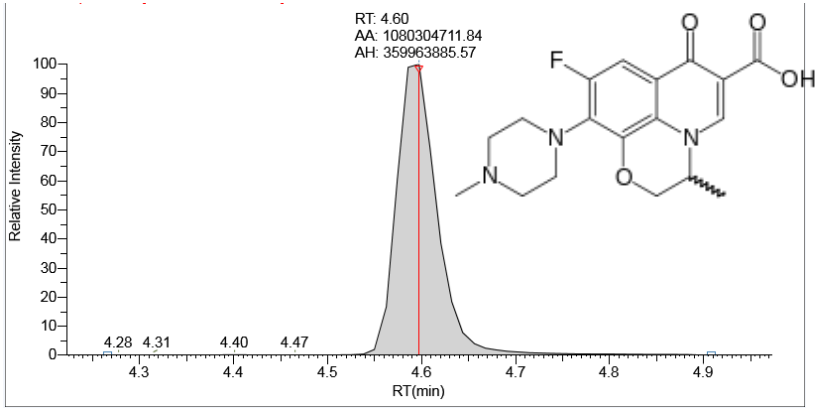
Data from imported fish sample



Enrofloxacin, > 3000 ng/g

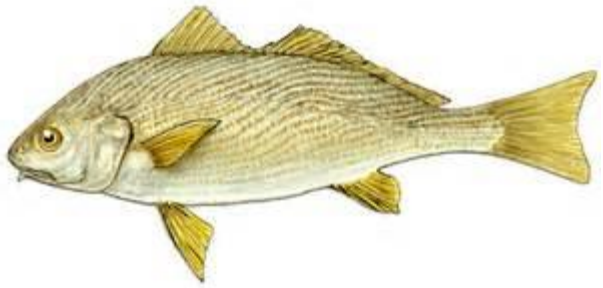


Ciprofloxacin, ~500 ng/g



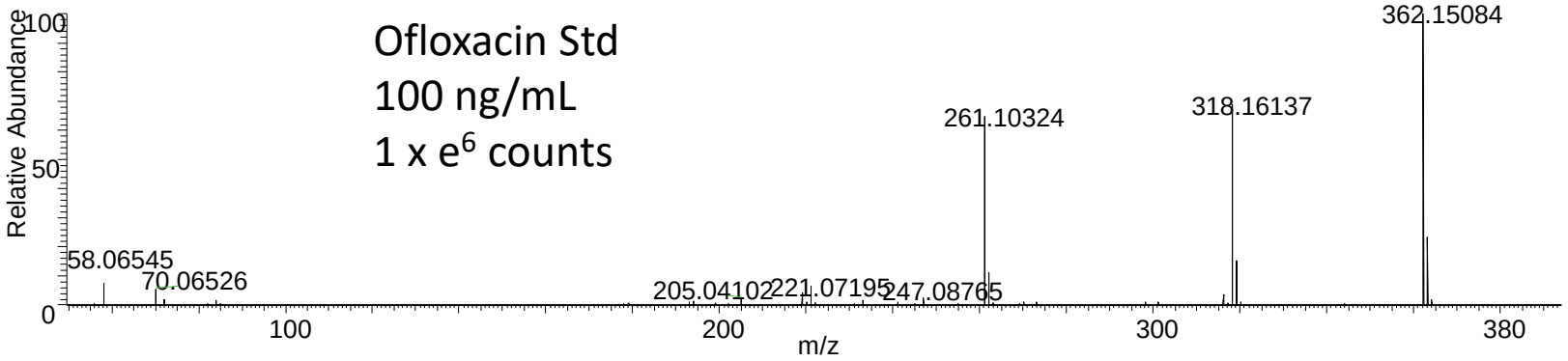
Ofloxacin, ? ng/g

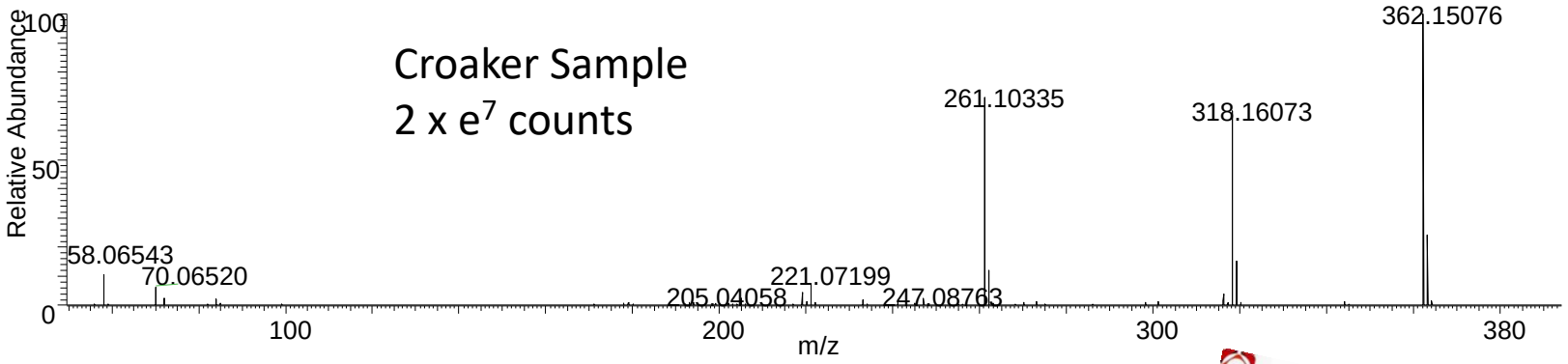
Similar area counts to ciprofloxacin



Yellow Croaker

Data from imported fish sample





Ofloxacin

Not typically used in aquaculture, although formulations are available on-line.

Residues in the environment

Table 1. Concentrations of the Four Typical Fluoroquinolone Antibiotics Detected in the Sewage Water and Surface Water Samples

	Sibao STP (ng/L)		surface water (ng/L)		
compounds	influent	effluent	site 1	site 2	average
ofloxacin	1405	429	51.6	45.7	48.7
norfloxacin	248	96	7.0	12.9	10.0
ciprofloxacin	268	199	9.3	11.0	10.2
enrofloxacin	108	54	10.5	18.7	14.6
total FQs	2029	778	78.4	88.3	83.5

Tong et al. *J Ag Food Chem* (2011) 59, 7303

- Ofloxacin has also been found in sewage water and surface water in China and many other parts of the world
- Environmental contamination could be another potential source of residues in fish

Expanding method

Validating for addition chemical contaminants

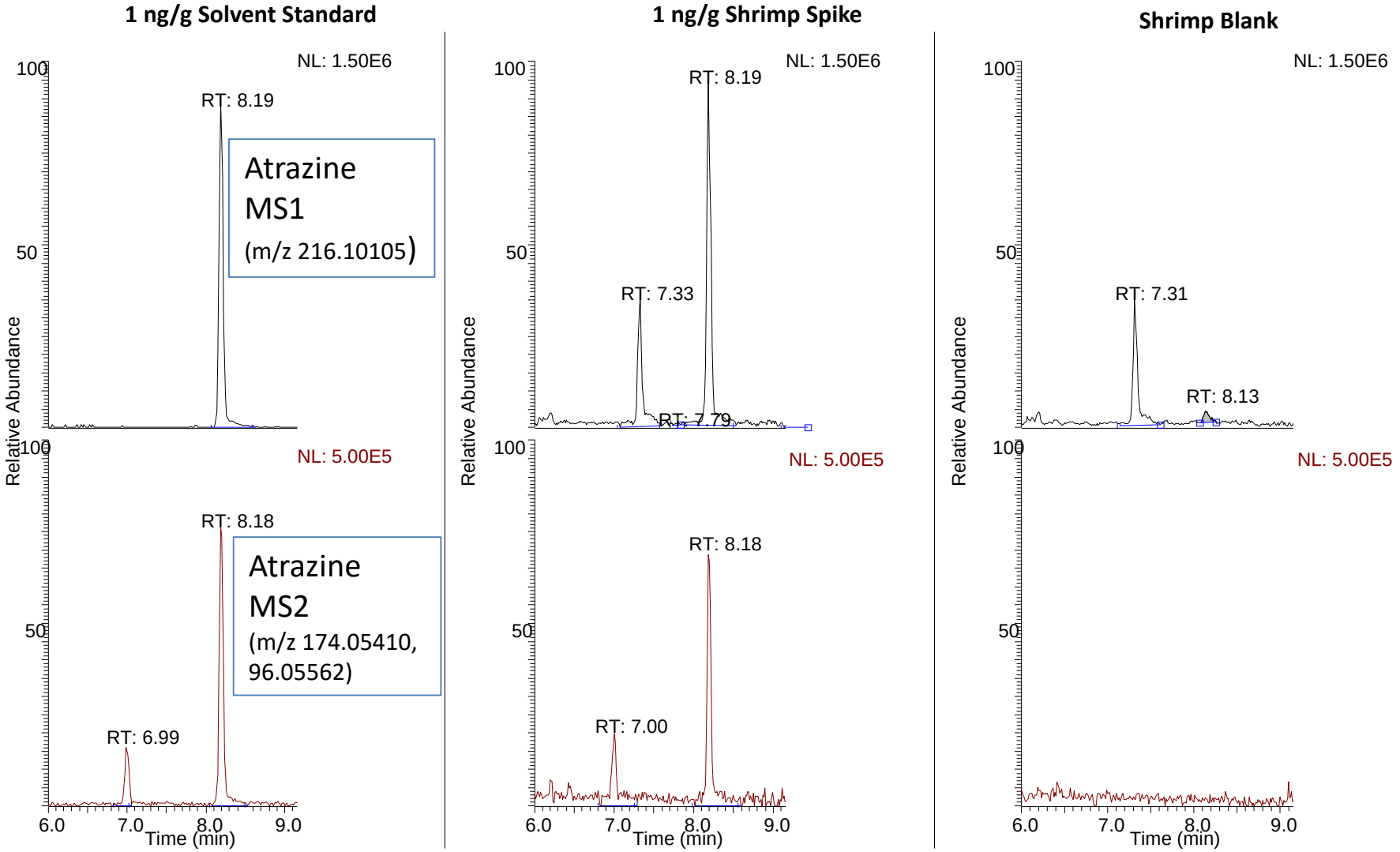
- **Disinfectants/Antimicrobial Soaps**
  - Benzalkonium chlorides, triclocarban, triclosan

- **Pesticides**
  - Few dozen likely to be found in aquaculture from agricultural run-off
  - LC-MS compounds

- **Human Pharmaceuticals/Emerging Contaminants**
  - Those commonly found in surface water
  - Includes drugs for depression, hypertension, pain

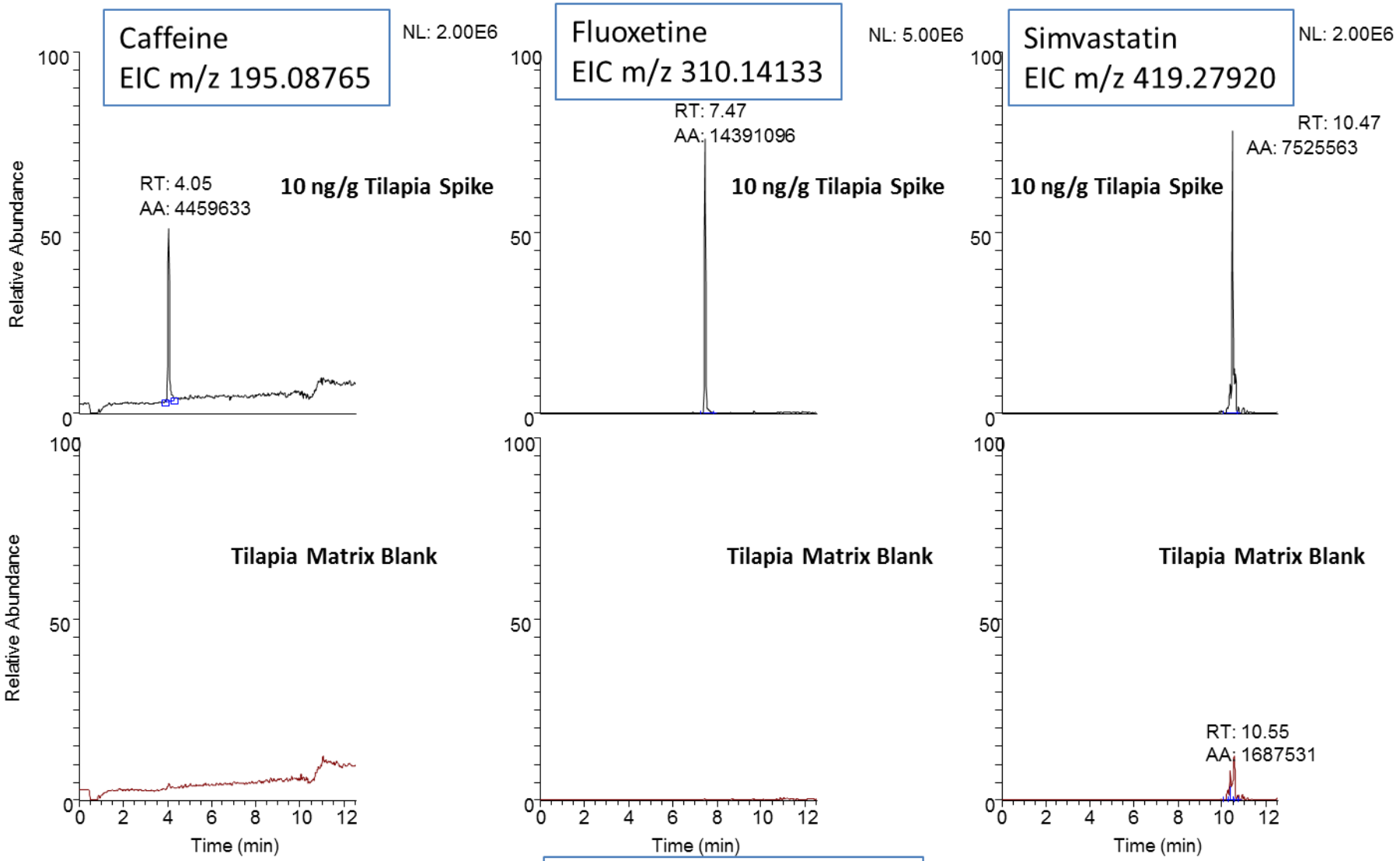
- **Additional Veterinary Drug Compounds**
  - More antibiotics, anti-wormers, etc.

Example: Atrazine in shrimp



(5 ppm window)

Example: Human drugs in tilapia



MS^1 (5 ppm window) AIF

Expanding method

Validating for additional chemical contaminants

~~1,3-Dibromo-5,5-dimethylhydantoin~~
~~1,3-Dichloro-5,5-dimethylhydantoin~~
Benzalkonium chlorides
Triclocarban
Triclosan
Amitraz (degradant)
Atrazine
Azadirachtin
Azamethiphos
Benzocaine
Carbaryl
Carbofuran
~~Cypermethrin~~
Dichlorvos
~~Etofenprox~~
Fipronil/Fipronil sulfone
Malathion
Phoxim
Praziquantel
Propazine
Quinalphos
Simazine
Trichlorfon
~~Trichloroisocyanuric acid~~
~~Trifluralin~~
Quinoclamine

Atenolol
Caffeine
Carbamazepine
Clarithromycin
Clofibric acid
Diclofenac
Diltiazem
Diphenhydramine
Fluoxetine

Gemfibrozil
Ibuprofen
Metformin
Naproxen
Propranolol
Ranitidine
Sertraline
Simvastatin
Sotalol
Valsartan

Rifampin
Aldicarb/Aldicarb sulfone/Aldicarb sulfoxide
Methylene blue
Acriflavine/Proflavine
Rotenone
Thiabendazole
Sulfisoxazole
Rifaximin
Roxithromycin
Marbofloxacin
Orbifloxacin
Baquiloprim
Virginiamycin M1

- Initially ~ 60 additional compounds
- The majority worked well through the method, some ~~were not detected~~, and others were detected only at higher levels
- Tested 4 different fish fortified at 100, 10 and 1 ng/g
- This increased the number of residues validated for our method and expands the scope of the type of contaminants we are monitoring for in aquaculture.

Food Addit. Contam. (2019)

Expanding method

Detection of additional chemical contaminants

Using HRMS screening method, several eel samples were initially presumptive positive for additional chemical contaminants. (HRMS identification criteria were met using non-targeted data acquisition)

- Further analysis (targeted MS^2 data acquisition, standard addition, analysis on separate QqQ method) **confirmed thiabendazole** (~ 6 ng/g) in one eel sample.

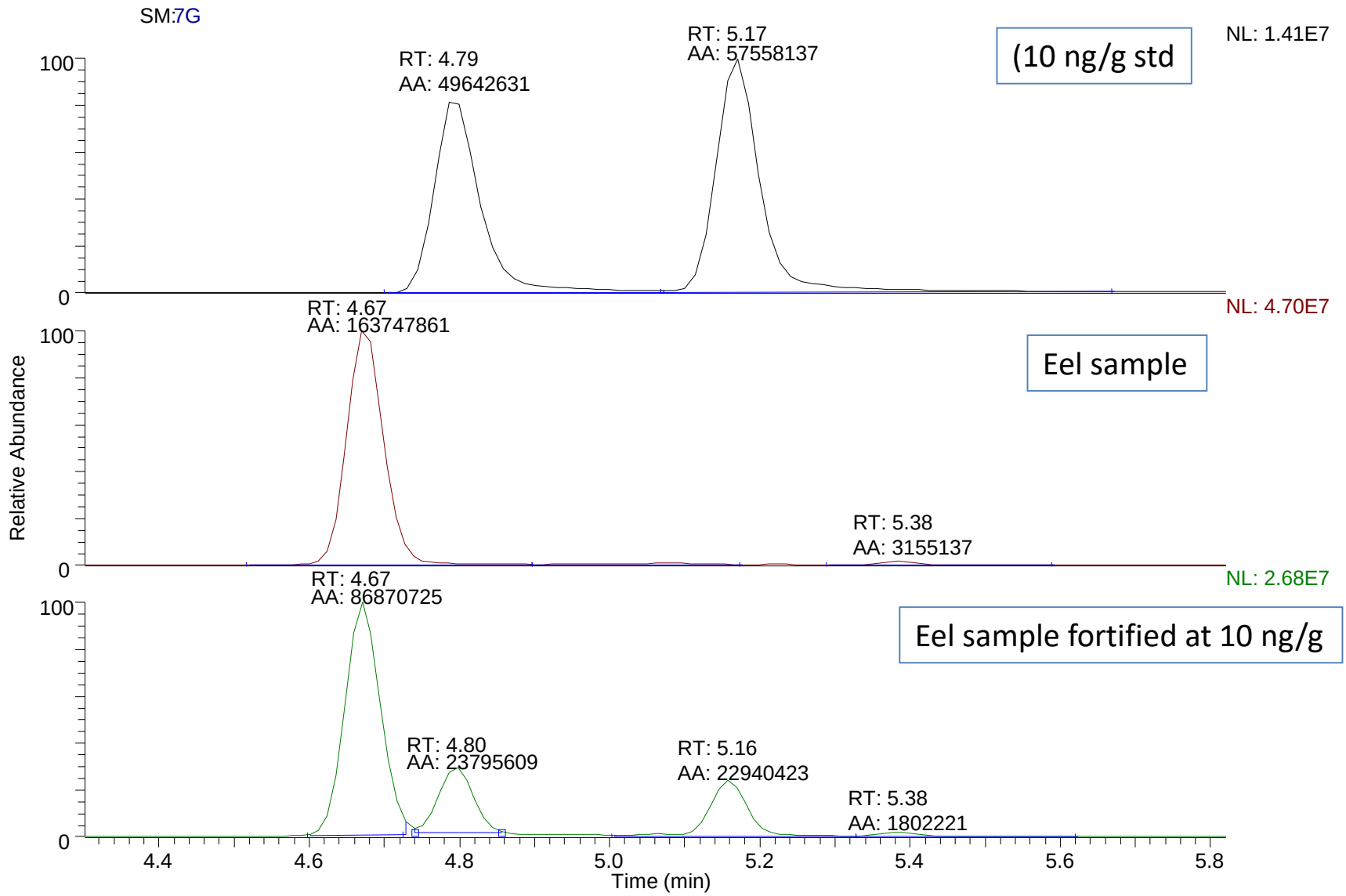
- **Acriflavine was** presumptive positive in many eel samples, but further analysis (targeted MS^2 data acquisition, standard addition) **ruled out** the presence of this compound.

- Trace levels (< 1 ng/g) of **diltiazem** were detected in another eel sample.

Expanding method

Acriflavine in Eel?

m/z 224.1182



HRMS screening method for aquaculture

- HRMS screening method was able to identify test compounds in aquaculture at or below their target testing level.
- FDA Office of Foods and Vet Medicine guidance documents were followed to develop and validate methods.
- Detection and identification of other residues including metabolites demonstrated ability to expand screening in aquacultured products.
- Will begin to look at more nontargeted data analysis workflow
- Continue working to implement HRMS technology to improve enforcement of food safety.

HRMS methods for antibiotics and chemical contaminants in animal feed

"Analysis of veterinary drug and pesticide residues in animal feed by high-resolution mass spectrometry: comparison between time-of-flight and Orbitrap" **(2015) Gómez-Pérez et al.,** *Food Addit Contam A* 32:1637

Table 4. Results obtained from the analysis of 18 feed samples. Concentrations expressed as μg kg^{-1}.

Compound	M1	M2	M5	M11	M15	M16	M17	MRL[a]
Chlorpyrifos	52 (65)[b]	18 (18)				75 (92)	148 (193)	5000[c]
Sulfadiazine			1053 (1114)	193 (217)				
Trimethoprim			311 (225)	157 (72)				
Robenidine		5912 (4186)		36 (12)				6600[d]
Monensin Na	144 (124)	715 (315)			142 (239)	141 (189)	100 (84)	1250[d]

Notes: [a] MRL, maximum residue level.
[b] Concentrations obtained with TOF are given in brackets.
[c] Value provided for *Codex Alimentarius* for primary animal feed commodities.
[d] EU MRL.

Similar strategies using HRMS have been used to monitor for chemical contaminants in animal feed

HRMS methods for antibiotics and chemical contaminants in animal feed

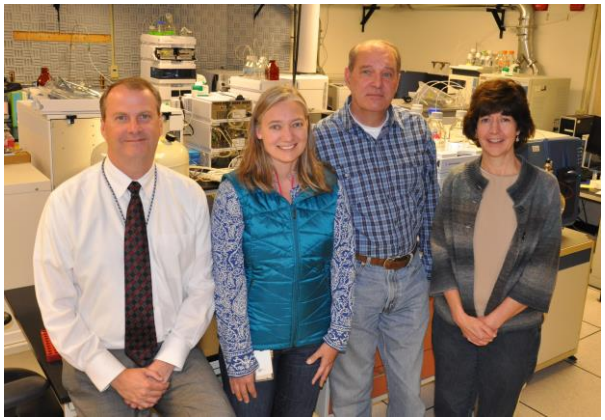
"Target analysis and retrospective screening of veterinary drugs, ergot alkaloids, plant toxins and other undesirable substances in feed using liquid chromatography–high resolution mass spectrometry" **(2016) León et al.** *Talanta* 149:43

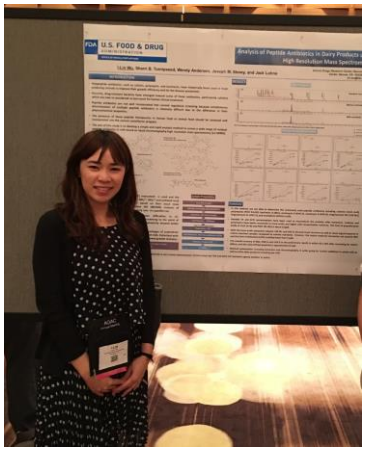
For post-target screening a customised theoretical database including the exact mass, the polarity of acquisition and the expected adducts was built and used for post-run retrospective screening. The analytical strategy was applied to 32 feed samples collected from farms of the Valencia Region (Spain). Florfenicol, zearalenone and atropine were identified and quantified at concentrations around 10 μg kg^{-1}. In the post-target screening of the real samples, Sulfadiazine, Thrimetoprin and Pirimiphosmethyl were tentatively identified.

Another example of HRMS been used to monitor for chemical contaminants in animal feed

Acknowledgements

Co-authors (Animal Drugs Research Center/FDA Denver Laboratory)





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