



Continued Progress in the Multiclass, Multiresidue Analysis of Veterinary Drugs in Animal Tissues

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Outline

1) Conclusions

2) Background

3) Progress

- Summation Integration Approach
- Analyte Identification
- Ion-Pairing Agent in the Final Extract

4) Future Possibilities

- TYT for TAT Interlab Method Validations
- Isocratic Fast (UHP)LC-MS/(HR)MS

Conclusions

- Current validated USDA ARS/FSIS methods have been demonstrated to be very effective and efficient for analysis of >170 targeted drugs in animal tissues (not milk).
- Ion-pairing reagent added to combined final extracts allows inclusion of aminoglycosides in the same UHPLC-MS/MS method with other common drugs.
- Automated data processing using summation chromatographic peak integration yields trustworthy quantification and identification without human review and re-integrations.
- Method files can be shared to easily and readily compare multiple methods in interlab trials of PT-like samples.

USDA Food Safety and Inspection Service

Link to US National Residue Program:

www.fsis.usda.gov/wps/portal/fsis/topics/regulatory-compliance/regulatory-enforcement!/ut/p/a1/04_Sj9CPykssy0xPLMnMz0vMAfGjzOINAg3MDC2dDbwMDIHQ08842MTDy8_YwMgYqCASWYG_paEbUEFYoL-3s7OBhZ8xkfpxAEcDQvq9iLDAqMjX2TddP6ogsSRDNzMvLV8_oig1vTQnsSS_qFI3FShQIJyam5pXoh-uH4XXPH8TdAVYPAxRgNtHBbmhEVU-acGe6YqKAPChfMA!/?1dmy¤t=true&urile=wcm%3Apath%3A%2Ffsis-content%2Finternet%2Fmain%2Ftopics%2Fdata-collection-and-reports%2Fchemistry%2Fresidue-chemistry

Blue Book – Annual Sampling and Analysis Plans

Red Book – Annual Monitoring Results

Link to FSIS Chemistry Laboratory Guidebook:

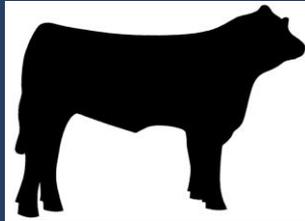
www.fsis.usda.gov/wps/portal/fsis/topics/science/laboratories-and-procedures/guidebooks-and-methods/chemistry-laboratory-guidebook/chemistry-laboratory-guidebook

CLG-MRM(#) - Multiclass, Multiresidue Method

CLG-AMG(#) - Aminoglycosides Method

FSIS Residue Monitoring Scheme since 2012

≈6,300
Slaughter
Houses



Inspector
Samples
n > 150,000/yr
>0.5% of cows



KIST™
Screen 3 hrs
Test

FSIS Labs

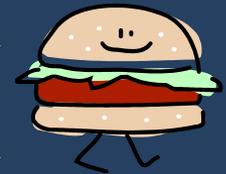
St. Louis, MO
Athens, GA
Alameda, CA

Shipment
24 – 48 hrs



24 hrs

UHPLC-
MS/MS*

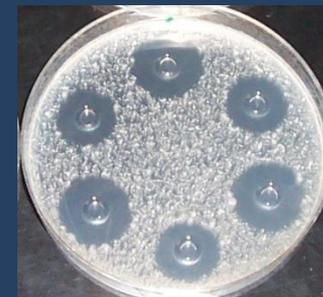
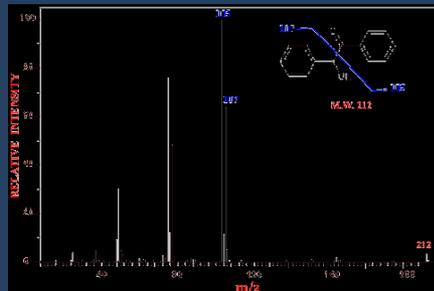


*Qualitative Screening:
Identification, Confirmation
and Semi-Quantification

FDA-CVM On-Farm
Enforcement of
Violations

Analytical
Confirmation and
Quantification

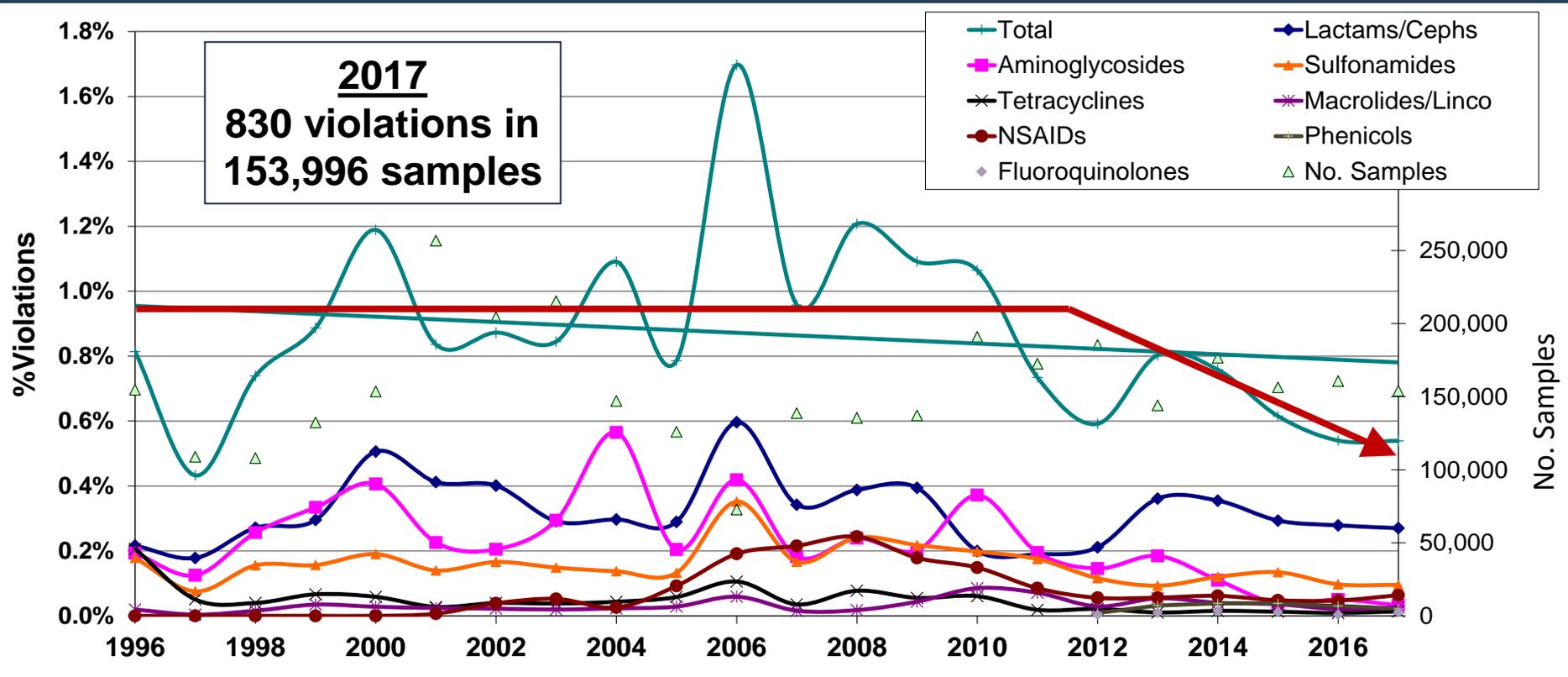
< 7 days



Quantitative
Bioassay

24 hrs

Violation Rate of Veterinary Drugs In Bovine Slaughter Classes



KIS test and UHPLC-MS/MS implemented in 2012

A Decade of Gradual Progress

Schneider and Lehotay, “A comparison of the FAST, Premi[®] and KIS[™] tests for screening antibiotic residues in beef kidney juice and serum” *Anal. Bioanal. Chem.* **390**, 1775-1779 (2008).

Schneider et al., “Comparison of screening methods for antibiotics in beef kidney juice and serum” *Anal. Chim. Acta* **637**, 290-297 (2009).

B. Kinsella et al., “New method for the analysis of flukicide and other anthelmintic residues in bovine milk and liver using liquid chromatography – tandem mass spectrometry” *Anal. Chim. Acta* **637**, 196-207 (2009).

Lehotay et al., “Development and validation of a streamlined method designed to detect residues of 62 veterinary drugs in bovine kidney using ultrahigh performance liquid chromatography – tandem mass spectrometry” *Drug Testing Anal.* **4 (S1)**, 75-90 (2012).

Geis-Asteggiane et al., “Ruggedness testing and validation of a practical analytical method for >100 veterinary drug residues in bovine muscle by ultrahigh performance liquid chromatography – tandem mass spectrometry” *J. Chromatogr. A* **1258**, 43-54 (2012).

Lehotay et al., “Rapid analysis of aminoglycoside antibiotics in bovine tissues using disposable pipette extraction and ultrahigh performance liquid chromatography - tandem mass spectrometry” *J. Chromatogr. A* **1313**, 103-112 (2013).

Identification and Confirmation by Mass Spectrometry:

Lehotay et al., “Identification and confirmation of chemical residues by chromatography-mass spectrometry and other techniques” *Trends Anal. Chem.* **27**, 1070-1090 (2008).

Lehotay et al., “Current issues involving screening and identification of chemical contaminants in foods by mass spectrometry” *Trends Anal. Chem.* **69**, 62-75 (2015).

Structural Characterization by Mass Spectrometry:

Geis-Asteggiante et al., “Structural characterization of product ions by electrospray ionization and quadrupole time-of-flight mass spectrometry to support regulatory analysis of veterinary drug residues in foods” *Rap. Commun. Mass Spectrom.* **28**, 1061-1081 (2014).

Nuñez et al., “Structural characterization of product ions by electrospray ionization and quadrupole time-of-flight mass spectrometry to support regulatory analysis of veterinary drug residues in foods. Part 2: Benzimidazoles, nitromidazoles, phenothiazines, and mectins” *Rap. Commun. Mass Spectrom.* **29**, 719-729 (2015).

Nuñez et al., “Structural characterization of product ions of regulated veterinary drugs by electrospray ionization and quadrupole time-of-flight mass spectrometry. Part 3: Anthelmintics and thyreostats” *Rap. Commun. Mass Spectrom.* **30**, 813-822 (2016).

Recent Developments

Schneider et al., “Validation of a streamlined multiclass, multiresidue method for determination of veterinary drug residues in bovine muscle by liquid chromatography – tandem mass spectrometry” *Anal. Bioanal. Chem.* **407**, 4423-4435 (2015).

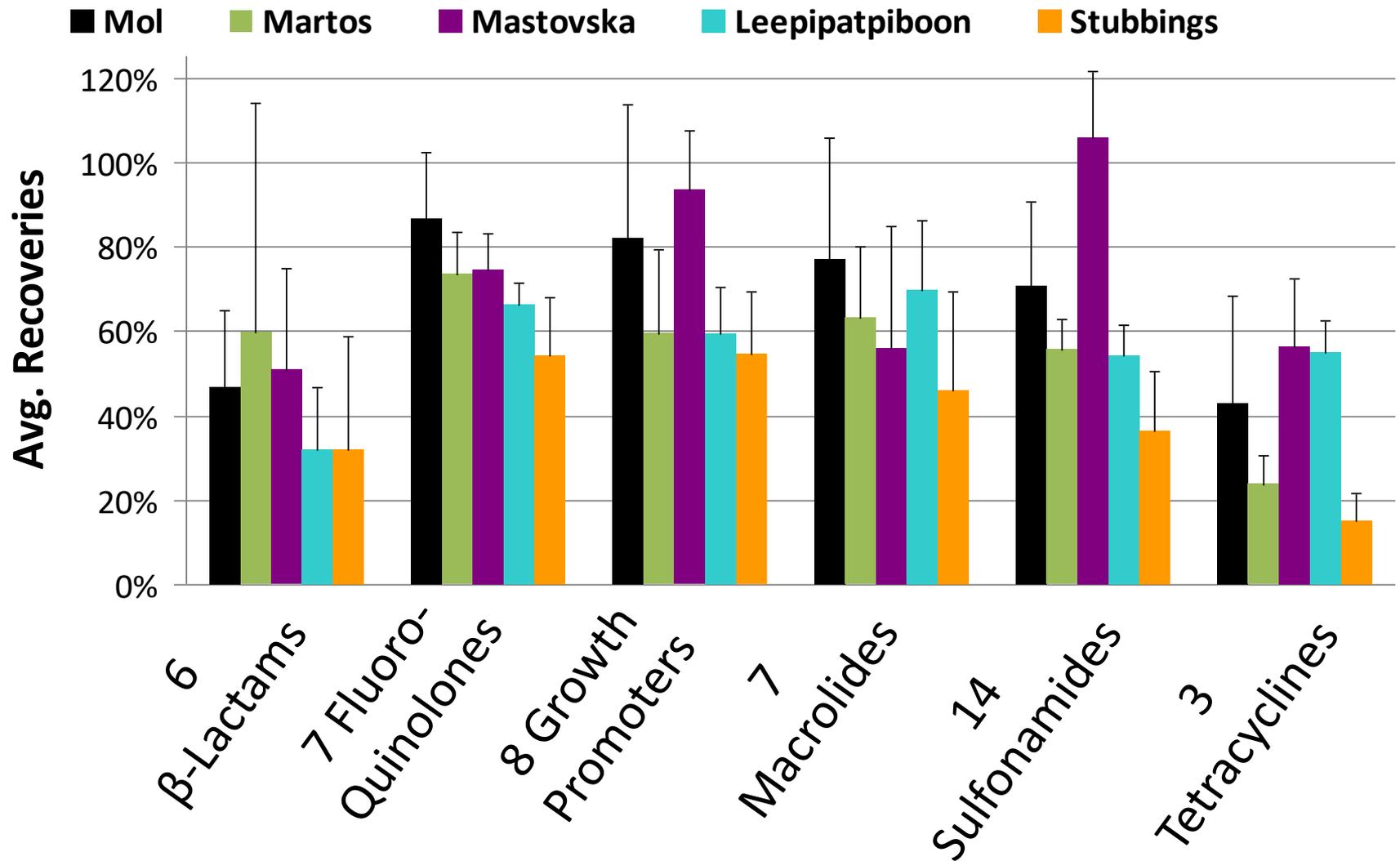
Anumol et al., “Comparison of veterinary drug residue results in animal tissues by ultrahigh-performance liquid chromatography coupled to triple quadrupole or quadrupole-time-of-flight tandem mass spectrometry after different sample preparation methods, including use of a commercial lipid removal product” *Anal. Bioanal. Chem.* **409**, 2639-2653 (2017).

Lehotay, “Utility of the Summation Chromatographic Peak Integration Function to Avoid Manual Reintegrations in the Analysis of Targeted Analytes” *LCGC North America* **35**, 391-402 (2017) and *LCGC Europe* **30**, 530-540 (2017).

Lehotay and Lightfield, “Simultaneous analysis of aminoglycosides with many other classes of drug residues in bovine tissues by ultrahigh-performance liquid chromatography – tandem mass spectrometry using an ion-pairing reagent added to final extracts.” *Anal. Bioanal. Chem.* **410**, 1095-1109 (2018).

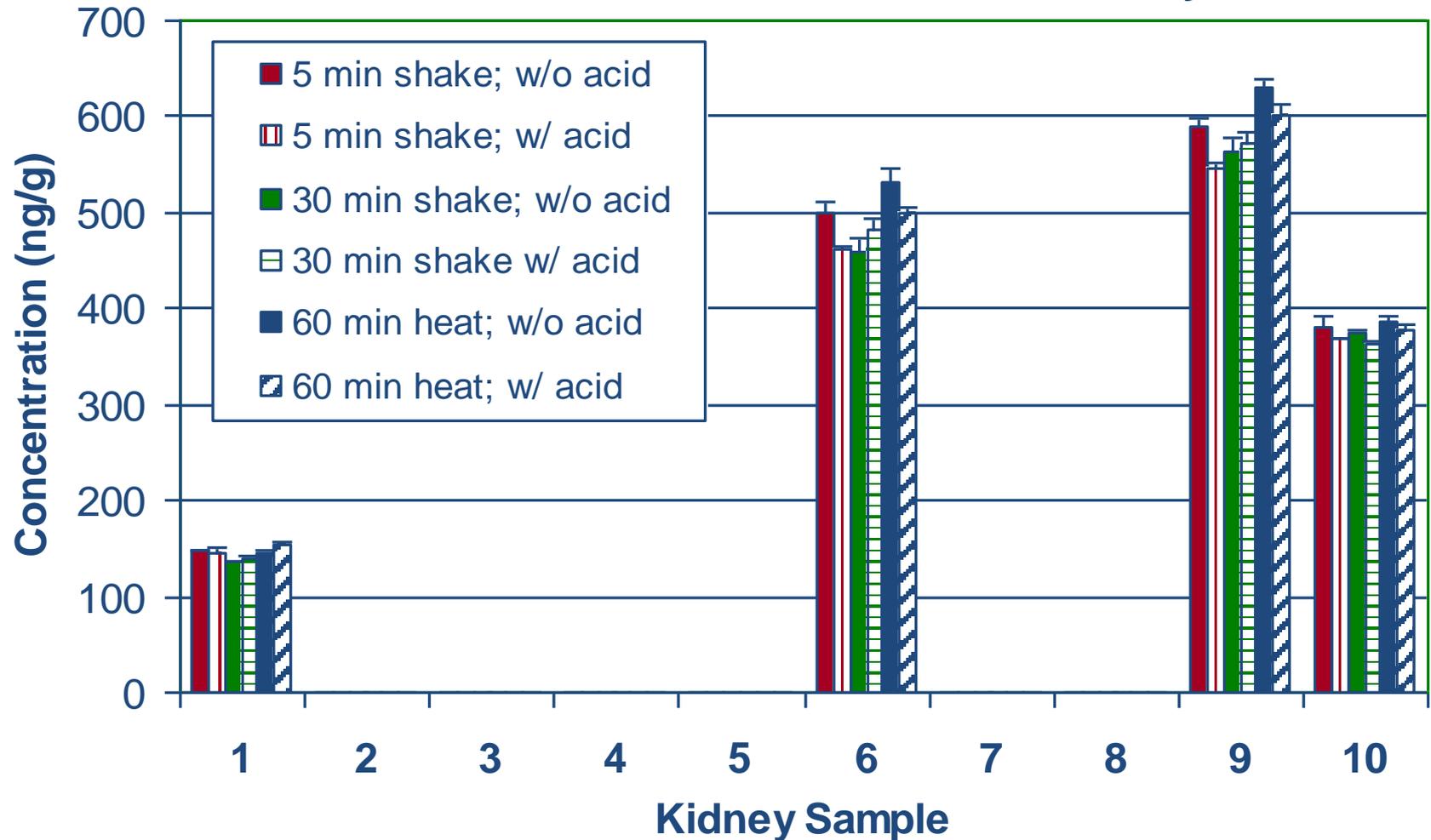
Comparison of 5 Vet. Drug Methods

Spiked $\frac{1}{2}x$, $1x$, and $2x$ Target Levels (n= 6 each) in Kidney, No I.S.



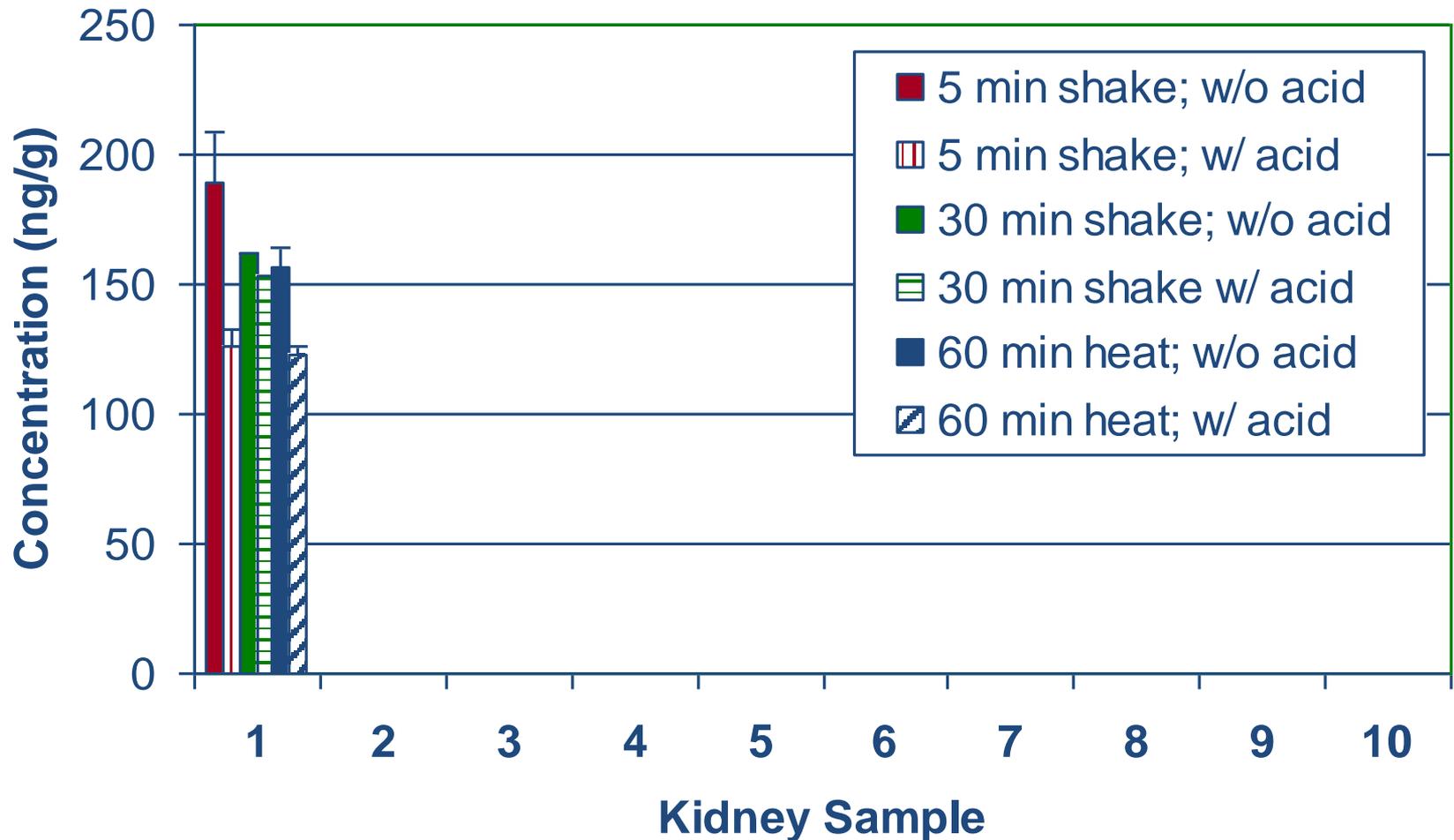
Analysis of Incurred Kidney (2 g)

Flunixin vs. Flunixin-d3 IS in Incurred Kidney



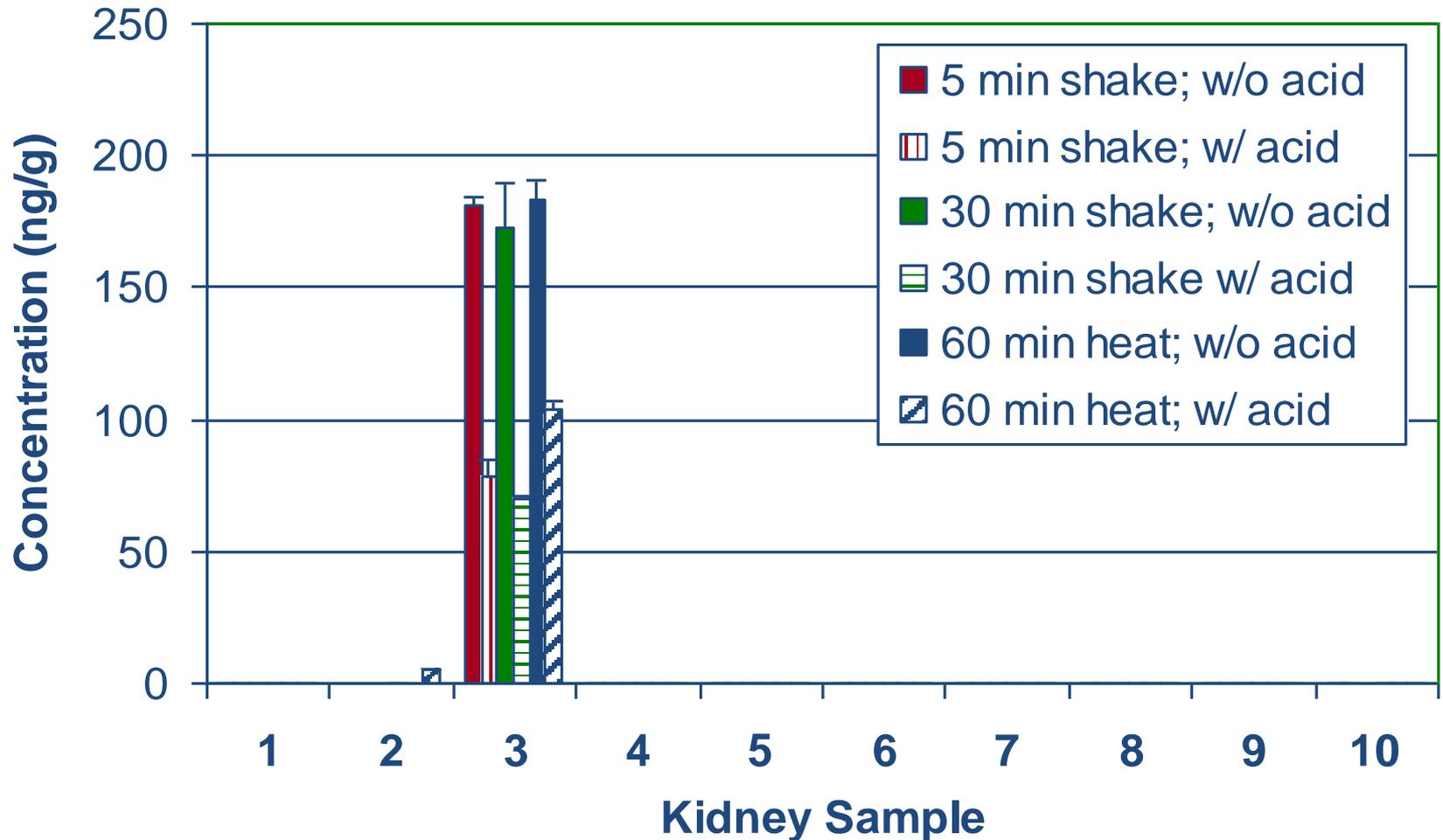
Analysis of Incurred Kidney (2 g)

Pirlimycin in Incurred Kidney (no IS)



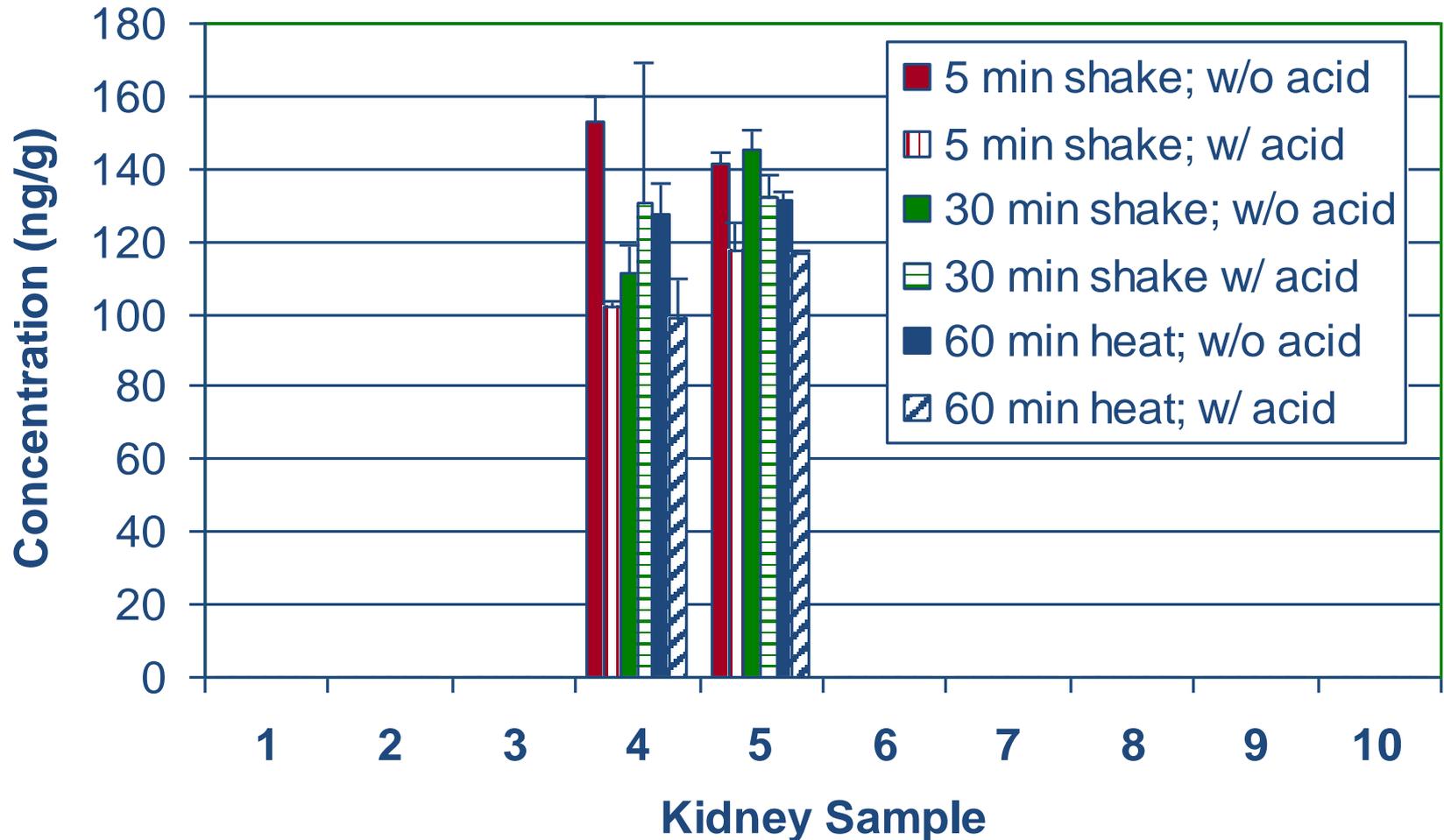
Analysis of Incurred Kidney (2 g)

Sulfadimethoxine in Incurred Kidney (no IS)



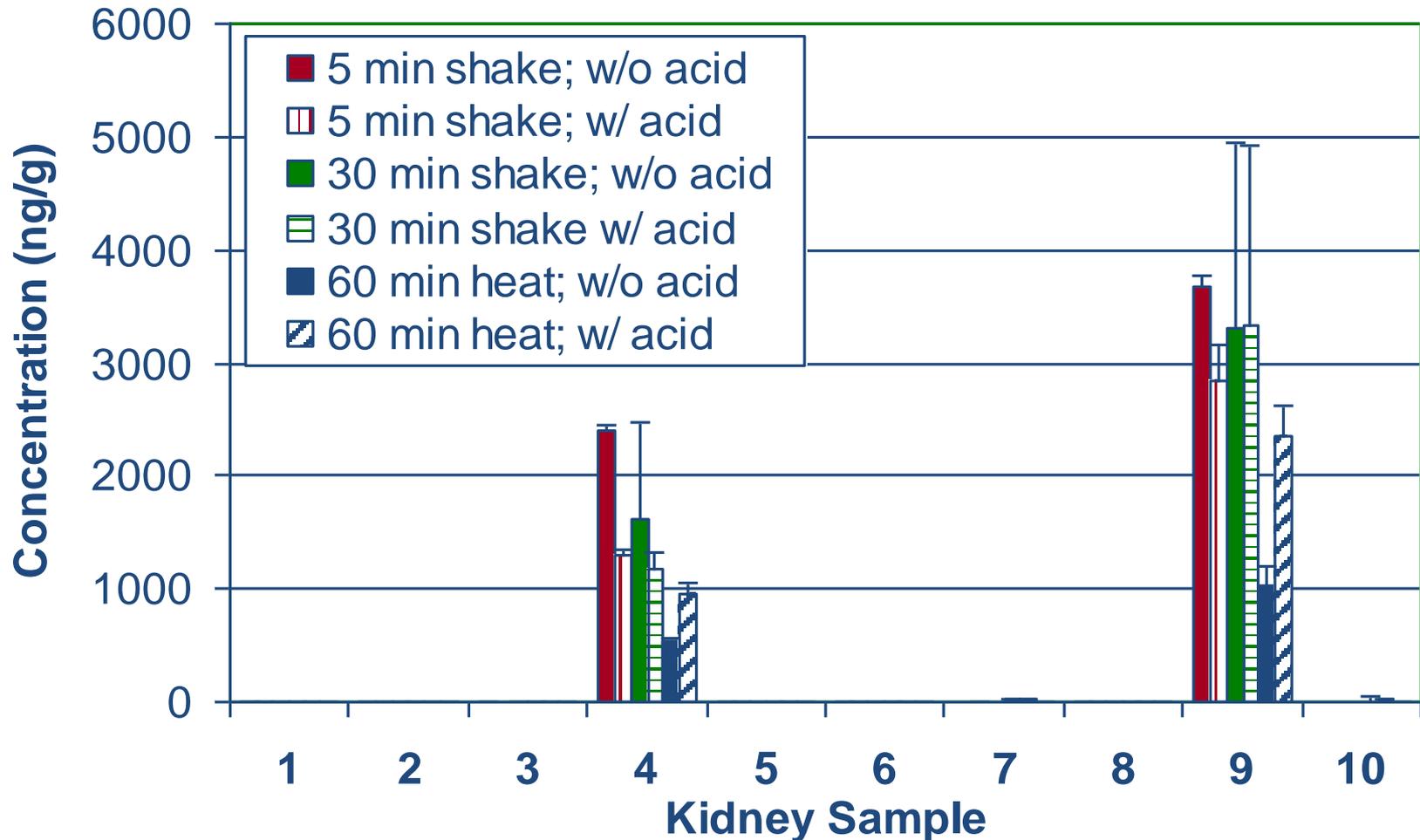
Analysis of Incurred Kidney (2 g)

Sulfamethazine in Incurred Kidney (no IS)



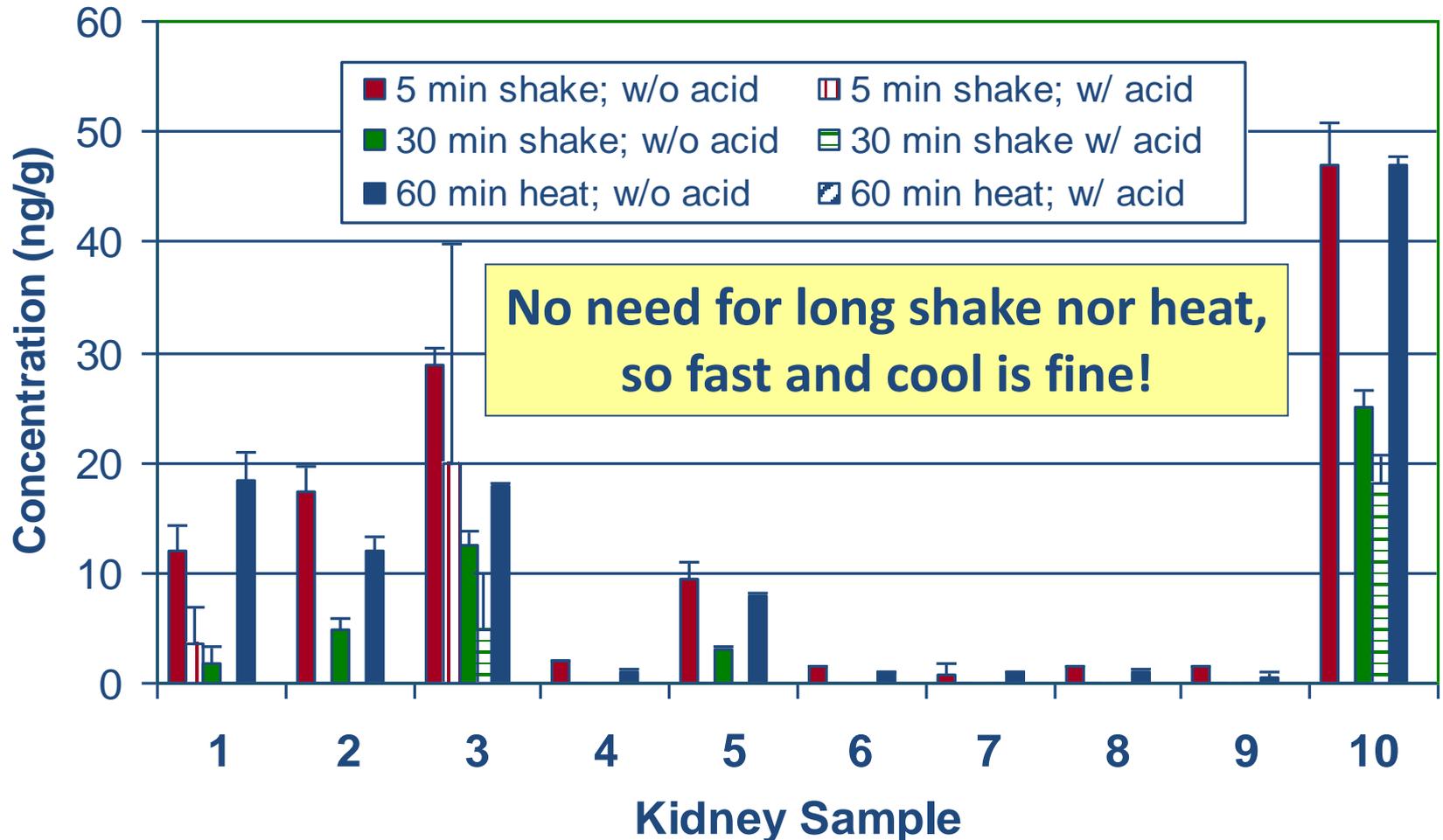
Analysis of Incurred Kidney (2 g)

Tetracyclines (Sum) in Incurred Kidney (no IS)



Analysis of Incurred Kidney (2 g)

Penicillin G vs. PenG-d7 IS in Incurred Kidney



The Case of Penicillin G

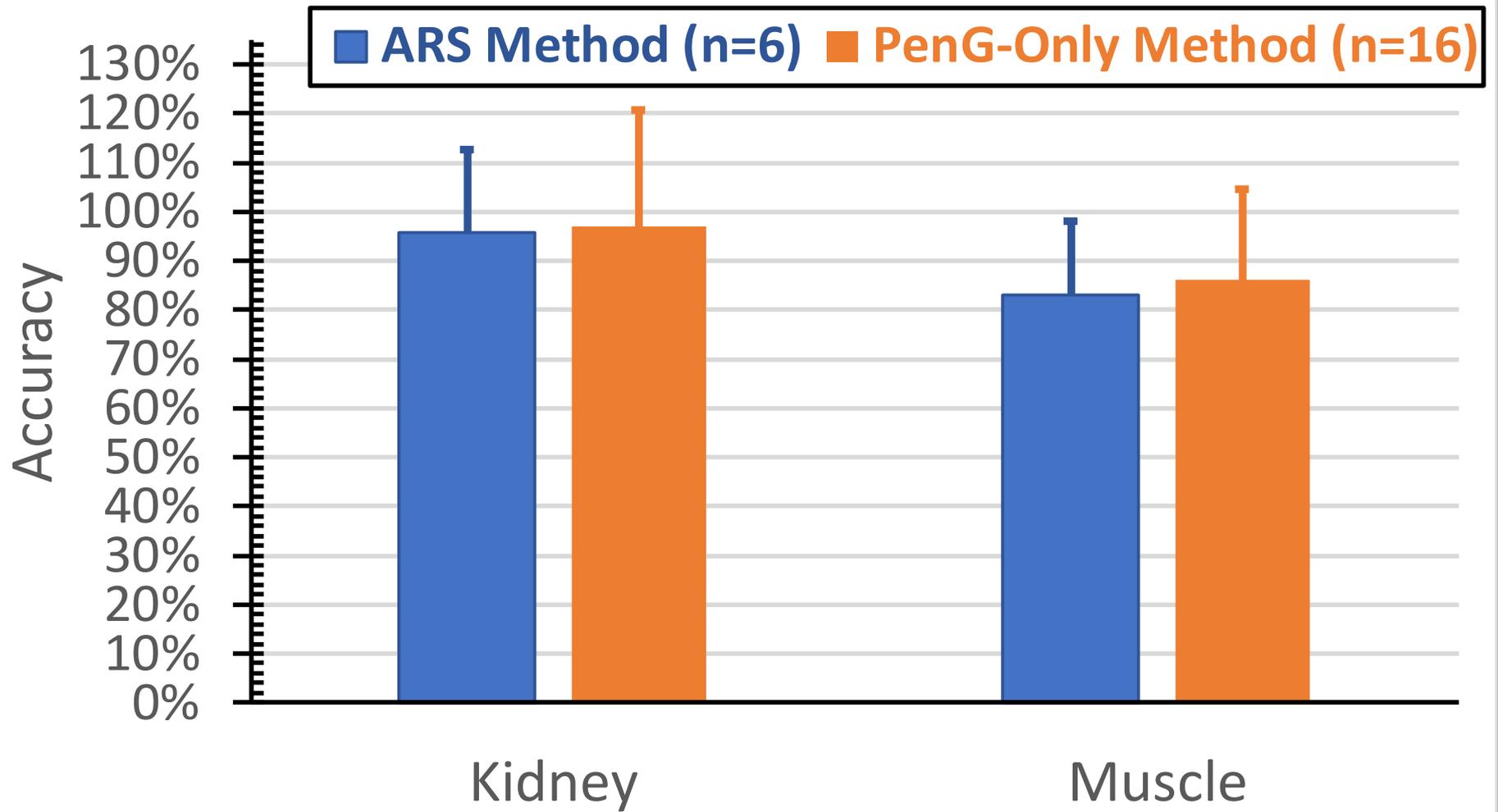
Due to degradation concerns, added main metabolites of penicillin G to the target list in the method.

In a degradation study, no degradation was observed when using fresh acetonitrile/water solutions and mobile phase, but older solutions caused degradation, presumably due to formation of acetamide, which was also monitored.

We still look for the PenG metabolites, but have only observed slight degradation of the PenG-d7 int. std. over time in the stock solution.

A key to the improved analysis of PenG is to use reasonably fresh extraction solution and mobile phase (made weekly).

Analysis of PenG in Prof. Test Samples



Freeze-dried PT samples analyzed among 4 labs monthly with blind conc. 20-200 ng/g (so far)

Phase I Validated Method (2012)

extraction

2 g tissue in a 50 mL tube
add IS mix (SMZ-IS; flunixin-d3; PenG-d7)

add 10 mL of 4/1 (v/v) MeCN/water
vortex briefly, shake for 5 min
centrifuge for 5 min >3500 rcf

clean-up

supernatant + 500 mg C18 + 10 mL hexane sat'd
w/MeCN; mix for 30 s, centrifuge for
5 min > 3500 rcf; aspirate hexane to waste

evaporate 5 mL extract to 1 mL final vol.

filter extract with the Mini-UniPrep™

UHPLC-MS/MS analysis

UPLC-TQD Parameters

- ✓ Column – Acquity UPLC HSS T3, 1.7 μm , 100 x 2.1 mm
- ✓ Mobile Phase A – 95% H_2O / 5% MeCN / 0.1% formic acid
- ✓ Mobile phase B – 100% MeCN / 0.1% formic acid
- ✓ Flow rate of 0.50 mL/min.
- ✓ Gradient:
 - 0.0 min – 0.2% B, 0.1 min – 0.2% B, 8.00 min – 99.8% B,
 - 9.5 min – 99.8% B, 9.6 min – 0.2% B, 12.8 min – 0.2% B
- ✓ Injection volume of **20 μL = 17.4 mg equiv. sample!**

Phase I **Method Logistics**

1 chemist was able to process 60 pre-homogenized samples in an 8-hr day for an overnight sequence

(longest step was 1 hr to evaporate MeCN)

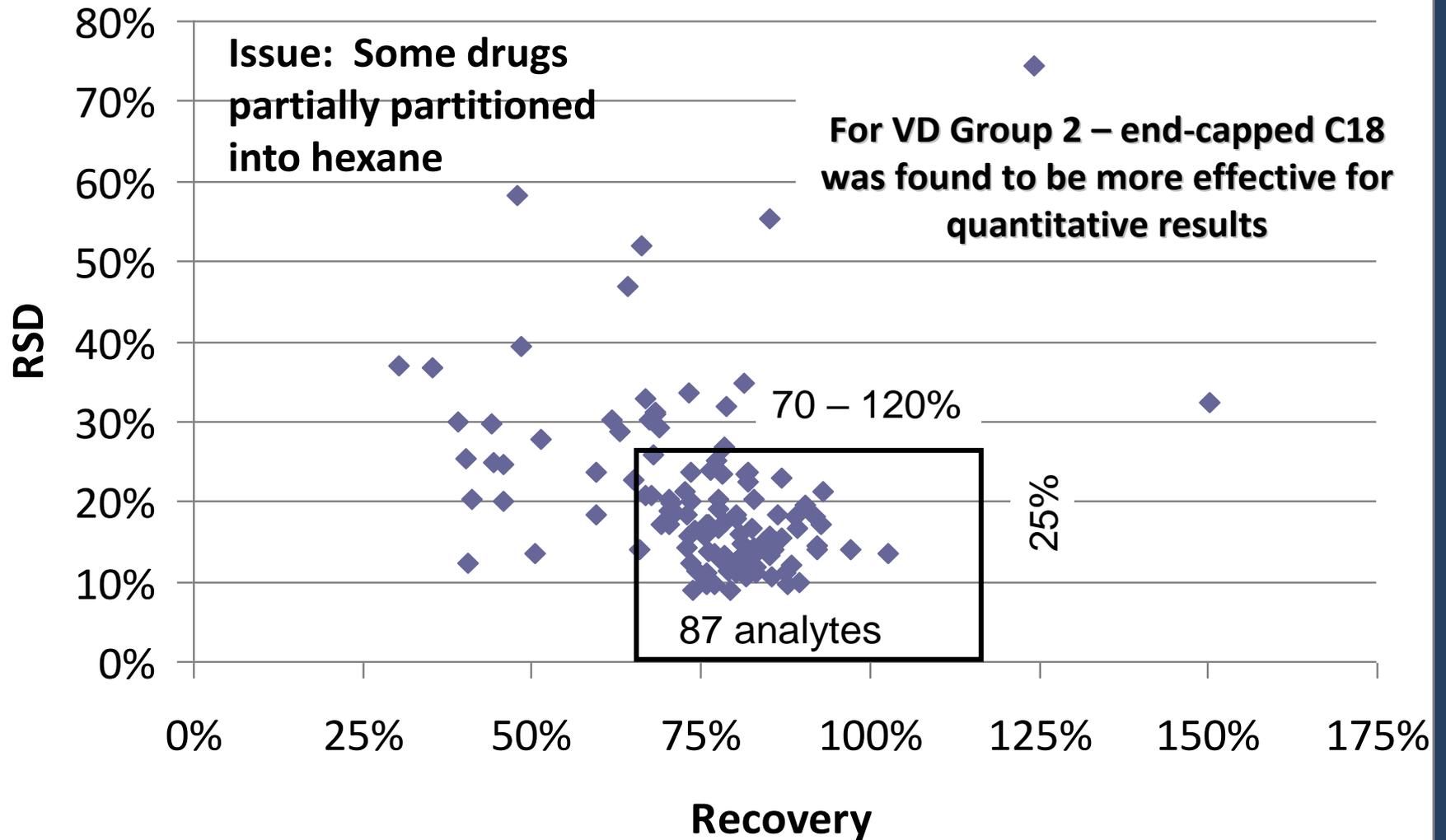
No glassware to be cleaned afterwards

Cost of materials \approx \$3/sample (using bulk C18)

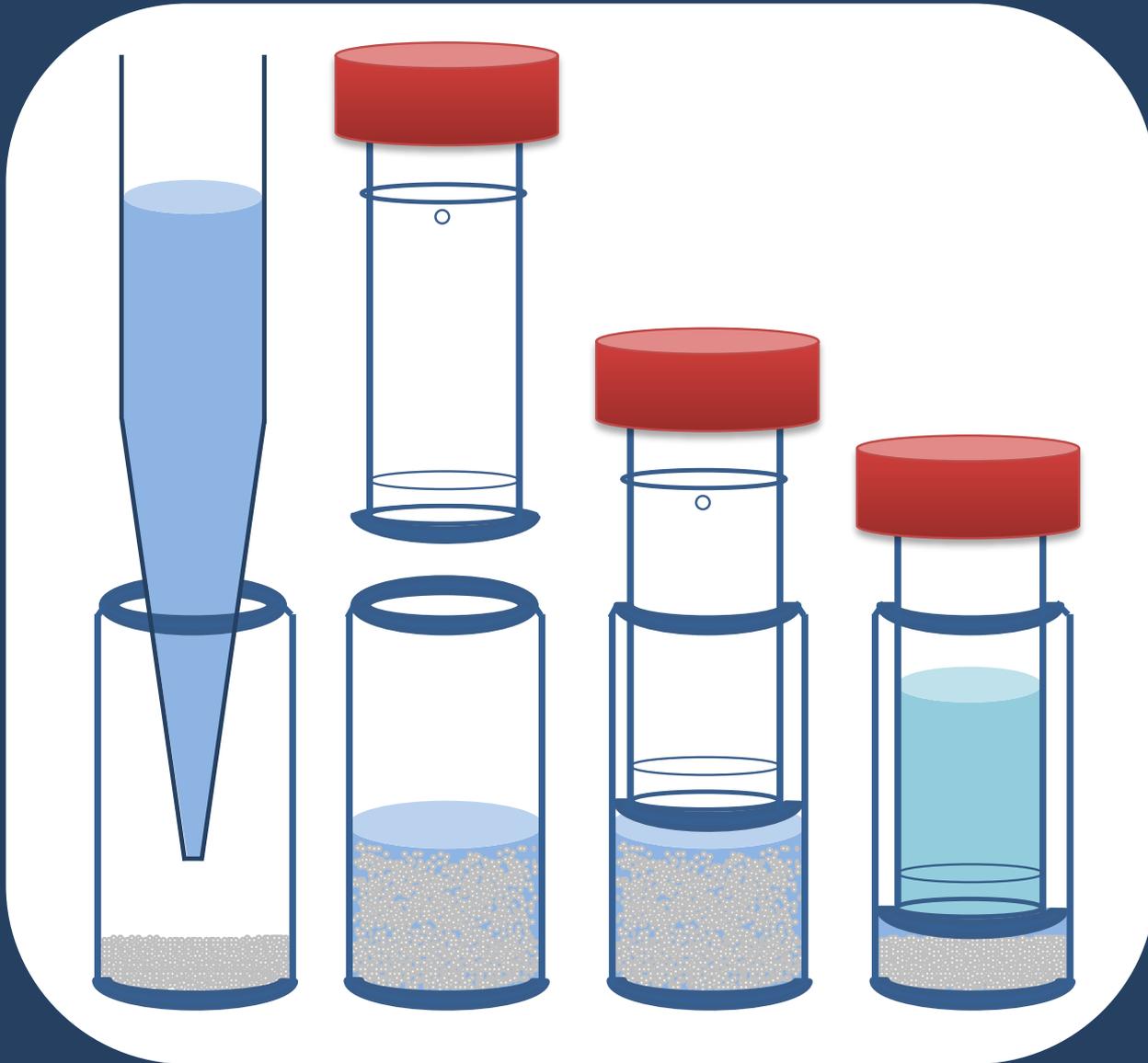
Waste = 10 mL hexane and 5 mL MeCN

(and two 50 mL and one 15 mL polypropylene tubes)

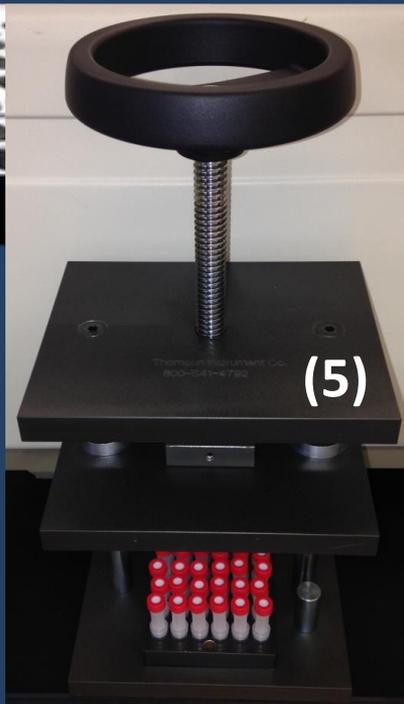
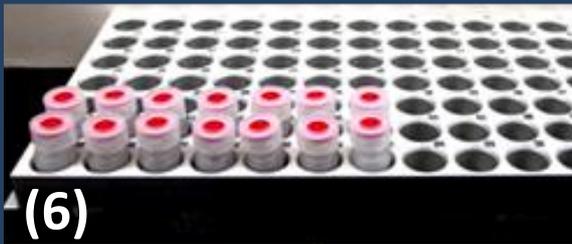
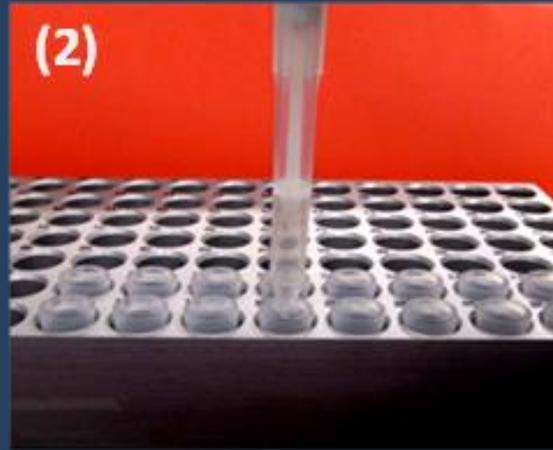
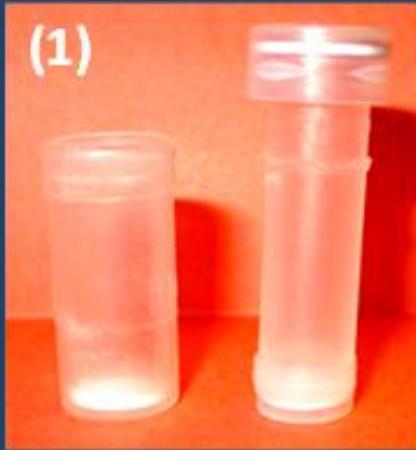
Average Recoveries and Reproducibilities



Phase II: Filter-Vial dispersive-SPE



Filter-Vial d-SPE in a Batch Process



9-tube press for 5-10 mL filter tubes, too

Phase II Method for Veterinary Drugs (2015)

extraction

2 g tissue in a 50 mL tube
add IS mix (SMZ-IS; flunixin-d3)

add 10 mL of 4/1 (v/v) MeCN/water
vortex briefly, shake for 5 min
centrifuge for 5 min >3500 rcf

clean-up

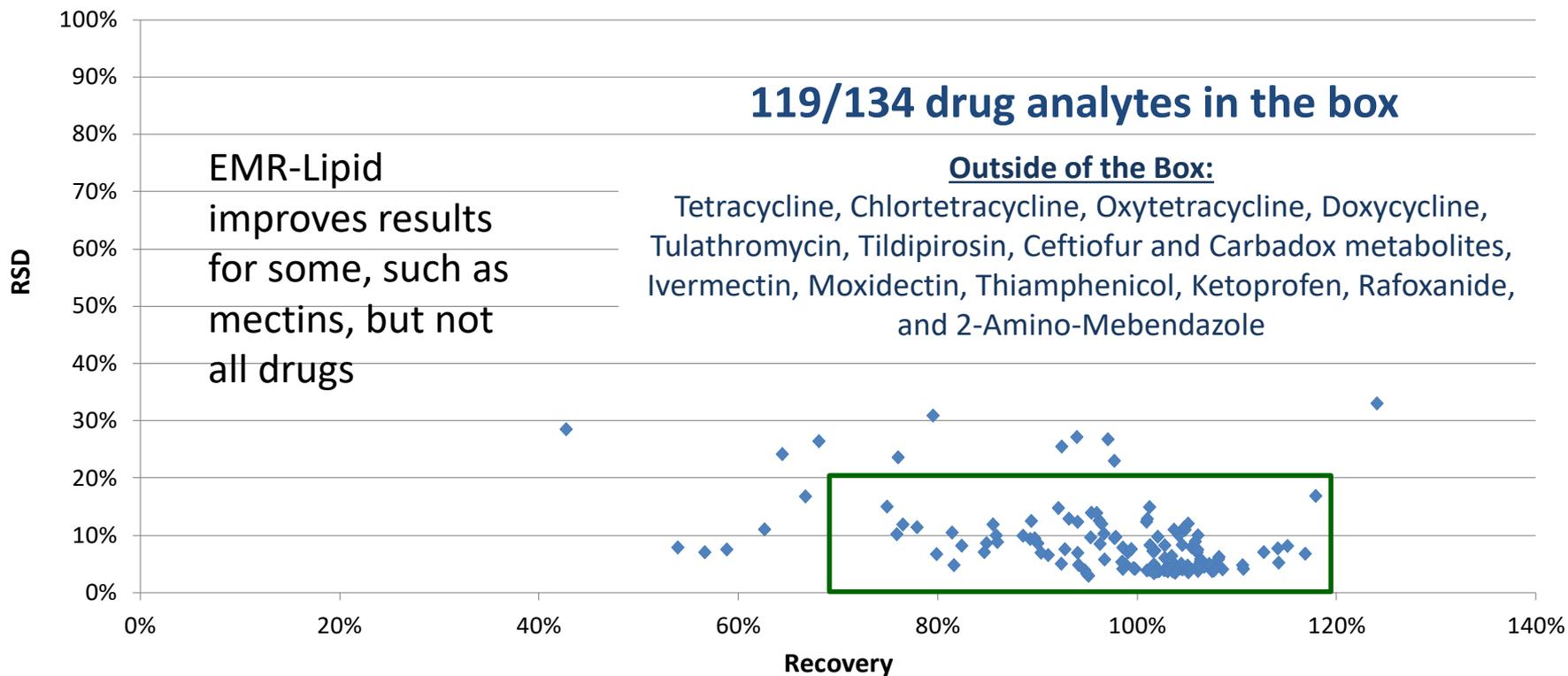
0.4 mL supernatant + 25 mg C18 in filter-vial d-SPE;
vibrate AS tray for 30 s and filter through 0.2 μ m
PVDF by pressing plungers to seal the vials

Inject 1 μ L in UHPLC-MS/MS

17.4 mg equiv. sample reduced to 0.174 mg
by using more modern instrument!

Phase II Method Performance

Results for Bovine Muscle in the Final Method (0.5, 1, 2X Levels, n=29, Day 2), no IS



**LC-MS/MS results based on matrix-matched calibration
- added int. stds not employed**

Phase II Method Logistics

1 chemist was able to process 60 pre-homogenized samples in 3 hours

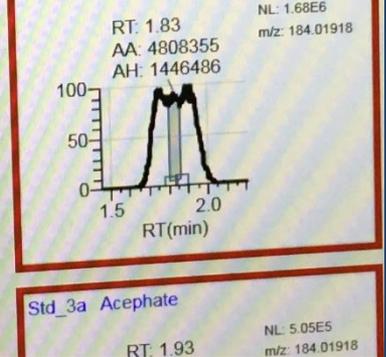
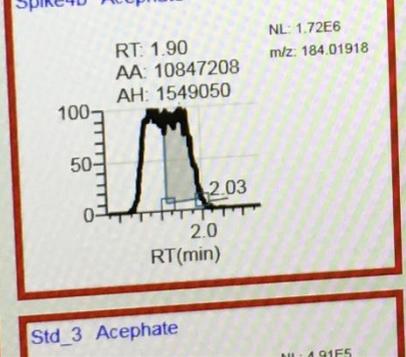
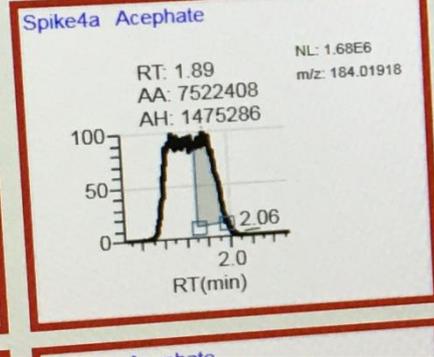
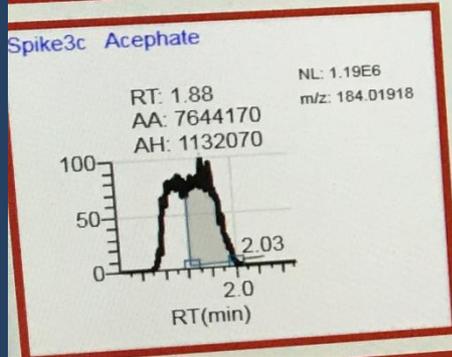
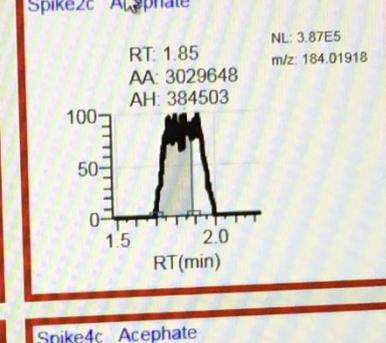
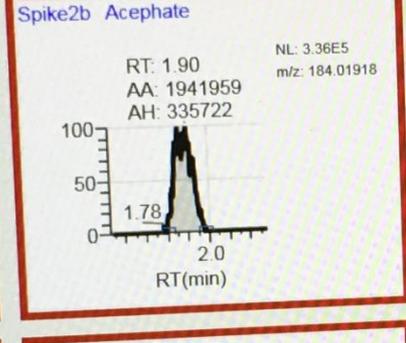
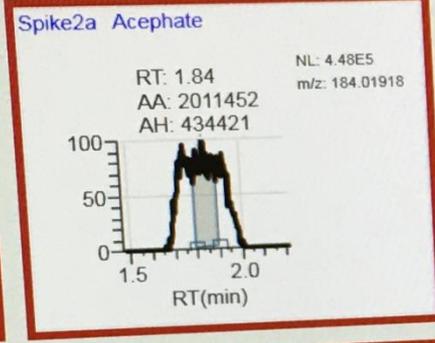
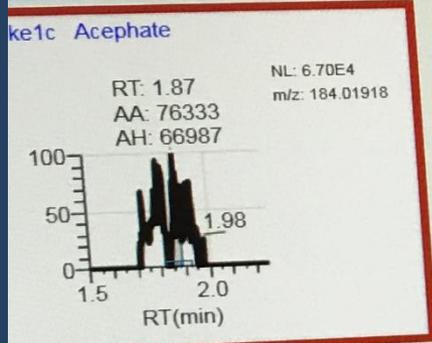
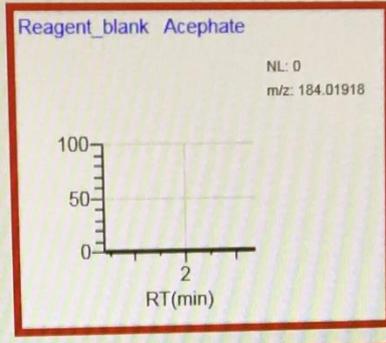
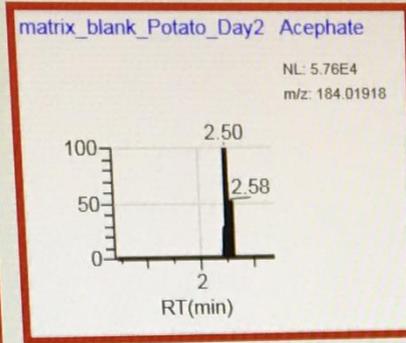
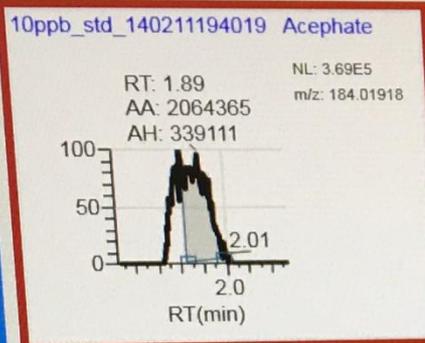
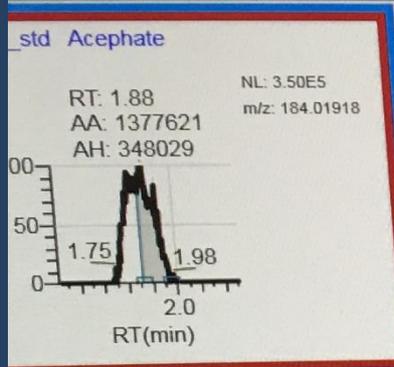
(longest steps involved labeling tubes/vials, weighing, and preparing calibration standards)

No glassware to be cleaned afterwards

Waste = 10 mL MeCN (and one 50 mL tube and an autosampler vial)

Review of results for 135 drugs x 3 transitions x 67 injections (>27,000 data points) took 8 hours

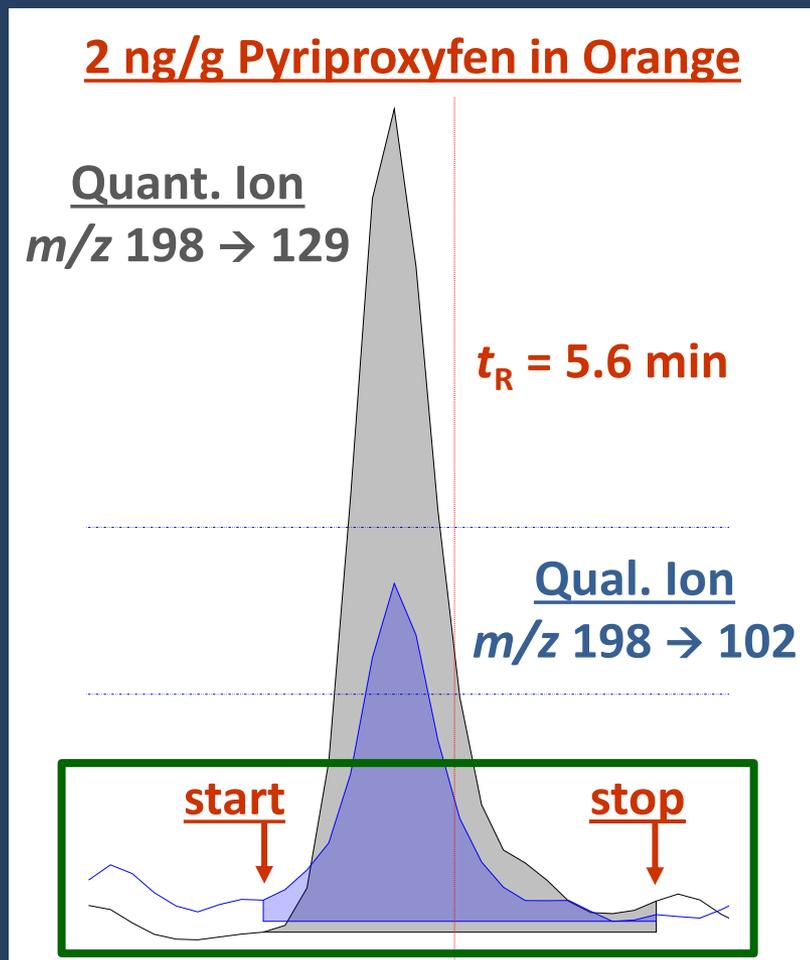
Poor integration undoes excellent detection



Summation Integration in Chromatography

SIMPLIFY, don't COMPLIFY!

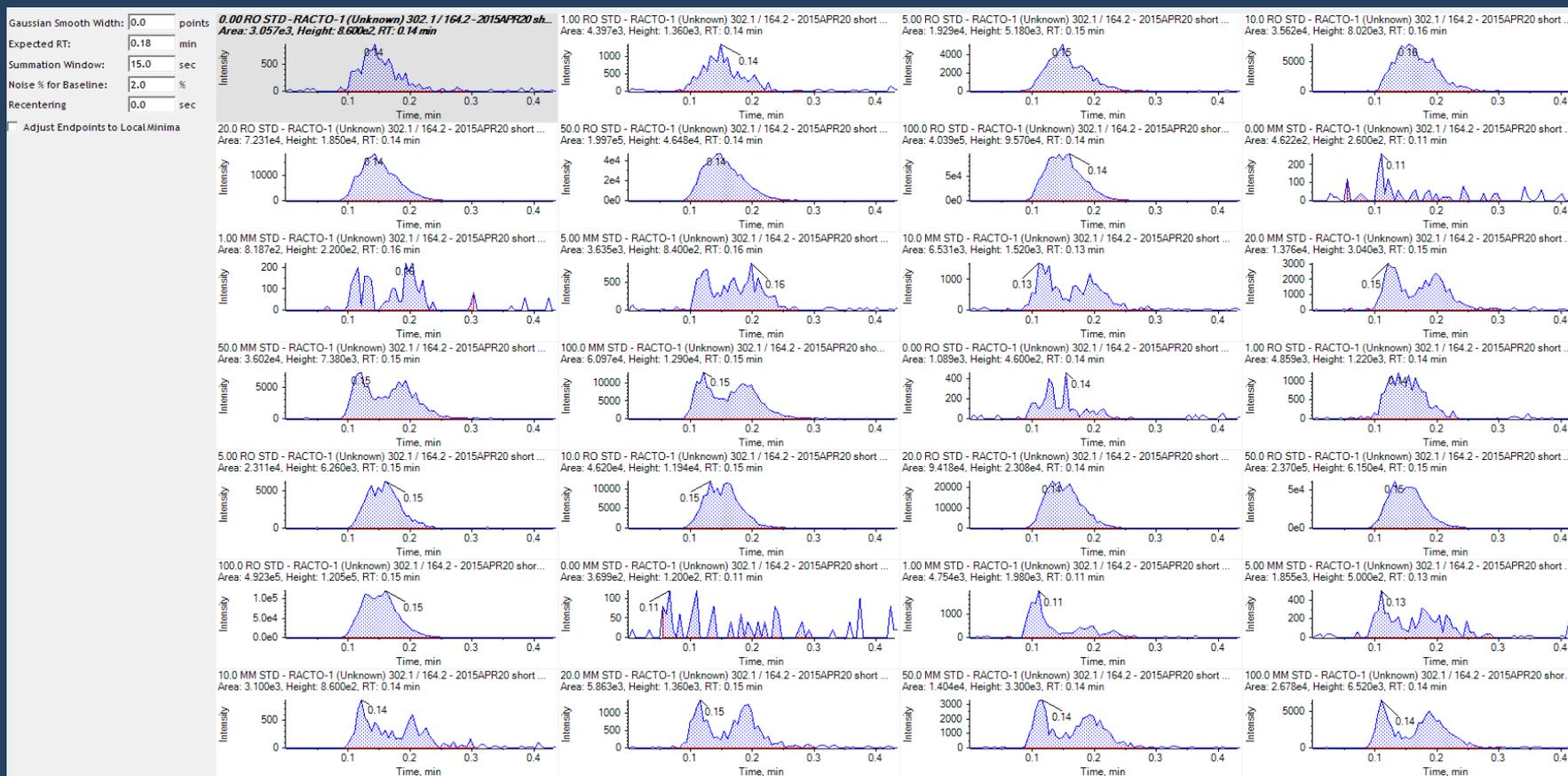
- Draw a straight line at the baseline just before the start of the expected peak to just after its expected end → **EASY PEASY!**
- See: Lehotay, *LCGC North America* 35 (2017) 391-402.
- **Advanced ≠ Better**
- **Function ≠ Beauty**
- **Time = Money**



**Quant. and Qual. Ions
Co-Elute with the Same t_R !**

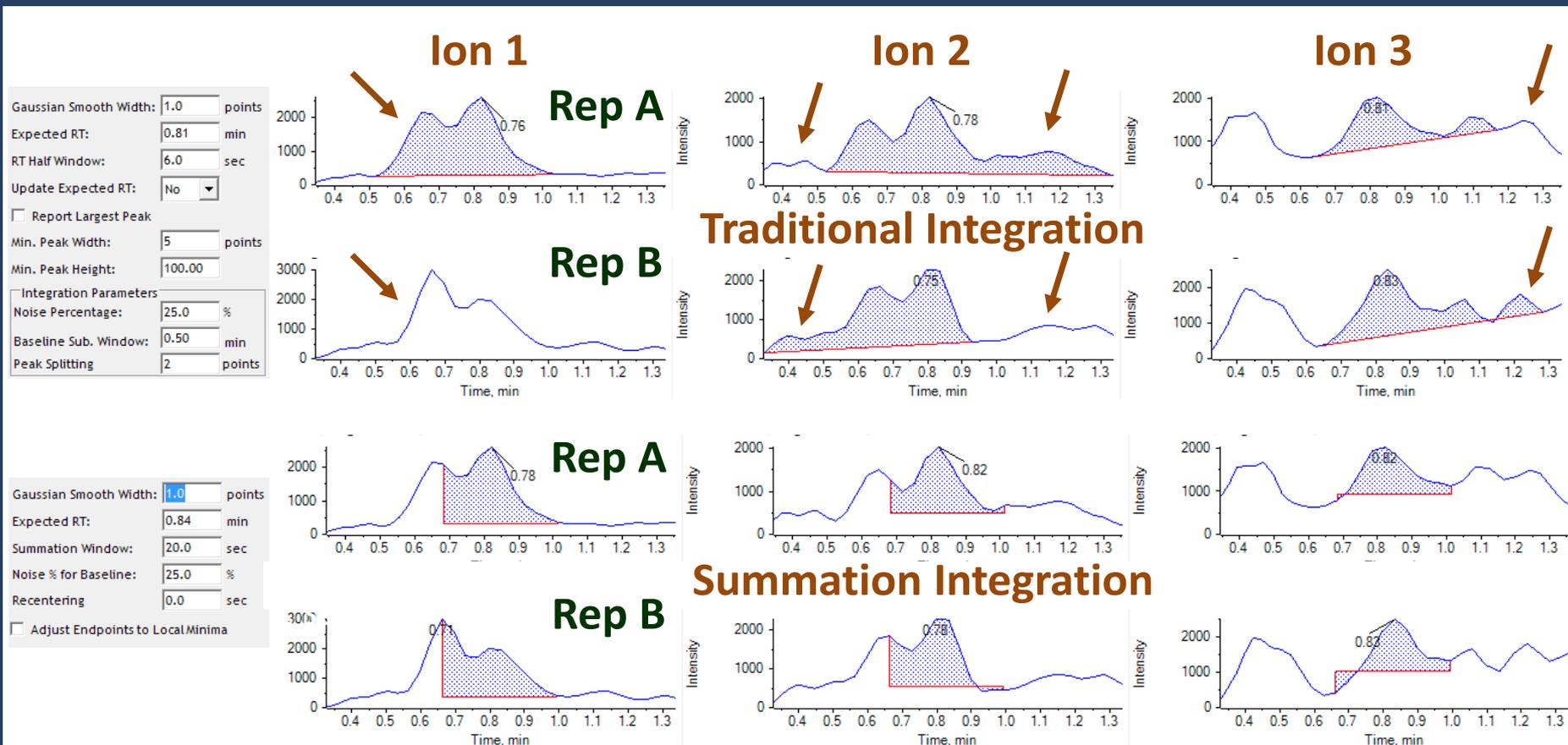
Summation Integration Function

- ≈ 1 min to integrate a batch of >60 samples of ≈ 660 MRMs per sample **WITHOUT REVIEW!**



- This is a >40 year-old integration function, but **LACKING IN SOME DATA PROCESSING SYSTEMS!**

Summation integration is consistent and reliable



The top two integrations were false negatives, but not when using summation integration.

USDA Rules in Automatic Post-Run Identification

(*e.g.* in Excel or Instrument Software)

Note: Any Set of Identification Criteria can be Applied

1) Ret. time (t_R) for each ion (Quant. and Qual.) must be $\leq |0.1|$ min from the contemporaneous $t_R(\text{ref.})$, which is the **avg t_R** from high conc. calibration stds in solvent in the same sequence.

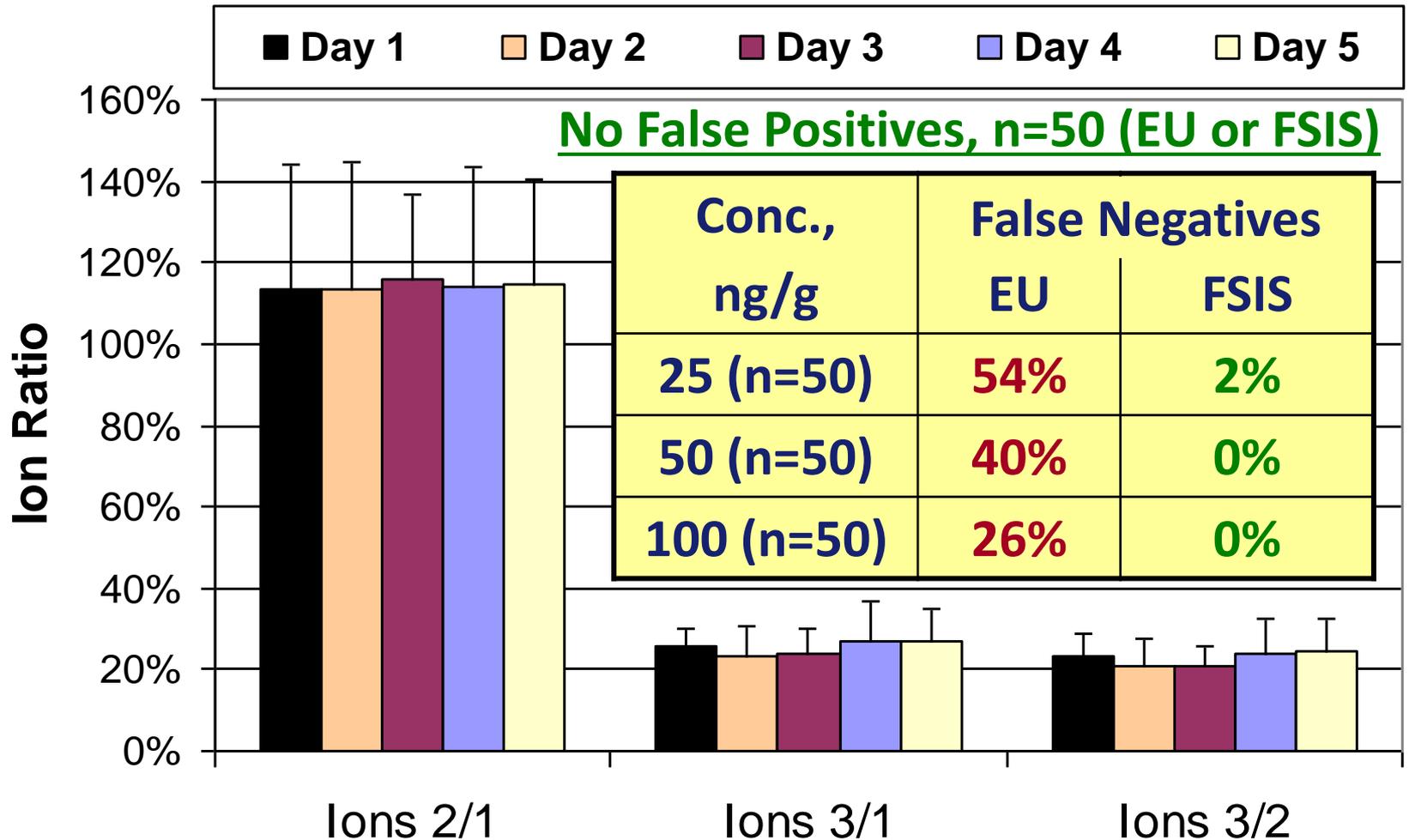
2) Ion Ratio (IR) = (signal ion 2)/(signal ion 1), 3/1, 3/2, *etc.* (in %);
IR(ref.) = **avg IR** of contemporaneous high conc. calibration stds in solvent [**note: IR(ref.) \leq 110%**]

Ident. requires $|\pm 10\%|$ for ≥ 1 IR or $|\pm 20\%|$ for ≥ 2 IRs vs. IR(ref.)

3) Conc. must be $>$ **reporting level** (*e.g.* LOQ, LOI, or MRL)

Ion Ratios for Ciprofloxacin in Kidney

Ion Ratios of Spks, n=30 each (10 x 3 levels)



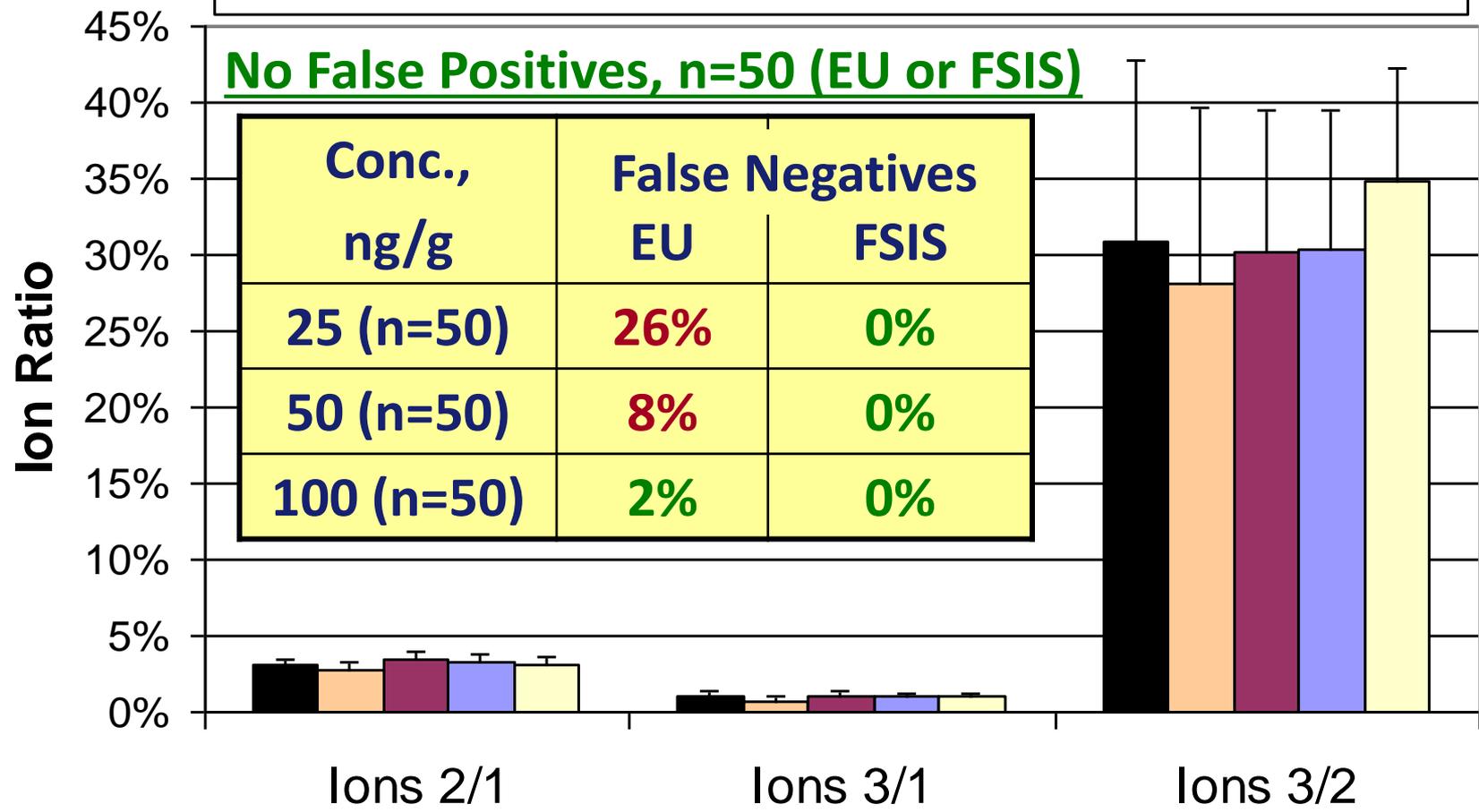
Ion Ratios for Lincomycin in Kidney

Ion Ratios of Spks, n=30 each (10 x 3 levels)

Day 1
 Day 2
 Day 3
 Day 4
 Day 5

No False Positives, n=50 (EU or FSIS)

Conc., ng/g	False Negatives EU	False Negatives FSIS
25 (n=50)	26%	0%
50 (n=50)	8%	0%
100 (n=50)	2%	0%



Bottom Line

There are many complicated opinions of “good enough” criteria to meet MS-based identification standards

But they are all based on generalizations, not scientific assessments at all actual conditions

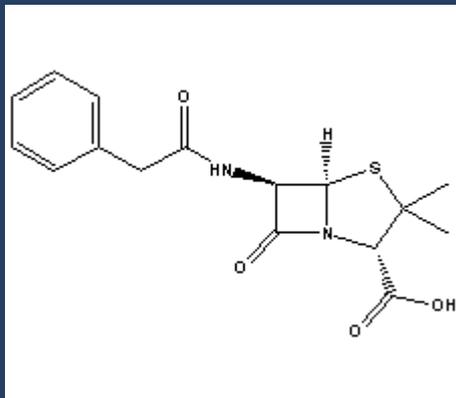
The bottom line is rates of false pos/neg

If analytical conditions shown to meet <5% false results in extensive validation (multi-matrix, multi-level, blind), then it should be acceptable

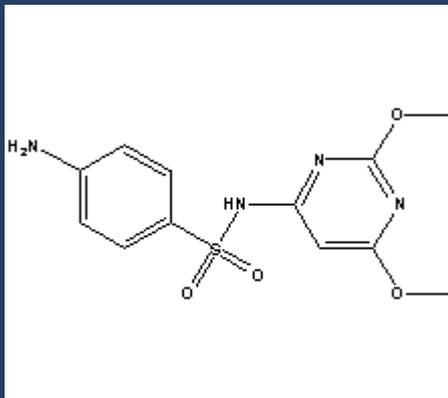
Rely on Orthogonal Confirmation Methods

Issue: What about Aminoglycosides?

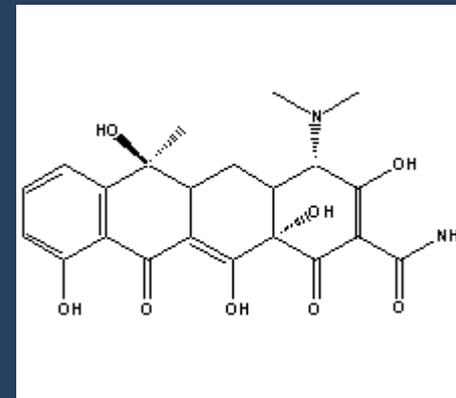
β -Lactams



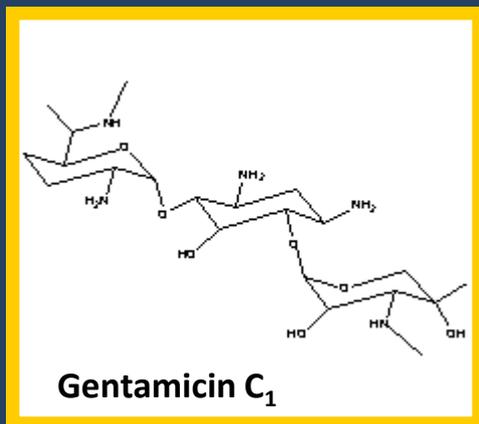
Sulfonamides



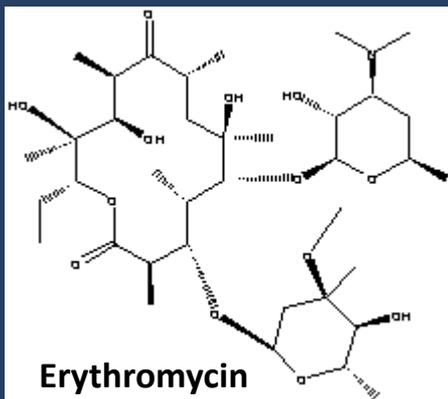
Tetracyclines



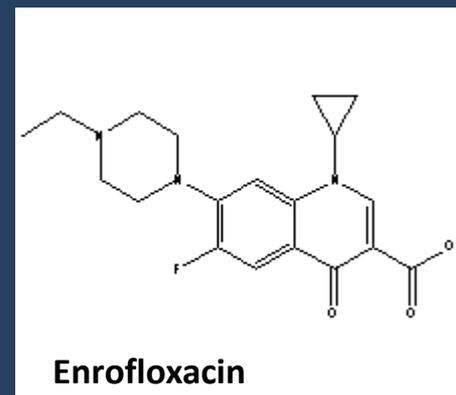
Aminoglycosides



Macrolides



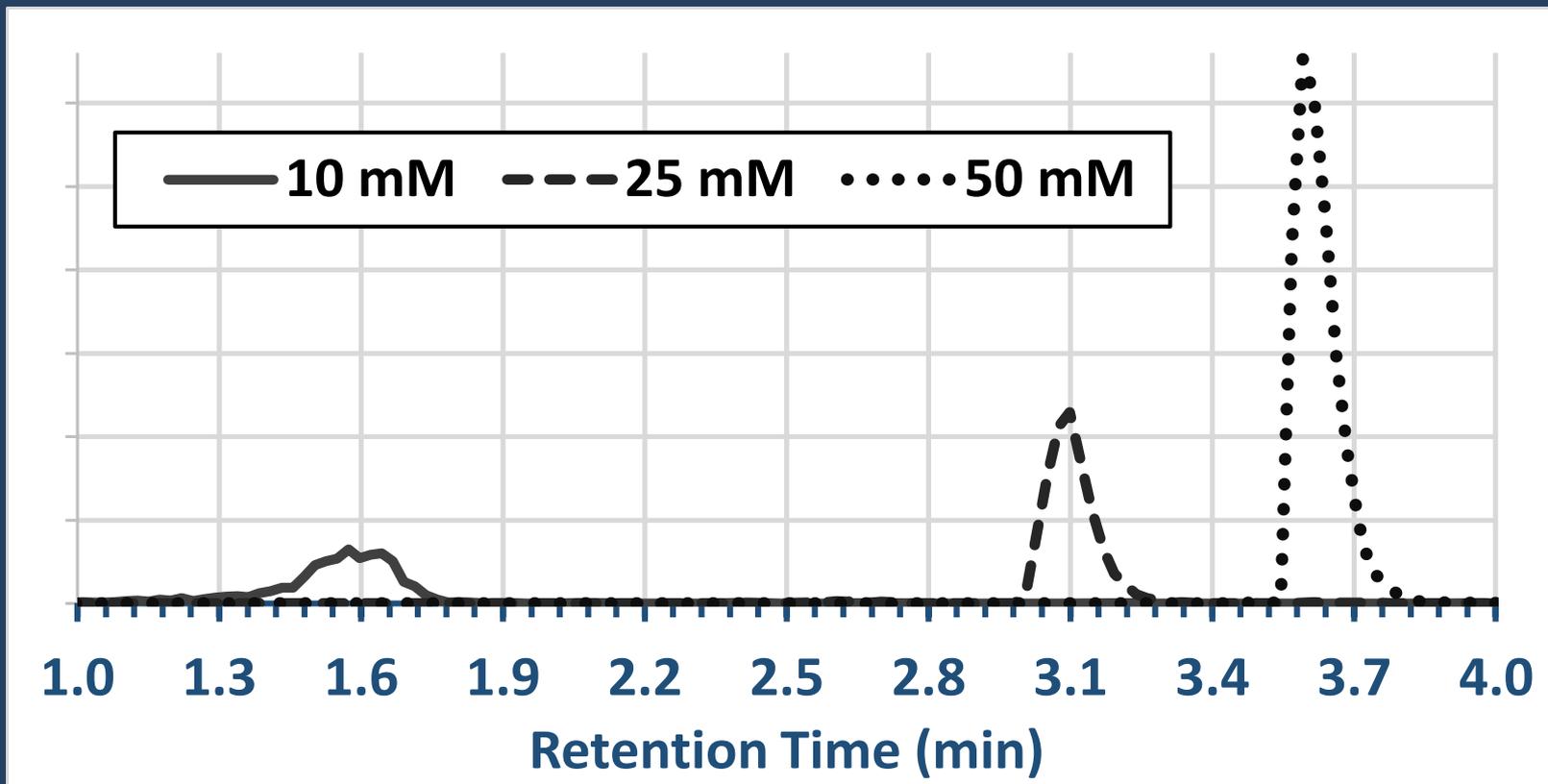
Quinolones



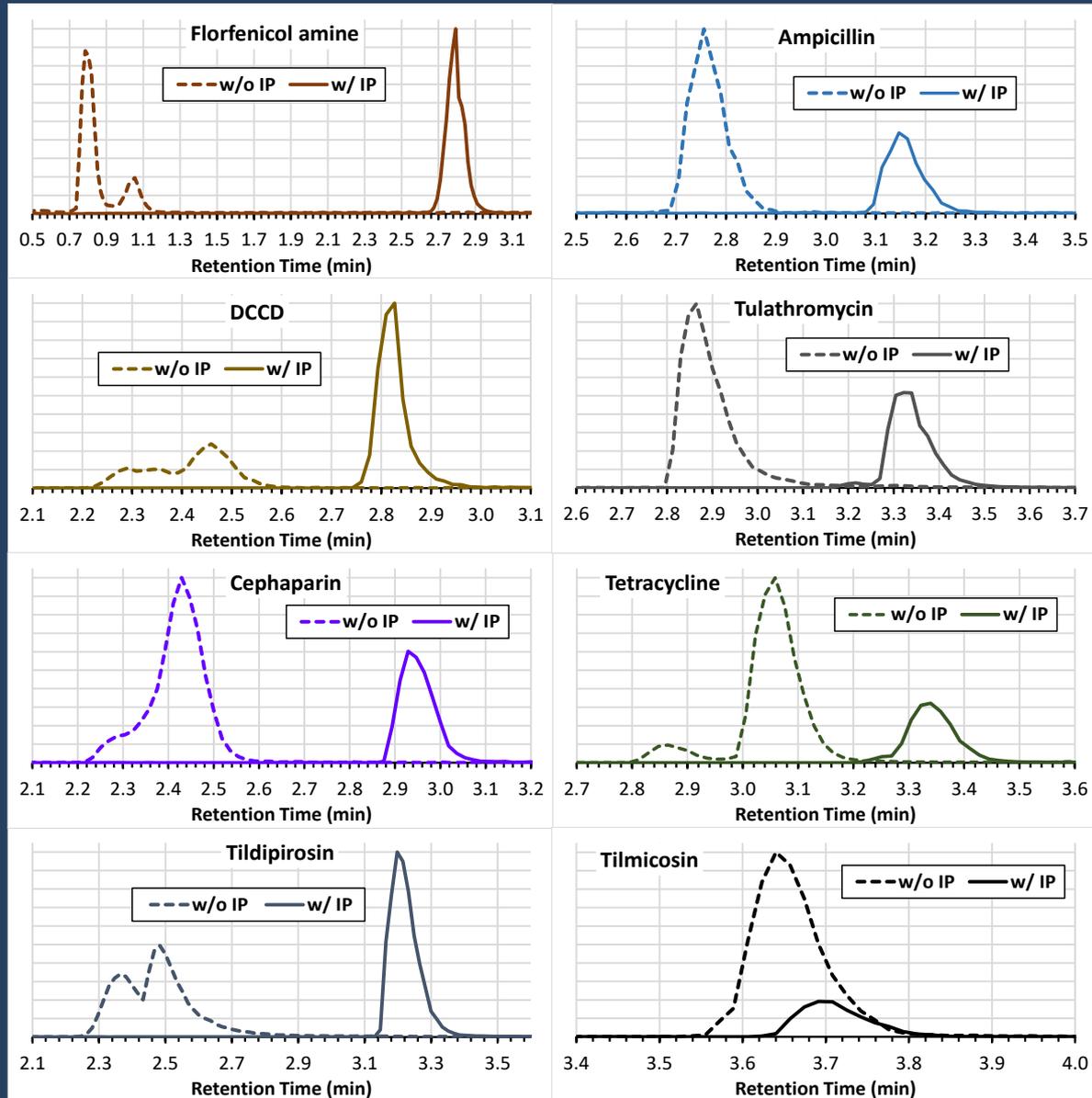
- Currently, 219 vet. drugs (including >100 antibiotics) are on our list, but have targeted and evaluated \approx 180 so far in (UHP)LC-MS/MS.

Sodium 1-Heptanesulfonate in Final Extract

UHPLC of apramycin and amount of IP agent

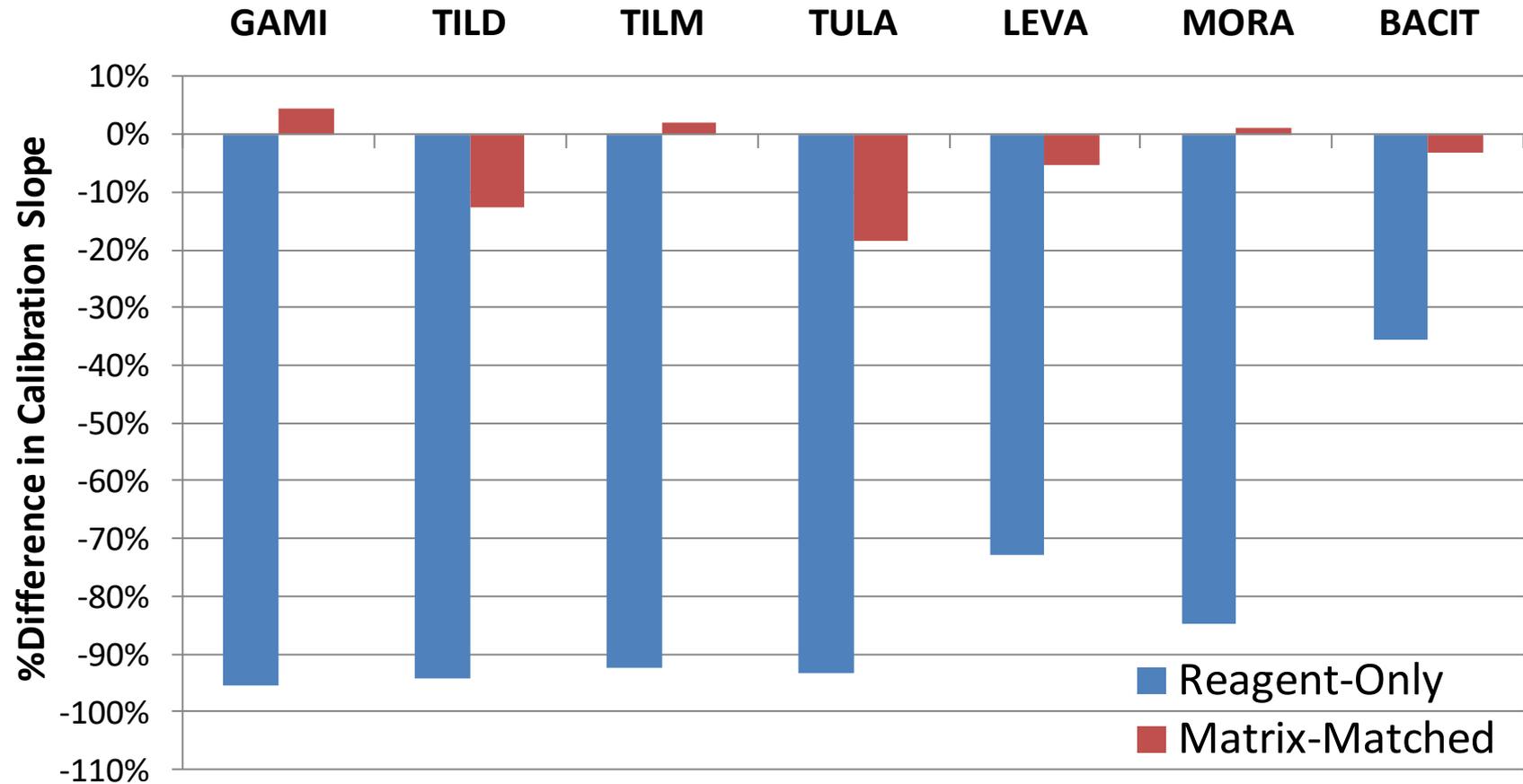


Effect of Ion-Pairing Agent in Final Extract



Issue: Losses due to Filtration w/o Matrix

Effect of Filtering (5:1 dil'n) for Macrolides/Others



Can avoid matrix effects by dilution, but still needed matrix-matching to compensate for losses of some analytes

Phase II Veterinary Drug Residue Method

Aminoglycosides

2 g tissue + 20 mL of 10 mM NH_4OAc , 0.4 mM EDTA, 2% trichloroacetic acid, and 0.5% NaCl in water + IS

Multiclass, Multiresidues

2 g tissue + 10 mL 4/1 (v/v) acetonitrile/water + IS

Shake 5 min on pulsed vortex platform shaker (80% setting, max pulsation)

Centrifuge 3 min at 3700 rcf

Transfer 10.75 mL (1 g equiv. sample) to 15 mL tube

Adjust pH to 6.5 ± 0.1 using a pH meter

Condition 50 mg WCX* DPX[†] tips with 3 mL each of methanol and water

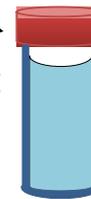
Load extract in 3 portions onto 50 mg WCX DPX tips

Wash DPX tips with 5 mL water

Elute DPX tips with 1 mL 10% formic acid in water

***WCX = weak cation exchange sorbent**
†**DPX = dispersive pipette extraction**

71 μL extract
(71 mg sample equiv.)



Tissue equivalence 0.174 g/mL

(no cleanup)

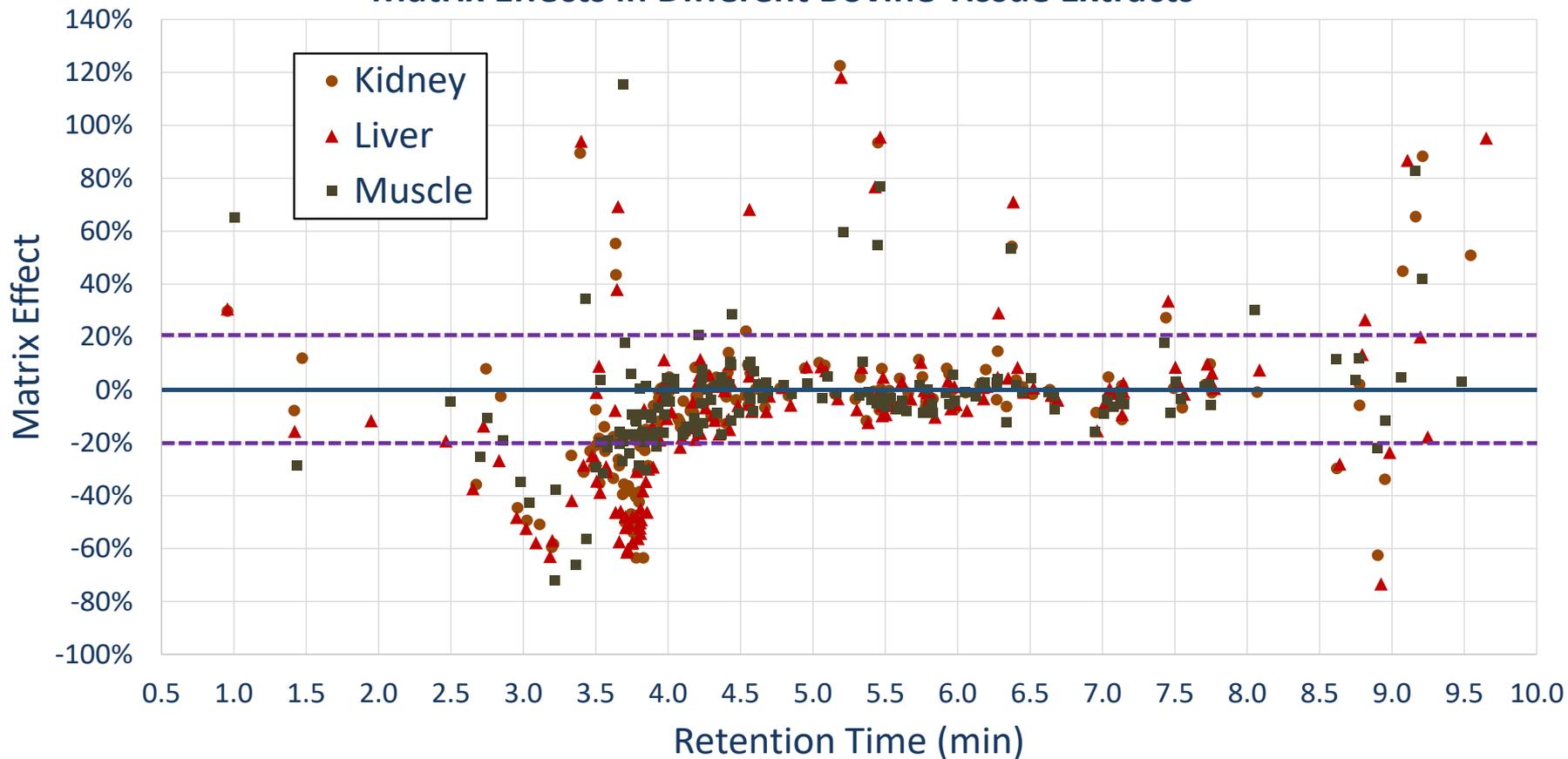
407 μL extract
(71 mg sample equiv.)

+ 272 μL 138 mM sodium 1-heptanesulfate ion-pairing (IP) reagent in water/acetonitrile

Yields 95 mg/mL final extract for each method in 34/66 (v/v) acetonitrile/water containing 50 mM IP reagent and 0.85% HO_2CH → 4 μL injection = 0.38 mg equiv. sample on column

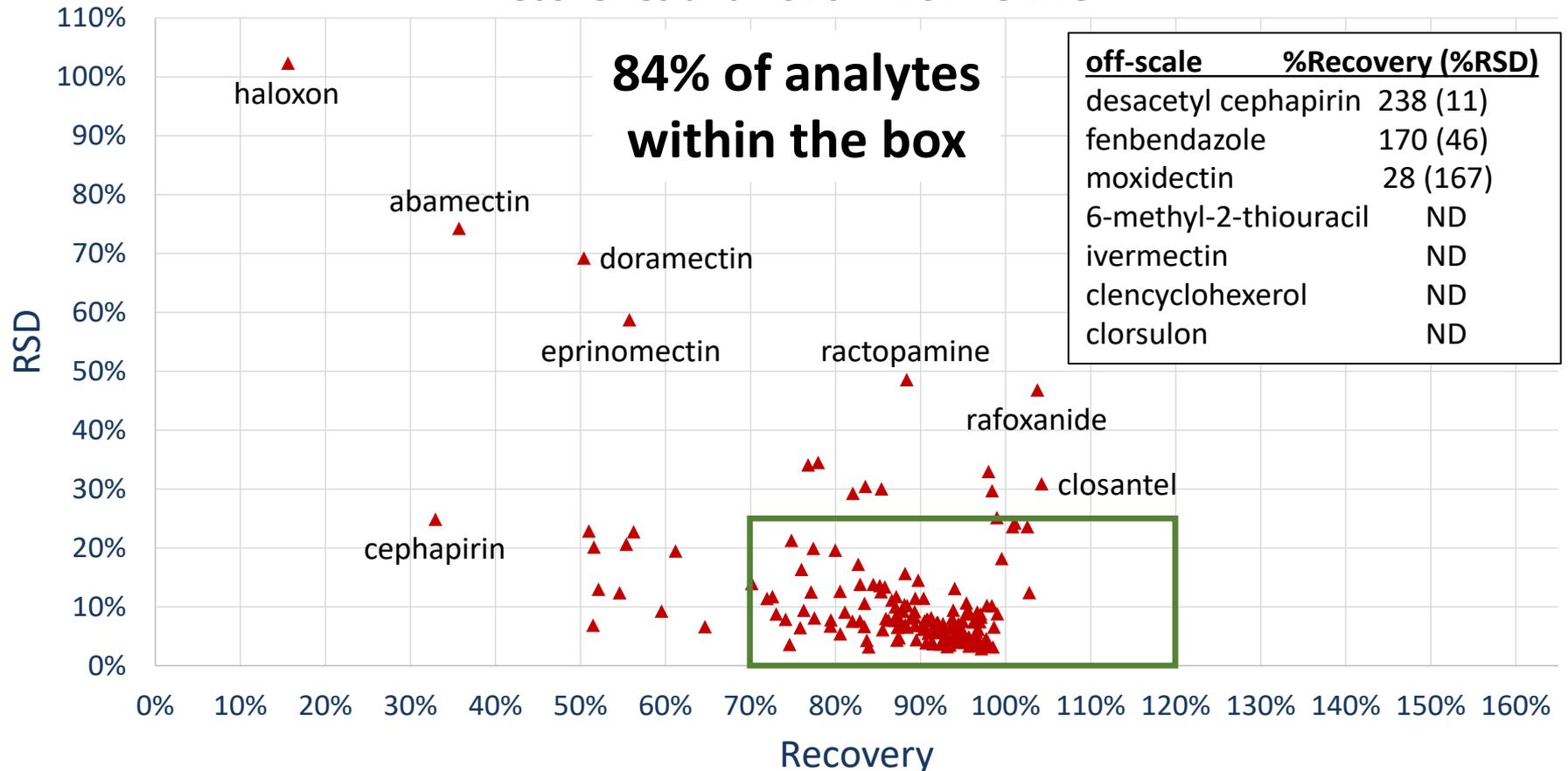
Matrix Effects

Matrix Effects in Different Bovine Tissue Extracts



Phase III Validation Results

Recoveries and RSDs in Bovine Liver



Validation of Liquid and Powdered Eggs

FSIS Validation Protocol followed for 168 Targeted Drugs

Criterion	Liquid Eggs	Powdered Eggs
Recoveries 70-120% *	156 (89%)	152 (87%)
RSD < 25% *	154 (88%)	155 (89%)
False Negatives <10%	154 (92%)	153 (91%)
Limit of Quant. < 10 ng/g	144 (86%)	97 (58%) **
Limit of Ident. < 10 ng/g	122 (73%)	72 (43%) **

* 175 targets including QC; ** dry weight sample

Subsequently, coccidiostats and ionophores were added to the list of drug analytes and similar validation results were achieved for catfish, chicken tenders, bacon, and sausage

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Waters

Sciex

Phenomenex

Disclaimer: USDA does not recommend any products over others of a similar nature.

New Developments

- 1) Added several coccidiostats to multiresidue method (MRM), and most give acceptable results with current method
- 2) Conducting proficiency test samples from FSIS for penicillin, and our MRM results match FDA single-analyte method so far
- 3) Studied penicillin degradation and need to avoid old aqueous acetonitrile solutions in which acetamide is generated
- 4) Comparing and validating our and other published MRMs for drugs in catfish and ready-to-eat meats
- 5) Analyzed proposed certified reference material from Canada in freeze-dried bovine muscle

Other Future Plans

- Comparing rates of false positives and negatives when using 2 ions vs. 3 ions in qualitative MS/MS analyte identifications.
- Investigations of HILIC and/or ion-pairing (and different IP agents) in simultaneous LC-MS analysis of diverse analytes.
- Can flow-injection analysis achieve acceptable results in multiclass, multiresidue monitoring?
- Improved sample preparation for better cleanup and wider scope, including problematic analytes and matrices.

Phase III Validation Results

Table 1: Results for the veterinary drugs spiked at 0.5X, 1X, and 2X levels, n=10 each, in the bovine tissues; (t_R = retention time); aminoglycosides in blue text. **Red = <50 or >150% Recovery or >40% RSD**

Gold = 80-110% Recovery, ≤15% RSD

Silver = 70-120% Recovery, ≤25% RSD

Bronze = 50-150% Recovery, ≤40% RSD

Drug Analyte	t _R (min)	1X Level (ng/g)	Kidney	Liver	Muscle	Drug Analyte	t _R (min)	1X Level (ng/g)	Kidney	Liver	Muscle
13C6-Sulfamethazine	3.75	200				Lincomycin	3.78	100			
2-Mercaptobenzimidazole	3.66	25				Mabuterol	4.42	10			
2-Mercapto-1-methylimidazole	1.95	200				Marbofloxacin	3.85	100			
Quinoxaline-2-carboxylic acid	3.82	100				Mebendazole	5.47	10			
2-Thiouracil	0.96	400				Mebendazole-2-amino	4.32	10			
Abamectin (Avermectin B1a)	8.80	50				Meclofenamic acid	7.53	200			
Albendazole-2-amino sulfone	3.81	50				Meloxicam	6.42	100			
Albendazole sulfoxide	4.13	50				6-Methyl-2-thiouracil	1.36	400			
Albendazole	5.45	50				Melengesterol acetate	7.57	25			
Albendazole sulfone	4.57	50				Morantel	4.22	100			
Amikacin	3.71	100				Moxidectin	8.93	100			
Amoxicillin	3.50	50				Metronidazole	2.83	10			
Ampicillin	3.89	20				Metronidazole-hydroxy	2.47	10			
Apramycin	3.78	100				Nafcillin	6.39	100			
Acetopromazine	5.09	10				Nalidixic acid	5.48	200			
Azaperone	4.21	10				Naproxen	6.35	100			
Bacitracin	4.68	1000				Neomycin	3.84	1000			
Beclomethasone	6.07	100				Niclosamide	7.76	10			
Betamethasone	5.96	100				Niflumic acid	7.15	200			
Bithionol	8.09	10				Nitroxynil	5.75	50			
Bromchlorobutanol	4.29	10				Norfloracin	3.91	50			
Brombuterol	4.35	10				Novobiocin	7.78	1000			
Cambendazole	4.55	10				Oxyphenylbutazone	6.18	100			
Chloramphenicol	4.72	50				Orbifloxacin	4.10	50			
Carazolol	4.43	10				Oxytetracycline	3.96	1000			
Carbadox	3.74	30				Oxacillin	5.98	100			
Carprofen	6.97	50				Oxbendazole	4.63	10			
Cefazolin	3.81	100				Oxyclozanide	7.46	10			
Cephapirin	3.48	100				Oxfendazole	4.70	800			
Cimaterol	3.57	10				Phenylbutazone	7.05	100			
Ciprofloxacin	3.96	50				Phenylbutazone-d10	7.02	200			
Clencyclohexerol	3.88	10				Penicillin G	5.47	50			
Clenbuterol	4.22	10				Penicillin G d7	5.43	200			
Clenbuterol-d9	4.20	200				6-Phenyl-2-thiouracil	4.23	400			
Clenpenterol	4.43	10				Pirlimycin	4.48	300			
Clindamycin	4.58	100				Piroxicam	5.77	100			
Clorsulon	4.54	100				Propionylpromazine	5.48	10			
Closantel	8.82	50				Prednisone	5.38	100			
Cloxacillin	6.20	10				Prednisolone	5.51	100			
Chlorpromazine	5.58	10				Promazine	5.06	10			
Cortisone	5.48	100				Procaterol	3.58	100			

Phase III Validation Results

Chlortetracycline	4.39	1000				Propyphenazone	5.80	100			
Danofloxacin	3.99	200				6-Propyl-2-thiouracil	3.53	50			
Dapson	3.86	100				Pyrantel	3.97	100			
DCCD	3.40	400				Ractopamine	3.98	30			
Desacetyl-cephapirin	2.65	100				Ractopamine-d3	3.96	200			
Desethylene ciprofloxacin	3.86	100				Rafoxanide	9.11	10			
Diclofenac	7.10	200				Ritodrine	3.76	10			
Dicloxacillin	6.53	100				Ronidazole	2.96	10			
Difloxacin	4.17	50				Salbutamol	3.51	10			
Dipyron (metabolite)	3.64	200				Sarafloxacin	4.18	50			
Dimetridazole	3.19	50				Sulfabromomethazine	5.54	100			
Dimetridazole-hydroxy	2.73	50				Sulfachloropyridazine	4.09	100			
Doramectin	8.99	100				Sulfadiazine	3.02	100			
Doxycycline	4.56	100				Sulfadimethoxine	4.79	100			
Dihydrostreptomycin	3.66	500				Sulfadoxine	4.26	100			
Emamectin B1a	7.14	50				Selamectin	9.20	200			
Enrofloxacin	4.03	100				Sulfaethoxyypyridazine	4.42	100			
Eprinomectin	8.64	100				Sulfisoxazole	4.35	100			
Erythromycin A	5.20	100				Sulfamethizole	3.72	100			
Fenbufen	6.46	50				Sulfamethoxyypyridazine	3.79	100			
Fenbendazole	6.18	400				Sulfamerazine	3.42	100			
Fenbendazole sulfone	5.17	400				Sulfamethoxazole	4.19	100			
Fenoterol	3.67	50				Sulfamethazine	3.76	100			
Florfenicol	4.31	300				Sulfanilamide	1.42	100			
Florfenicol Amine	3.09	300				Sulfantran	5.49	100			
Flubendazole	5.68	10				Spectinomycin	3.52	100			
Flubendazole-2-amino	4.43	10				Sulfapyridine	3.34	100			
Flumethasone	5.85	100				Sulfaquinolaxine	4.85	100			
Flumequine	5.62	300				Streptomycin	3.65	500			
Flunixin	6.69	25				Sulfathiazole	3.20	100			
Flunixin-d3	6.69	200				Thiabendazole	3.87	100			
Gamithromycin	4.56	100				5-Hydroxythiabendazole	3.71	100			
Gentamicin C1	3.80	300				Tetracycline	4.03	1000			
Gentamicin C1a	3.81	300				Triclabendazole	7.51	50			
Gentamicin C2+C2a	3.81	300				Triclabendazole sulfoxide	7.15	50			
Haloperidol	4.96	10				Triflupromazine	5.79	10			
Haloxon	6.65	100				Tildipirosin	3.90	500			
Hygromycin	3.64	100				Tilmicosin	4.64	100			
Indoprofen	5.94	50				Tiamulin	5.31	600			
Ipronidazole	4.58	10				Tobramycin	3.78	500			
Ipronidazole-hydroxy	3.95	10				Tolfenamic acid	7.73	200			
Ivermectin	9.25	50				Tulathromycin	4.11	1000			
Josamycin	5.82	100				Tylosin	5.34	200			
Kanamycin	3.72	100				Virginiamycin M1	6.28	100			
Ketoprofen	6.28	50				Xylazine	4.22	10			
Lasalosid A	9.65	100				Zeranol	5.99	100			
Levamisole	3.83	100				Zilpaterol	3.51	12			

Ion Ratio Criteria in 2002/657/EC (EU)

Rel. Abundance
vs. Base Peak

>50%
>20-50%
>10-20%
≤10%

Acceptable Diff. vs. Ref.
API-MS

±20% RSD
±25% RSD
±30% RSD
±50% RSD

<u>Ref. Ratio</u>	<u>EU Range*</u>	<u>FSIS (1 ion)</u>	<u>(2 ions)</u>
70%	56% – 84%	60% – 80%	50% – 90%
24%	18% – 30%	14% – 34%	4% – 44%
12%	8.4% – 15.6%	3% – 23%	>0% – 33%
4%	2% – 6%	>0% – 14%	>0% – 24%

* 2 ion transitions needed to achieve 3 ident. points in MS/MS

Guidelines in SANCO/12571/2013

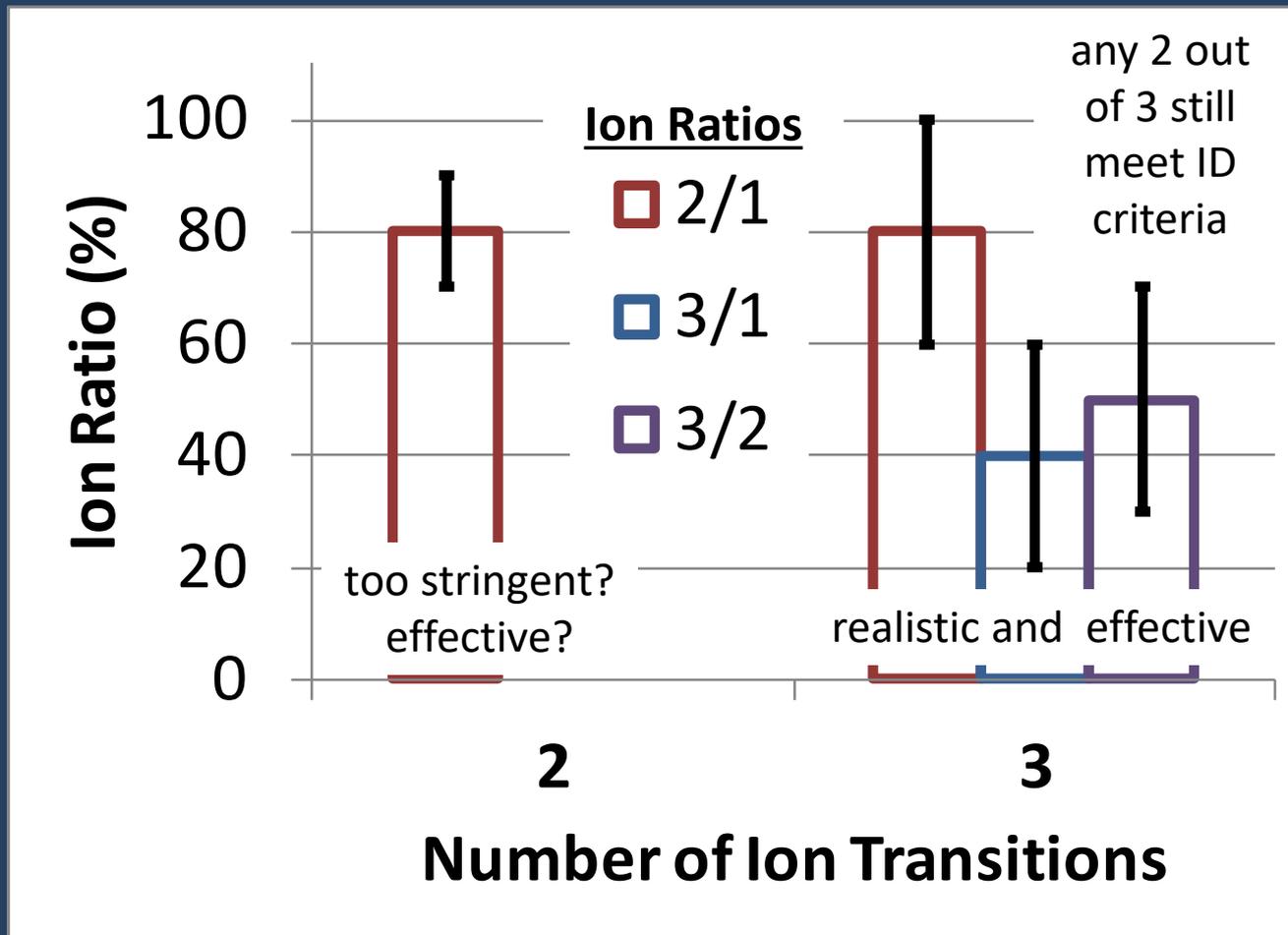
Rel. Abundance <u>vs. Base Peak</u>	Acceptable Diff. vs. Ref.	
	<u>EI-MS ($\geq 3^*$ ions)</u>	<u>MS/MS (≥ 2 ions)</u>
>50%	$\pm 10\%$ Rel	$\pm 30\%$ Rel
>20-50%	$\pm 15\%$ Rel	$\pm 30\%$ Rel
>10-20%	$\pm 20\%$ Rel	$\pm 30\%$ Rel
$\leq 10\%$	$\pm 50\%$ Rel	$\pm 30\%$ Rel

<u>Ref. Ratio</u>	<u>EI-MS Range</u> *	<u>MS/MS</u>
70%	63 – 77%	49 – 91%
24%	20.4 – 27.6%	16.8 – 31.2%
12%	9.6 – 14.4%	8.4 – 15.6%
4%	2 – 6%	2.8 – 6.2%

* ≥ 2 ions in high resolution MS with mass accuracy ≤ 5 ppm

Example MS/MS or SIM Ion Ratio Ranges

(how many ions/transitions to collect?)



Evaluation of Incurred Samples

- FSIS provided 10 kidneys found in their monitoring program to contain drug residues.
- We analyzed the samples in blind fashion (unknown drugs and unknown levels).
- These were each analyzed in duplicate using the different features of 3 methods to compare and assess their performance on real samples.