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Contaminants of Emerging Concern in WNS Bats

High Performance Liquid Chromatography in Environmental Monitoring

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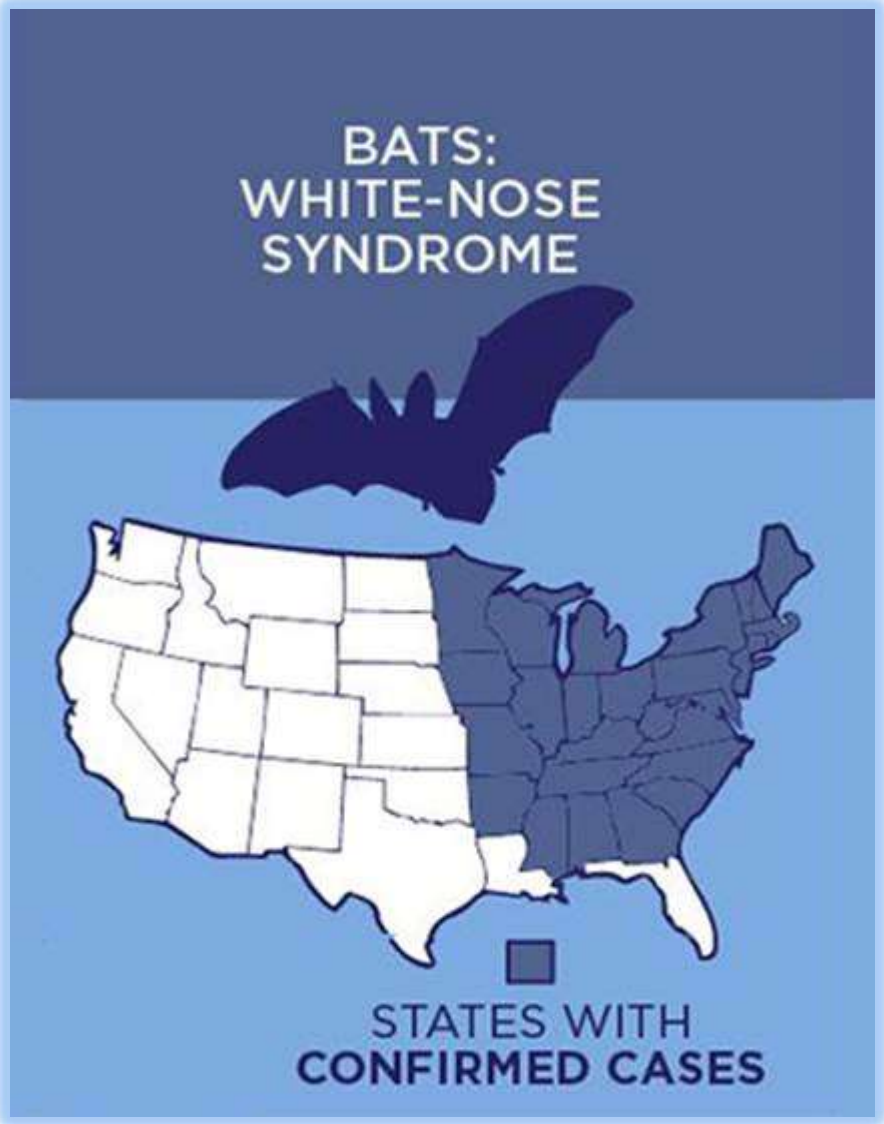
Presentation Outline

- Introduction
- Hypothesis
- Analytical Scope
- Prep Procedures
- Analysis Overview
- Analytical Procedure
- Analytical Results
- Complex Chromatography Challenges
- Analytical Retrospective



Bat Habits

Habits = increased risk of exposure to bioaccumulating chemicals

- Forage in aquatic and terrestrial habitats
- Live long lives
- High metabolic rates
- High food intake
- Insectivorous diet

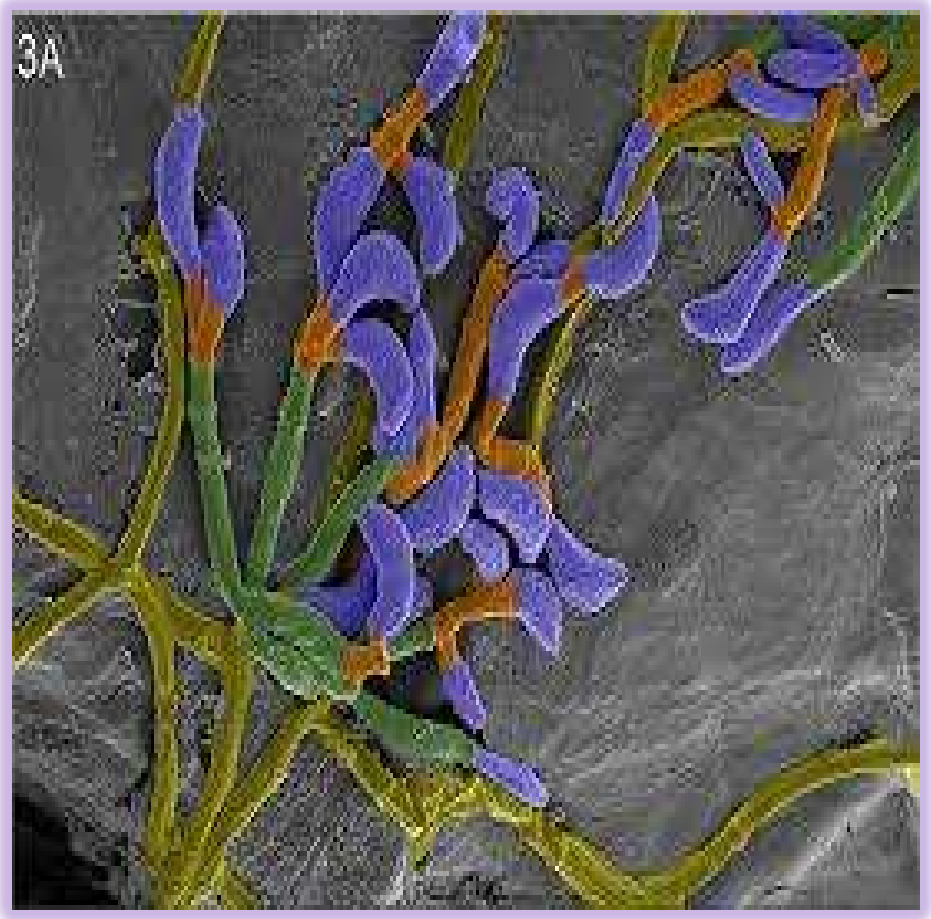
Bat Hibernation

Hibernation = more susceptible to effects of low doses

- Not true hibernators
- Annual hibernation cycle
- Extreme fat deposition
- Followed by extreme depletion
- High cave population density

White Nose Syndrome

- First identified in Feb of 2006
- Emerging disease in North American Bats
- Named for fungal growth on muzzles and wings
- No obvious treatment or prevention



Pseudogymnoascus destructans

WNS Impact

- ~ 7 million bat deaths in North America
- Some species declined >90%
- 11 bat species catastrophically affected
- At least 2.4 million pounds of insect go uneaten
- Farmers spend $3B dollars on pesticides
- Loss of pollination and seed dispersal

What is a chemical stressor? It is a stimuli that disturbs homeostasis.

Systems impacted:

- Hibernation
- Immune function
- Disease susceptibility
- Response to WNS

Chemical stressors:

- Organochlorine pest and PCBs
- Detergents/surfactants
- Antibacterials
- PPCPs
- PBDEs
- Many others

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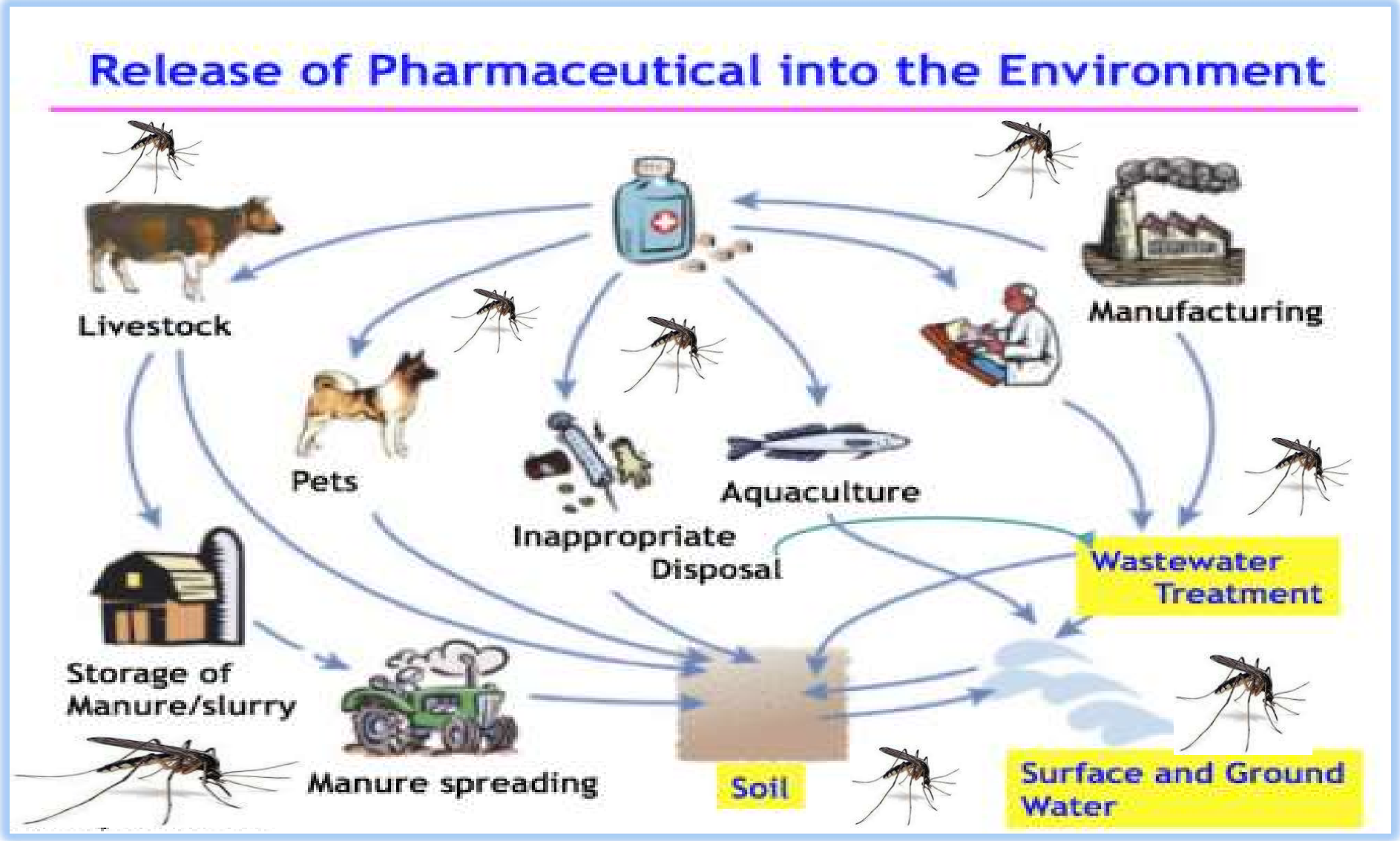
Hypothesis – Specific Antibiotic Stressors

- Healthy natural flora defends bats
- Chemical stressors (antibiotics, etc.) degrade this defense
- Consequence is WNS

Examples include:

- Cloxacillin
- Lincomycin
- Penicillin V
- Triclosan
- Sulfathiazole

CEC Exposure Pathway



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Analytical Scope

What chemical stressors were evaluated?

- PBDEs
- Antibiotics
- Pharmaceuticals (non-antibiotics)
- Endocrine disruptors
- Other PPCPs

Pre-prep Procedures

Phase 1

Proof of concept – whole bats were milled with dry ice

Phase 2

Resected wings - milled with liquid nitrogen

Prep Procedures

Complex matrix requires complex prep procedures

1. Bat carcasses were collected from various locations (2008 - 2010). All were frozen soon after collection and shipped to the lab on dry ice.

2. One or more carcasses – nominal 4 g mass were composited and cryogenically homogenized. Then spiked with isotopically labeled target analytes.

3. Each 4 g sample was split into 2 g fractions for acid and base buffered extractions. Each sample acid and base fraction was sonicated for 30min followed by microwave-assisted extraction.

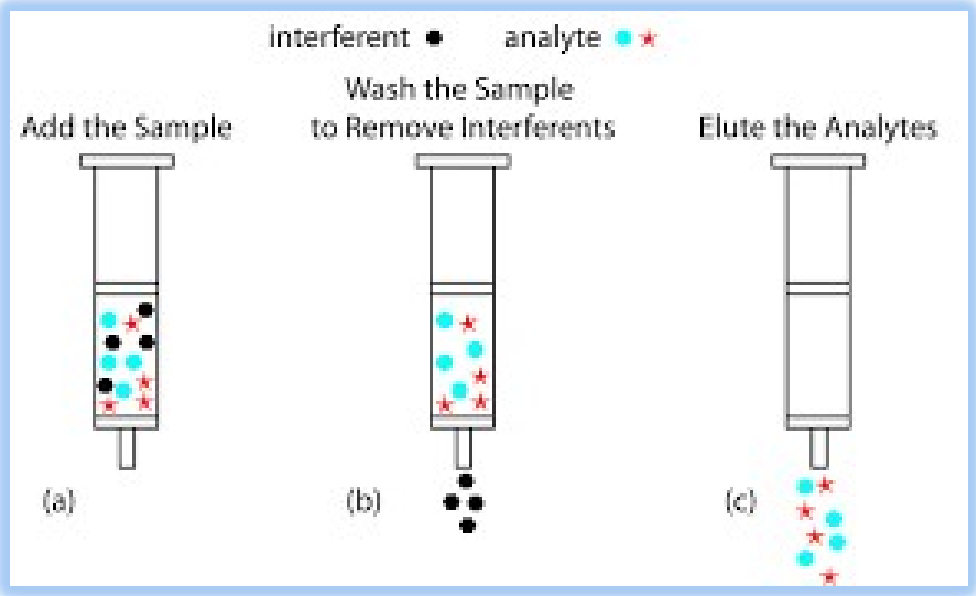
Prep Procedure - Continued

Complex matrix requires clean up procedures

4. SPE sample clean up. Prepare SPE, load sample and elute PPCP off the cartridge with 8 ml of MeOH for acid fractions and 8 ml of acid/MeOH for base fractions.

5. Each acid and base extract was concentrated by rotary evaporation and transferred to 2 ml auto vials.

6. Analyze by LCMSMS using either a C18 or C8 column with a gradient program using water, methanol and ACN. Mass spec is operated in (ESI) both negative positive ion mode.



Analysis Overview

- **Separate by HPLC**
  - 4 extracts per sample
  - Selected analytes on different LC columns
  - Selected analytes on different solvent and gradient systems
- **Determine by MS/MS**
  - Characteristic ion transitions developed by infusion
  - Multipoint calibration curve
  - Quantitate by isotope dilution or internal std

Analysis Procedures - Conditions

Parameter	Configuration
Column	C18- 100 x 2.1mm, 3.5um particle size (Waters XTerra C18 MS (PN 186000404), or equivalent)
Column Temperature	40 °C
Injection Volume	25 uL loop with partial overflow (Injection volume is consistent with the volume used for the initial calibration).

Time (minutes)	A %	B %	Flow Rate mL/min	Curve
0.00	95	5	0.20	Initial
1.00	95	5	0.20	6
25.00	0	100	0.20	6
27.00	0	100	0.20	6
27.10	95	5	0.20	End of Run

Capillary Voltage	2.95 kV	Desolvation Gas Flow	500 L/hr
Cone Voltage	Per analyte	Collision Energy	Per analyte
Extractor Voltage	4.00 V	Multiplier Voltage	750 V
RF lens	0.3 V	Collision Gas Flow	0.35 mL/min
Source Temperature	140 °C.	Pressure	~3E-3 mbar
Desolvation Temperature	380 °C.	Scanning Conditions	Per Analyte
Cone Gas Flow	20 L/hr	Dwell/Delay Times (Both Ions)	0.20 sec / 0.05 sec

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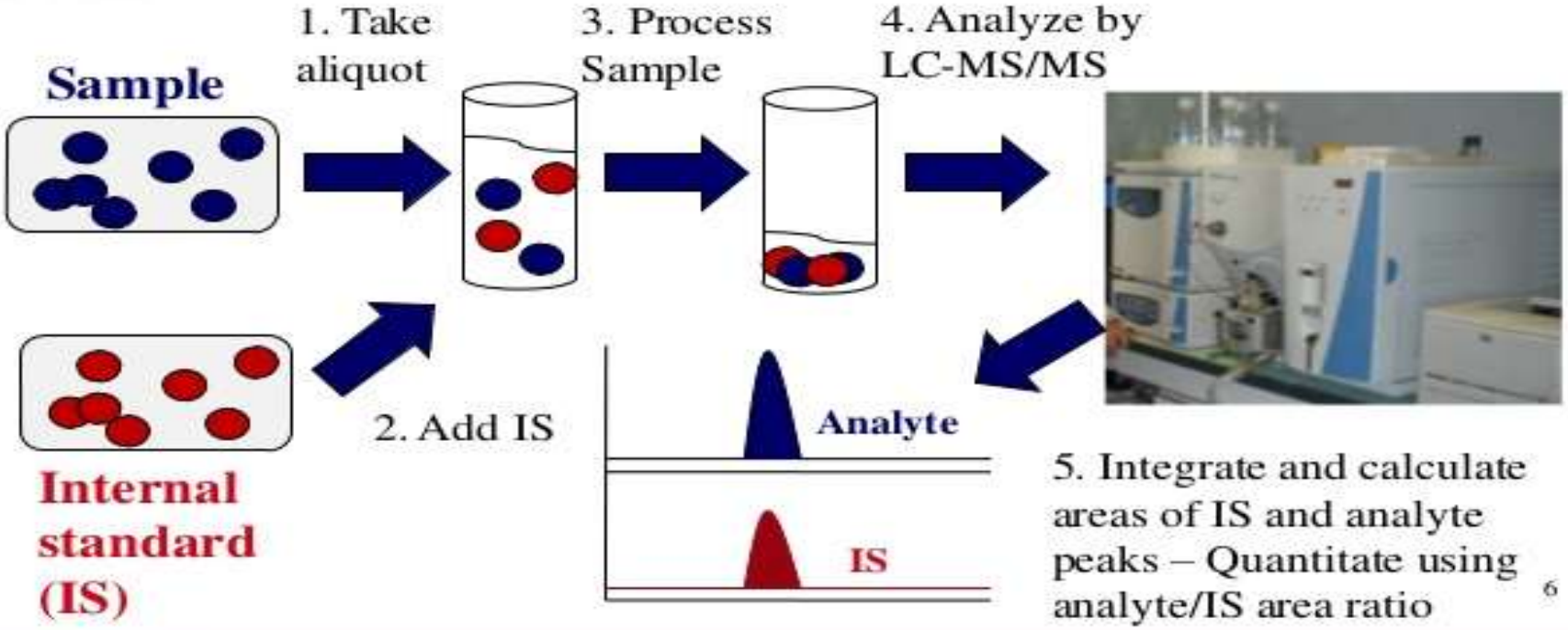
Mass Spectrometer Conditions

MS Conditions and Grouping							
Pharmaceuticals, Personal Care Products and Antibacterials							
Analyte	ESI Mode	Transition Precursor>Product	Dwell (Sec)	Cone Voltage	Collision Voltage	Delay (sec)	Group
Acetaminophen	pos	152.00 > 110.10	0.02	30	17	0.06	1
Albuterol	pos	240.3 >148.2	0.02	15	18	0.01	5
Amoxicillin	pos	366.10 > 114.10	0.02	14	20	0.01	1,3
Atenolol	pos	267.30 > 145.20	0.02	35	24	0.01	1,3
Atorvastatin (Lipitor)	neg	557.50 > 397.50	0.02	38	28	0.01	2,4
Atrazine	pos	216.2 > 174	0.002	18	18	0.01	
Azithromycin	pos	749.10 > 591.10	0.02	60	32	0.01	1,3
Bisphenol A	neg	227.2 > 212.20	0.02	36	20	0.01	2,4
Caffeine	pos	195.00 > 138.00	0.02	30	20	0.01	1
Carbadox	pos	263.1 > 231.1	0.02	30	14	0.01	1
Carbamazepine	pos	237.00 > 194.00	0.02	28	20	0.01	1,3,5
Cefotaxime sodium salt	pos	456.1 > 396.1	0.02	23	12	0.01	1
Chlortetracycline (CTC)	pos	478.90 > 462.20	0.02	26	25	0.01	1
Cimetidine	pos	253.40 > 158.90	0.02	23	15	0.01	1,3,5
Ciprofloxacin	pos	332.10 > 314.10	0.02	22	24	0.01	1,3
Clarithromycin	pos	748.6 > 158.2	0.02	25	30	0.01	1,3
Clinafloxacin	pos	366.2 > 348.1	0.02	30	20	0.01	1
Cloxacillin	pos	468 > 160.1	0.02	25	19	0.01	
Cotinine	pos	177.00 > 79.80	0.02	27	23	0.01	1,3,5
Codeine	pos	300.2 > 215.2	0.02	38	24	0.01	1
Demeclocycline	pos	465 > 430.1	0.02	28	27	0.01	1
Diazepam	pos	285.50 > 222.50	0.02	30	25	0.01	1,3

Application of Isotope Dilution

Ideal MS Quantitative Method

Isotope Dilution Mass Spectrometry (IDMS) – use of a isotopically labeled internal standard.



LCMSMS Advantages

Specificity/Selectivity - A target analyte's response is highly characteristic of its identity.

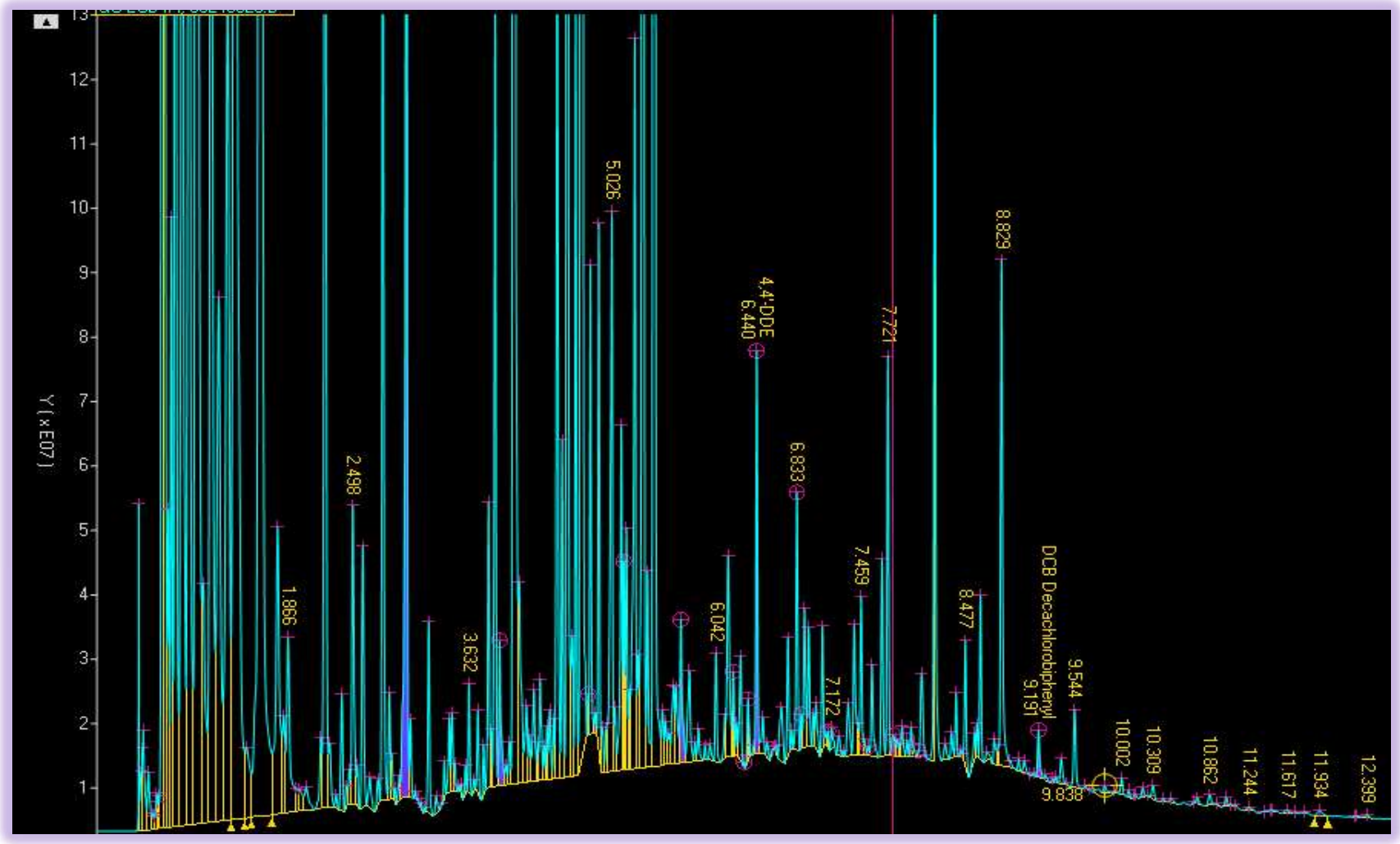
Sensitivity - Softer ionization than Electron Impact (EI) GCMS – allows for **thermally labile** analytes to be detected

Ruggedness – improved reproducibility for a wide variety of parameters and matrices and improved productivity

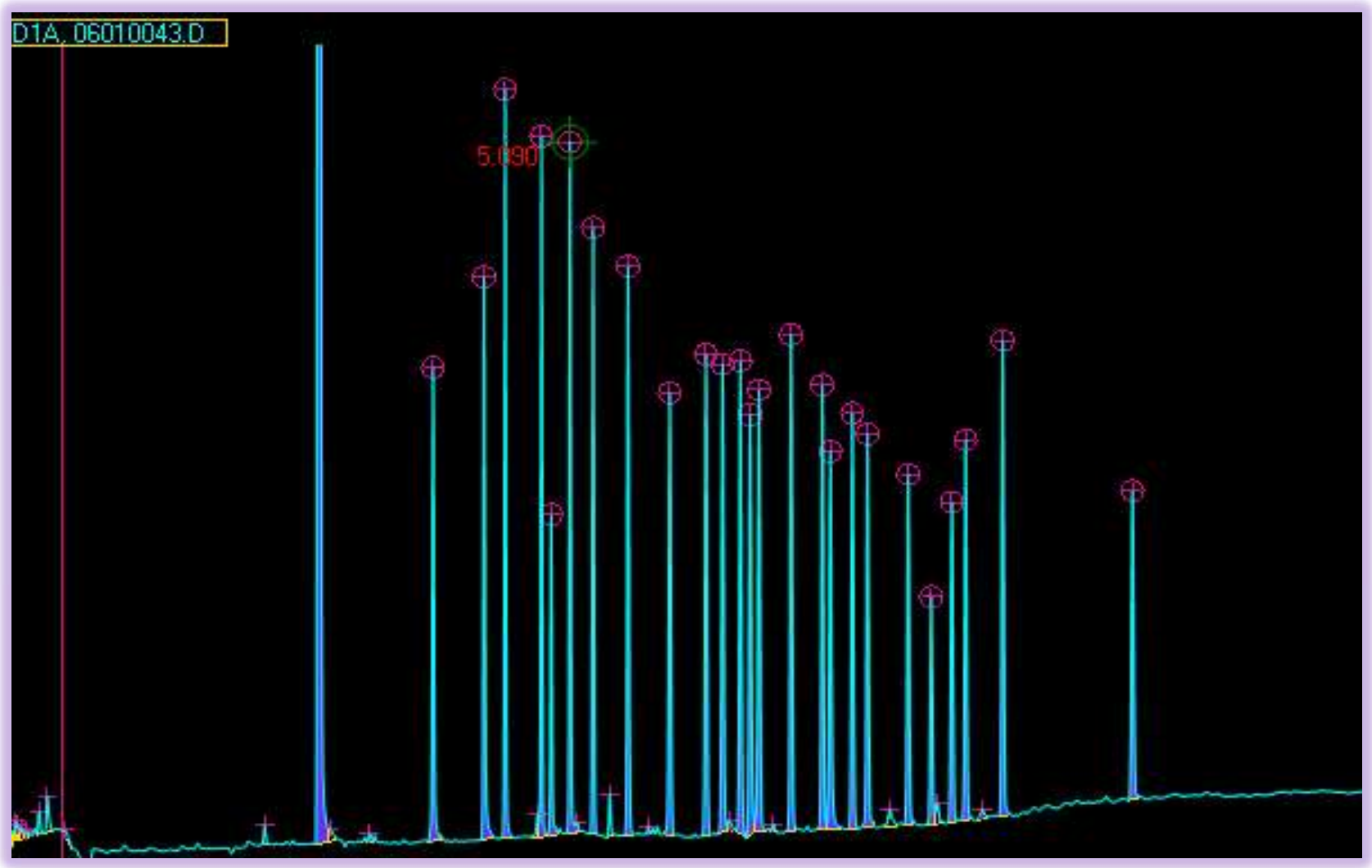
Quality Control Elements

- MBs (3) – Measures background lab artifacts
- LCS (3)– Measures target analyte recovery in simple matrix
- MS (6) – Measures target analyte recovery in complex matrix
- Duplicates (4) – Measures reproducibility
- 28 samples

Chromatographic Complexity in Tissue



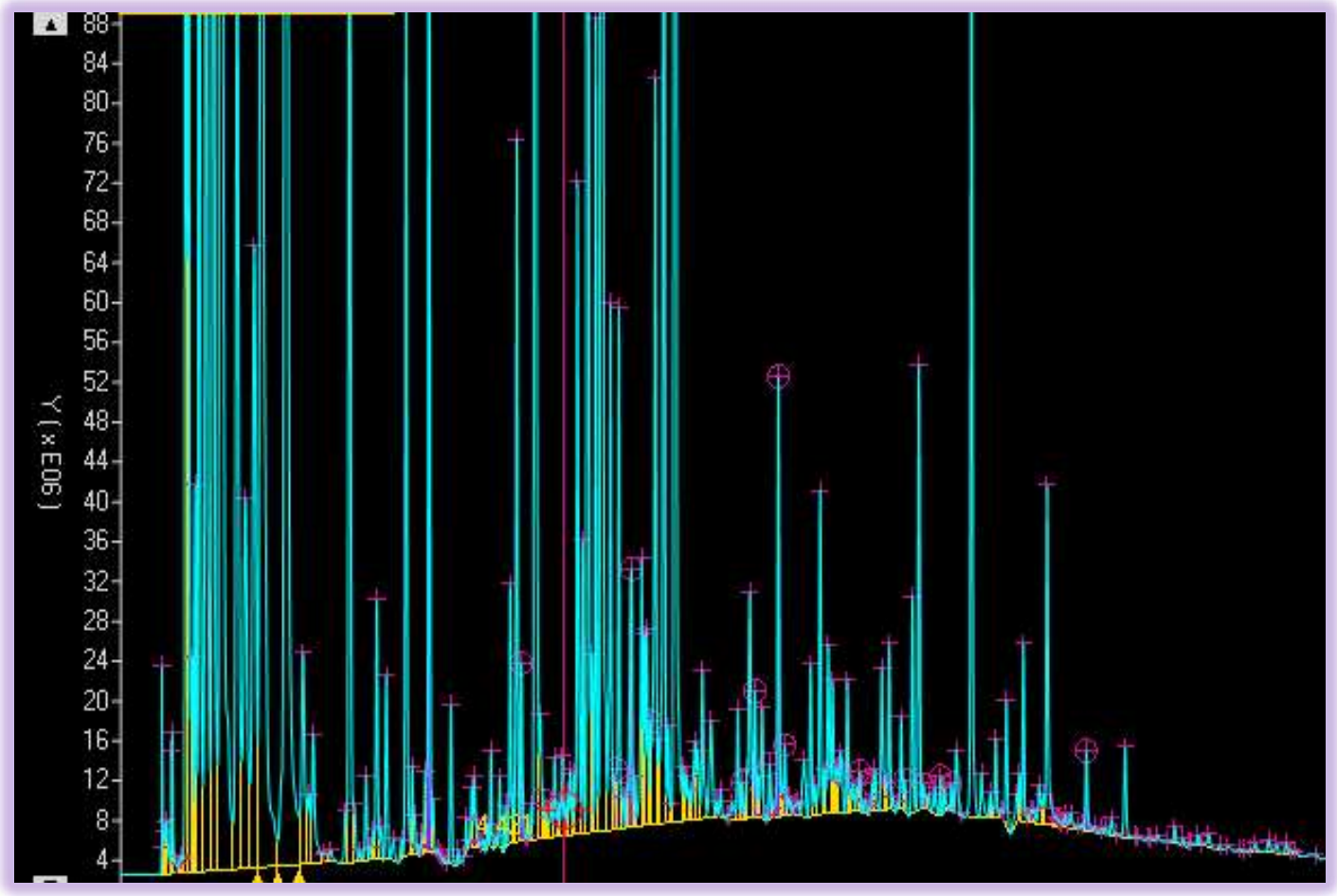
Chromatograph of Matrix Spike Mix



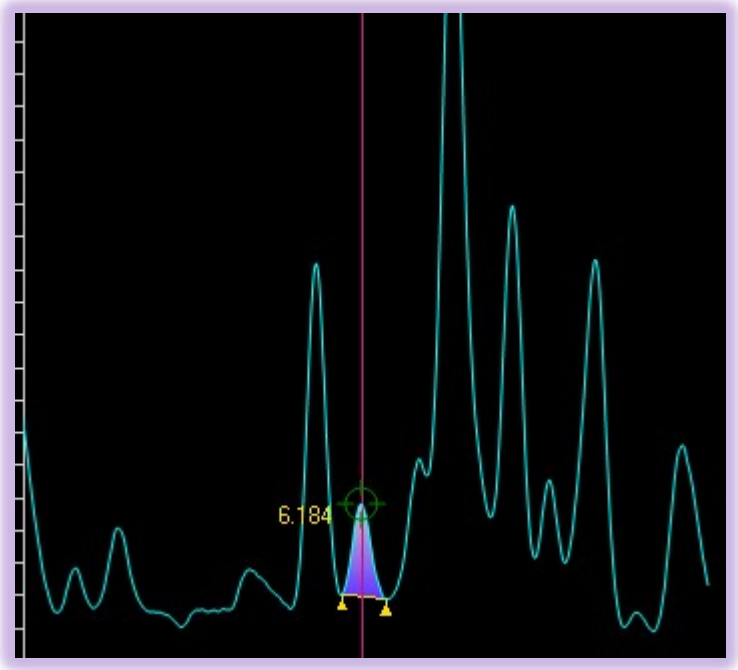
Same Spike in Tissue Sample

Same spike
6 false negatives

Un-spiked sample
8 hits (possible false positives)

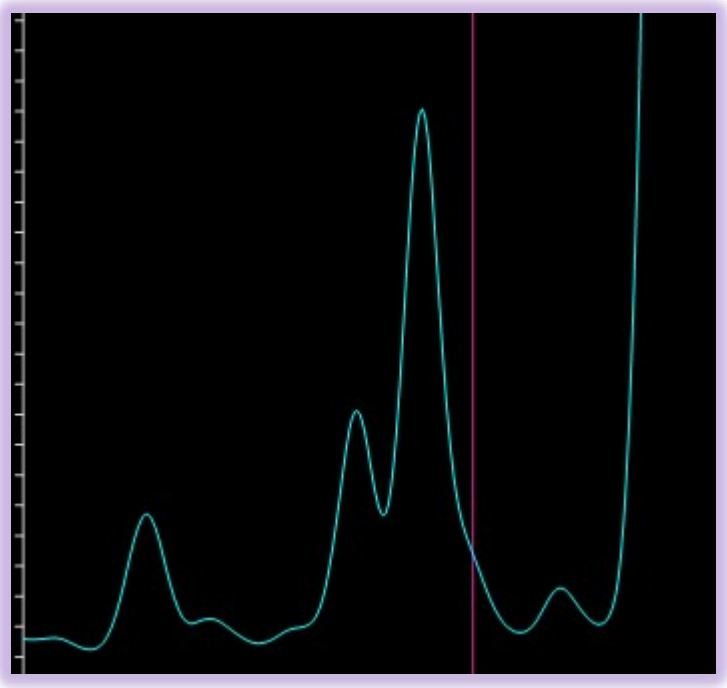


RT Shift or Obscuration?



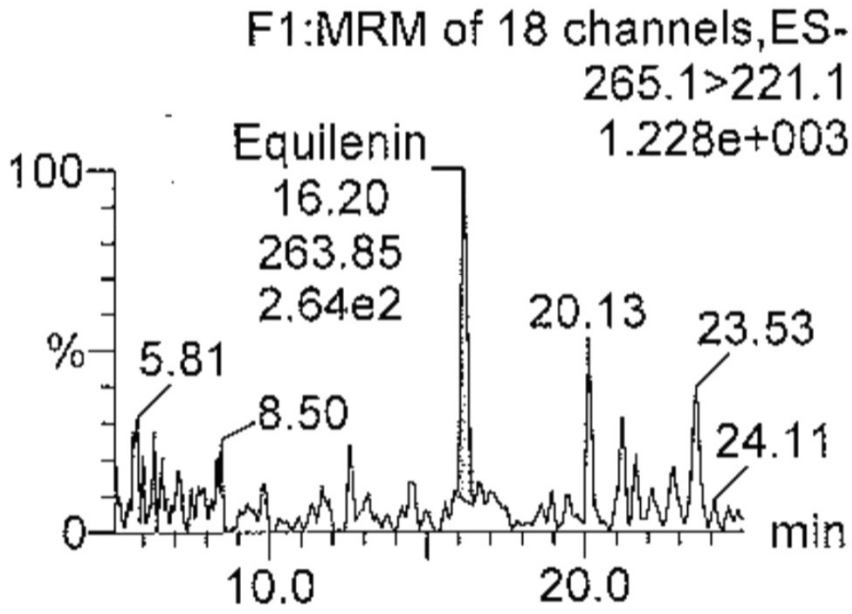
Non-interfered peak
No RT shift

Interfered peak
No RT shift
Masked by non-target compound

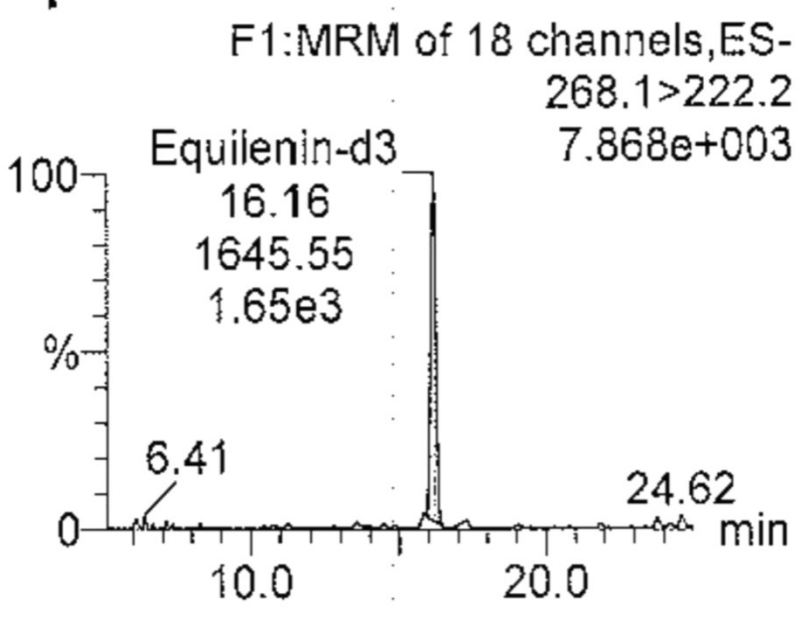


ID Enhanced by IDA

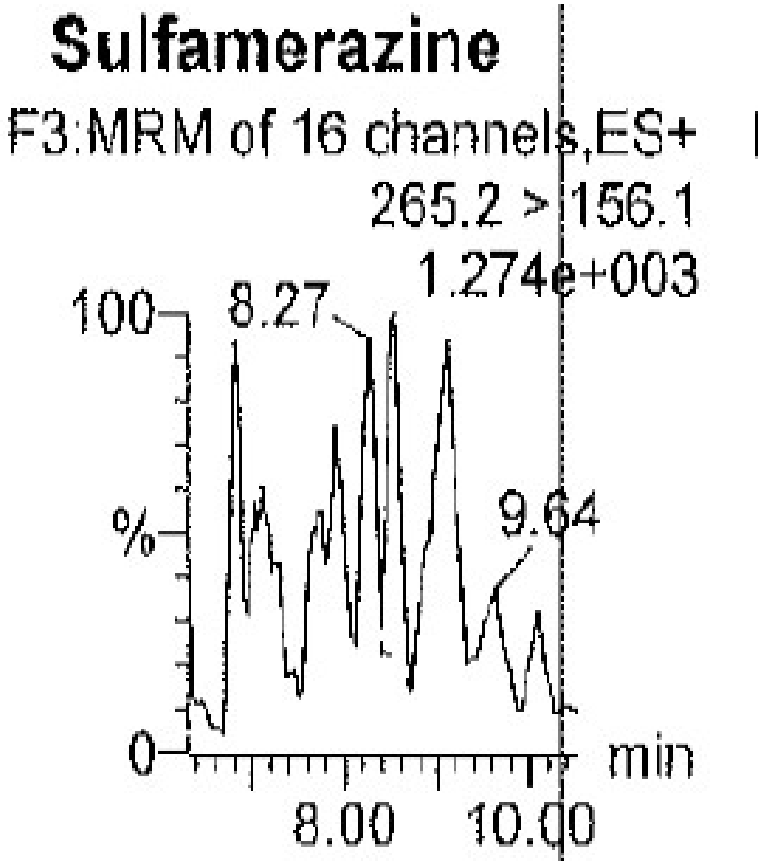
Equilenin

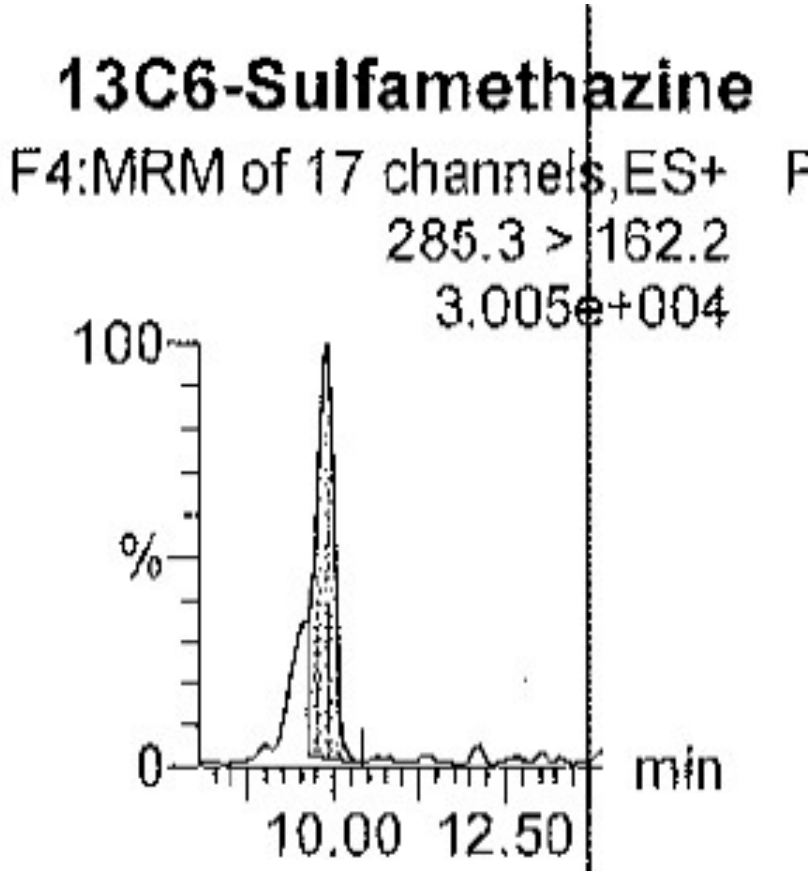


Equilenin-d3



Limited Facilitation from Labeled IS





Multiple interferences present
No RT shift
Possible masking by non-target compound

Use of Rigorous RT

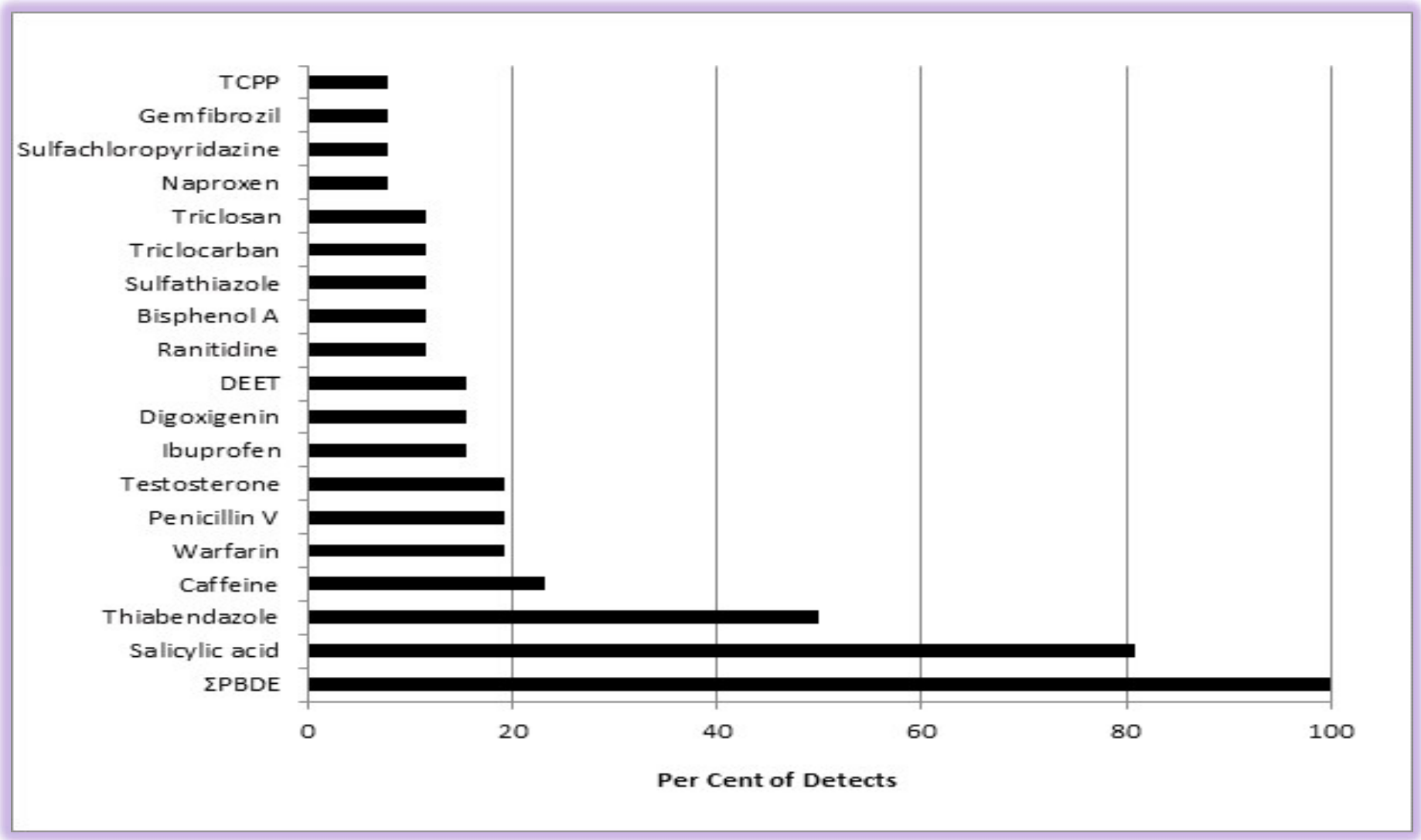
- RT is critical/only ID element for non-selective detector
  - Narrowest possible effective RT window
  - TestAmerica recommends 0.01 RT min window non-selective detectors
- RT criteria frequently relaxed for selective detectors
  - 1694 specifies 0.25 min for LC/MS (25X wider)
  - TestAmerica used 0.02 min window for LC/MS in complex matrix

Use of QC Elements

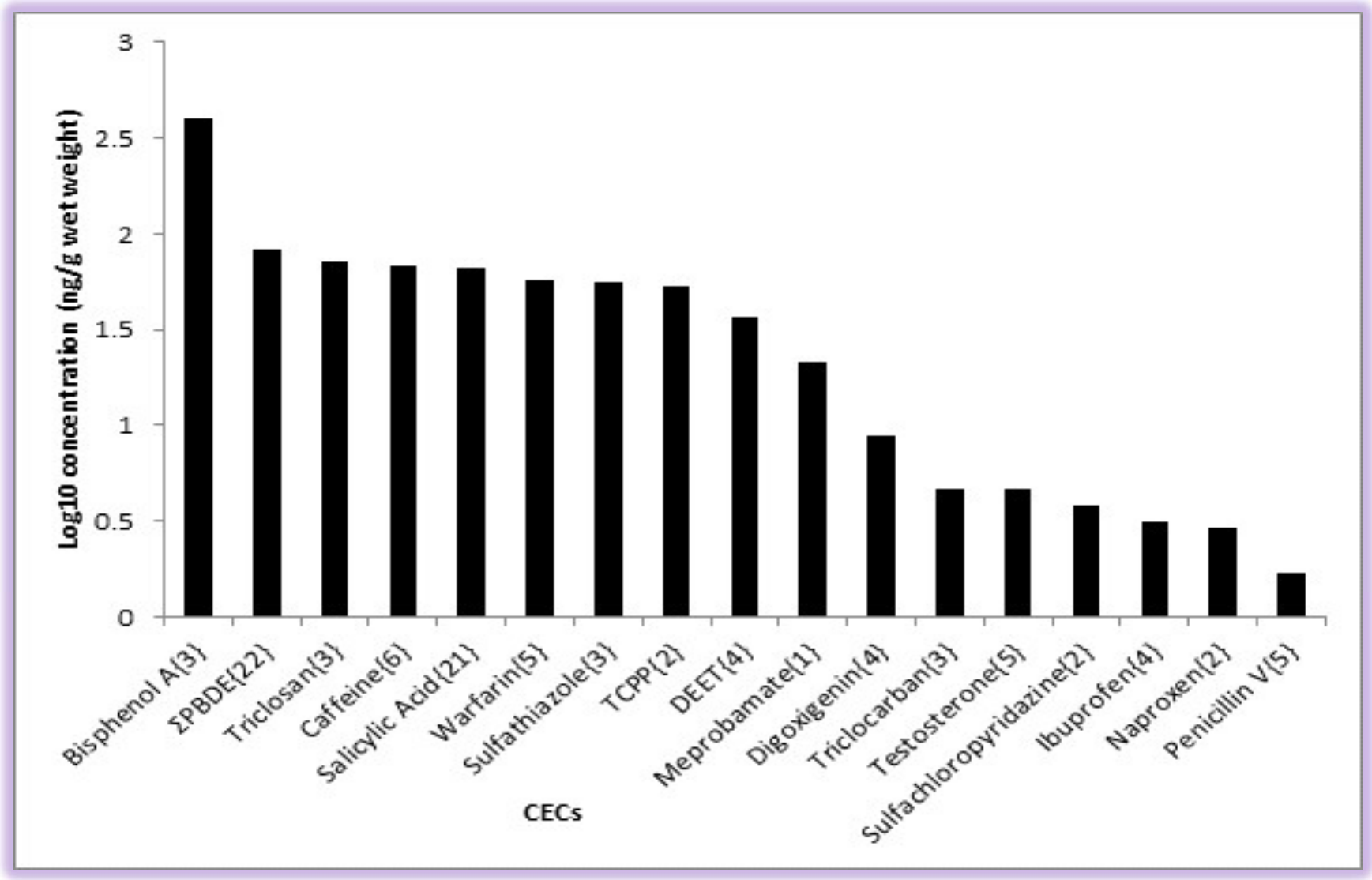
- Standard
- Method blank
- Laboratory control spike
- Matrix spike
- Sample duplicate
- IDA/Surrogate recovery

	CCV	MB Batch 2052182		LCS Batch 2052182			PAWNS15		PAWNS15-MS			PAWNS15-MSD			PAWNS19		PAWNS19-DUP	
Analytes (Results in ng/g)	%Recovery	RL	Result	Spike Amount	Result	%Recovery	RL	Result	Spike Amount	Result	%Recovery	Spike amount	Result	%Recovery	RL	Result	RL	Result
Name																		
1694 - PPCP compounds																		
1,7-Dimethylxanthine	77.4	20.0	ND	1000	989.52	99	16.2	ND	810	1030	127.2	810	1756	216.9	17.1	ND	17.1	ND
Sulfadiazine	113.4	10.0	ND	500	572.42	114.5	8.10	ND	405	2493	615.7	405	1089	269	8.55	ND	8.55	ND
Cotinine	161.1	2.5	ND	50	59.12	118.2	2.02	ND	40.5	28.3	70	40.5	20.7	51.2	2.14	22.2	2.14	39.6
Hydrocodone	94.1	1.0	ND	50	55.49	111	0.81	ND	40.5	43.1	106.4	40.5	41.8	103.4	0.85	ND	0.85	ND
Caffeine	134.4	2.5	ND	50	42.22	84.4	2.02	61.7	40.5	45.5	0.0	40.5	50.1	0.0	2.14	2.22	2.14	ND
Sulfamerazine	134.9	5.0	ND	50	55.81	111.6	4.05	ND	40.5	108	265.8	40.5	46.7	115.2	4.27	5.88	4.27	ND
Trimethoprim	89.1	2.5	ND	50	34.59	69.2	2.02	ND	40.5	29.5	72.8	40.5	35.1	86.7	2.14	ND	2.14	ND
Sulfamethizole	99.6	5.0	ND	250	261.06	104.4	4.05	9.63	202	391	188.3	202	134	61.5	4.27	ND	4.27	ND
Ormetoprim (IA)	96.1	1.0	ND	50	51.98	104	0.81	ND	40.5	56.0	138.2	40.5	40.5	100.1	0.85	ND	0.85	ND
Sulfamethazine	90.5	5.0	ND	250	268.12	107.2	4.05	ND	202	153	75.7	202	180	88.8	4.27	ND	4.27	ND
Pentoxifylline	103.2	1.0	ND	50	50.46	100.9	0.81	ND	40.5	38.0	93.8	40.5	45.1	111.4	0.85	ND	0.85	ND
Meprobamate	88.2	1.0	ND	50	56.86	113.7	0.81	ND	40.5	28.8	71.1	40.5	40.5	99.9	0.85	ND	0.85	ND

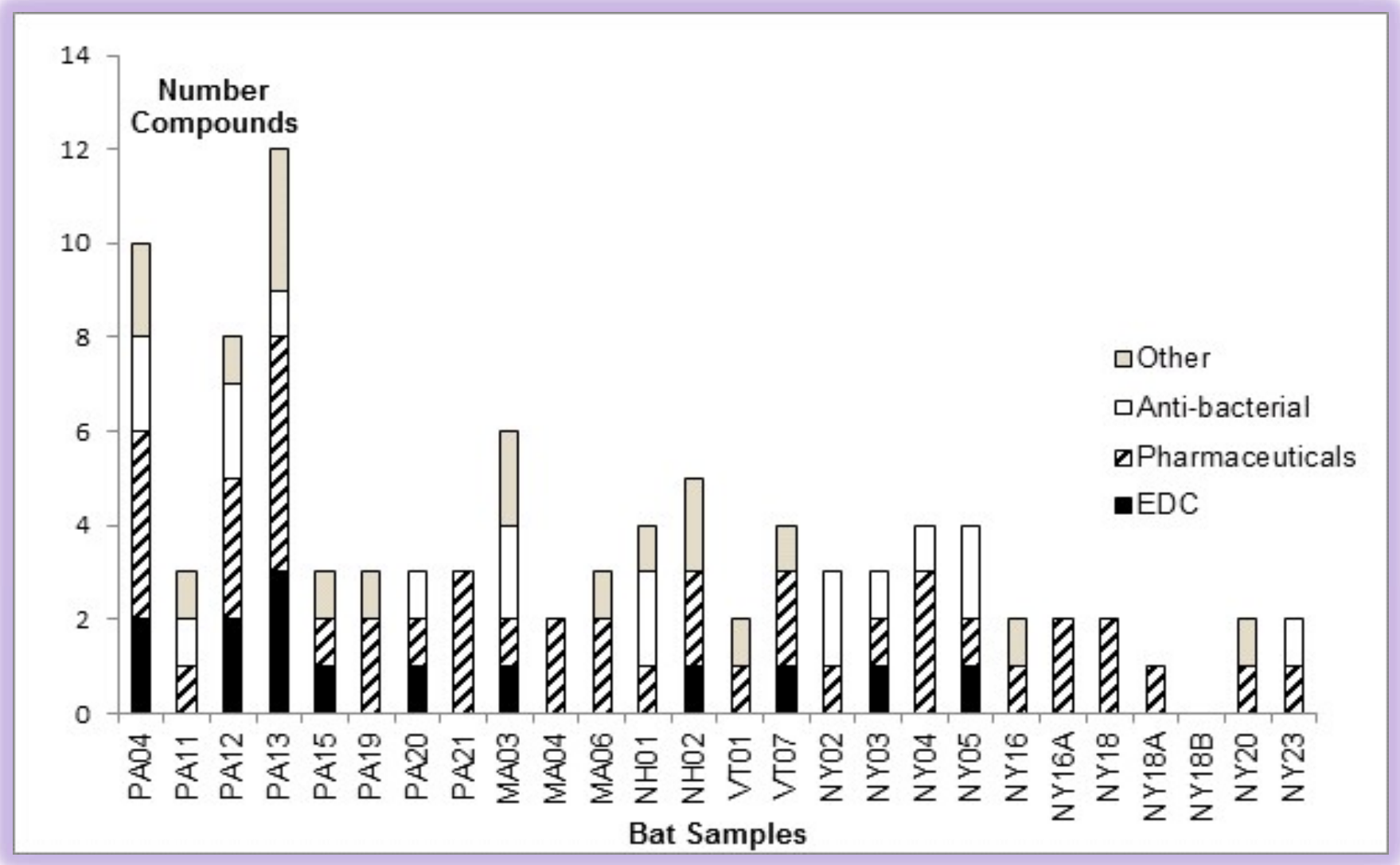
Analyte Frequency



Average Analyte Concentrations



Analyte Class per Bat



Analytical Retrospective

- Narrow the scope (fewer analytes)
- Add IDAs to 1:1 correspondence
- Optimize RT windows
- Add 2nd mass transitions
- Increase dwell time
- Multi-column review
- Optimize clean-up for selected analytes
- Balance extraction procedure to limit unintended interference
- Improved 'sampling'
  - Age/sex differentiation
  - Desiccated/decomposed vs harvested
  - Lipid normalization

Overall Conclusions

- Bats are exposed to xenobiotics
- Bats are exposed to antibacterials
- Hypothesis still plausible
- Analytical procedure can be improved
- Further study is needed

Contact and Acknowledgements

- Eric Redman, Eric.Redman@TestAmericainc.com (916) 374-4342
- Anne Secord, U.S. Fish and Wildlife Service, 3817 Luker Road Cortland, New York 13045
- Karla Buechler, TestAmerica Laboratories Inc., 880 Riverside Parkway, West Sacramento CA, 95605

In Honor of Dr. Charlie Carter

- Dr. Charlie Carter had a deep personal interest in the WNS phenomenon and was excited to cross paths in 2011 with Dr. Anne Secord from the Environmental Contaminants Division of the Fish and Wildlife Service. This initial encounter led to collaborative discussion and development of a working hypothesis that was amenable to support from analytical chemistry. Charlie spent countless hours poring over the analytical results generated in this study and subsequent studies of insects. We know that his dedication to this effort has contributed to the scientific community's understanding of WNS.

Questions???