

Recent Studies on Veterinary Drug Residue Analysis Methods in My Lab

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Wyndmoor, Pennsylvania; USA

HPLC Determination and MS Confirmation of Malachite Green and Gentian Violet and Their Leuco Metabolites Residues in Catfish Muscle

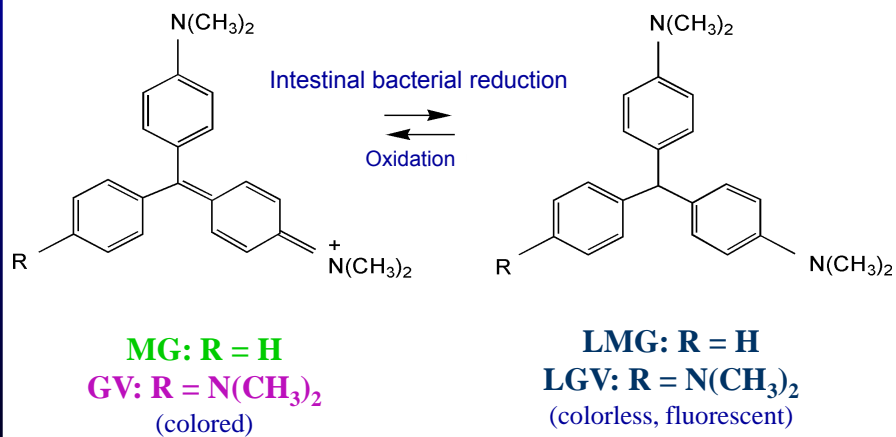
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Structures of GV, MG, LGV & LMG



The Goals

1. To achieve sensitivity required by FDA (1 ng/g) and EU (2 ng/g)
2. To simplify sample preparation
 - Extraction only once
 - No LLE
 - One SPE cartridge
3. To simultaneously determine MG, GV, LMG and LGV
 - MG and GV by HPLC-visible spectrometry
 - LMG and LGV by HPLC-fluorescence
4. To obviate a Pb₂O oxidation reactor
5. To confirm by HPLC-MS/MS

Sample Preparation

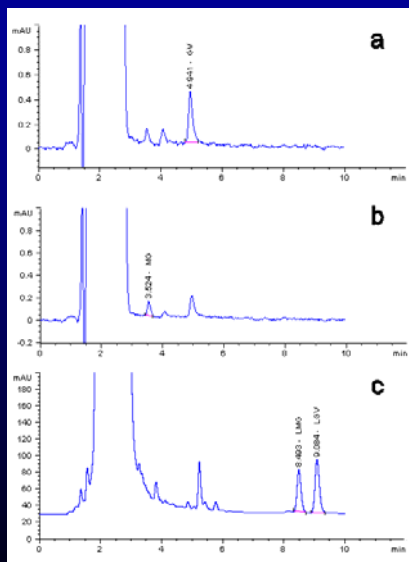
Extraction (once)

- Spike 5 g fish muscle to desired level, vortex
- Extract in 4 mL of pH 3 McIlvaine buffer, 100 μ L of 1 M p-TSA, and 100 μ L of 1 mg/mL TMPD, vortex for 30 s
- Add 25 mL of MeCN, vortex for 1 min
- Add 5 g NaCl, vortex for 20 s
- Centrifuge @4150 rpm for 5 min

SPE Cleanup (1 cartridge)

- Add ~2 g neutral alumina onto 150 mg/6 mL Oasis MCX columns
- Add quickly 2 sequential 3-5 mL of MeCN
- Load organic supernatant at ~2mL/min
- Wash with 5 mL of MeCN at ~1mL/min
- Blow off alumina with compressed air, rinse with MeCN
- Elute with 6 mL of freshly made SPE eluent
- Evaporate eluate at 40 $^{\circ}$ C to dryness under N_2
- Dissolve in 1 mL of 7:3 (v/v) MeOH-pH 4.1 acetate buffer

HPLC-DAD-Fluorescence Performance

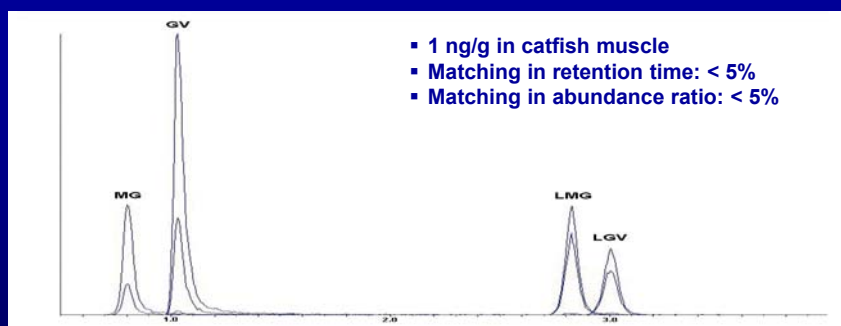


Detector	Analyte	r	LOD
		(0.5-16.0 ng/g)	(ng/g, S/N=3)
DAD	MG	0.9998	0.38
	GV	0.9994	0.26
FD	LMG	0.9999	0.10
	LGV	0.9994	0.09

Recovery (n=6)

Analyte	Concentration (ng/g)	Recovery (%)	RSD (%)
MG	10	44.5	5.3
	2	48.1	12.0
	1	49.2	7.6
GV	10	97.7	3.4
	2	92.2	3.7
	1	101.4	4.6
LMG	10	74.3	5.2
	2	81.5	5.7
	1	84.0	5.8
LGV	10	80.6	3.5
	2	85.9	5.0
	1	86.5	9.2

HPLC-MS/MS Confirmation



Analyte	m/z	Abundance Ratio	
		Reagent Standard	Matrix matched Standard
MG	(329-313)/(329-208)	3.78	3.73
GV	(372-356)/(372-340)	2.80	2.86
LMG	(331-239)/(331-316)	1.39	1.36
LGV	(374-358)/(374-239)	1.40	1.42

Conclusions

- Environmental responsible and user friendly protocol
 - **Pb₂O reactor eliminated**
 - **Dichloromethane eliminated**
- Assay time cut by 1/3
 - **Extraction only once**
 - **LLE eliminated**
 - **One commercial SPE column**
- LOD well below FDA and EU requirements
- Quantitation and confirmation

Flukicides/Anthelmintics

<i>ESI +</i>		<i>ESI -</i>
Triphenyl Phosphate (IS)	Albendazole	2,4-D (IS)
Cyprodinil (QC spike)	Albendazole-Sulfoxide	
	Albendazole-Sulfone	Bithionol
Abamectin	Albendazole-Amino-Sulfone	Clorsulon
Doramectin	Cambendazole	Closantel
Emamectin	Flubendazole	Nicosamide
Eprinomectin	Amino-Flubendazole	Nitroxylin
Moxidectin	Hydroxy-Flubendazole	Oxyclozanide
Ivermectin	Fenbendazole	Rafoxanide
Selamectin	Fenbendazole-Sulfone	Triclabendazole
	Oxfendazole	
Dichlorvos	Mebendazole	
Coumaphos	Amino-Mebendazole	
Coumaphos-Oxon	Hydroxy-Mebendazole	
Haloxon	Oxibendazole	
Morantel	Thiabendazole	
Levamisole	5-Hydroxy-Thiabendazole	
	Triclabendazole	
	Triclabendazole-Sulfoxide	

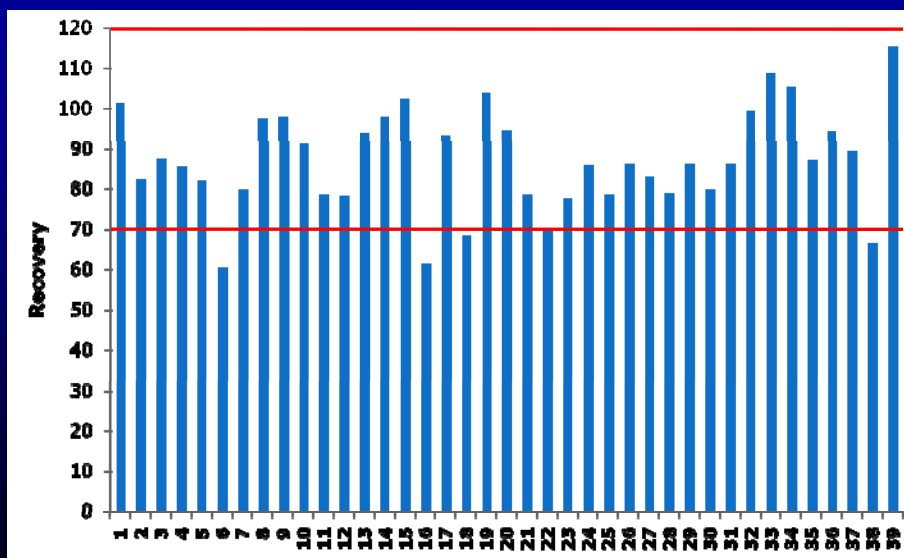
Slide adapted from Brian Kinsella, Teagasc

QuEChERS for Anthelmintics

- Extraction*
- Place 10 g tissue / 10 mL milk into tube
 - Add 10 mL MeCN + 4 g MgSO₄ + 1 g NaCl
 - Add internal standard (Cyprodinil + 2,4-D)
 - Shake for 1 min
 - Centrifuge for 3 min
- Clean-up*
- Place 1 mL supernatant into 2 mL tube w/ 150 mg MgSO₄ and 50 mg C₁₈
 - Mix for 1 min
 - Centrifuge for 1 min
- Analysis*
- Place 0.5 mL into autosampler vial
 - Add QC spike (TPP)
 - Inject onto LC-MS/MS (ESI+ and ESI-)

Slide adapted from Brian Kinsella, Teagasc

Recoveries of the Analytes

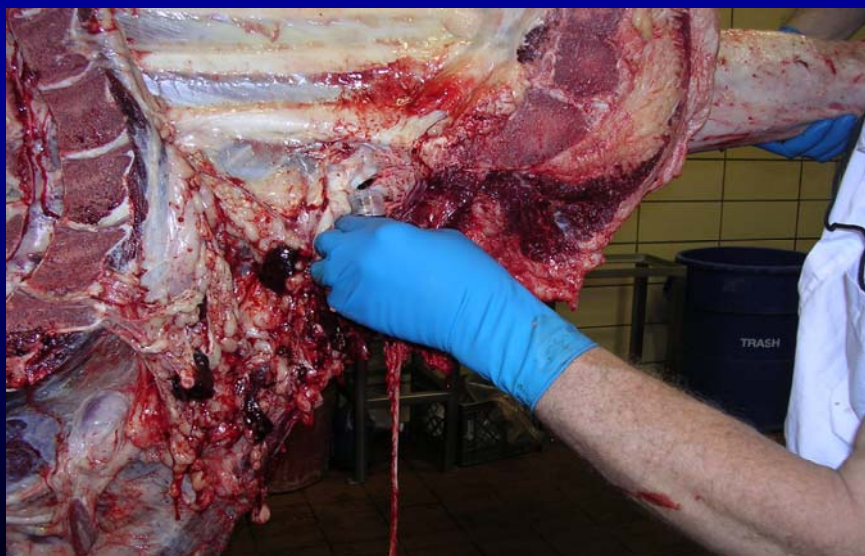


Slide adapted from Brian Kinsella, Teagasc

Status of Anthelmintics Method

- Teagasc (Ireland) has adapted the method to UPLC-MS/MS with fast ESI+/- switching to achieve high throughput with a single injection.
- It has been further validated according to EU criteria.
- Proficiency test sample results with the method have been exceptional.
- Other labs are starting to use the method, too.

Screening of Antibiotics in Beef



Two-Tiered Approach for Antibiotics

Tier 1: Screening Test Performed by the FSIS Inspectors in the Slaughterhouse.

Currently with Microbial Inhibition Tests

Tier 2: Presumptive Positive Samples Sent to the FSIS Laboratory for Quantitative and Qualitative Analysis.

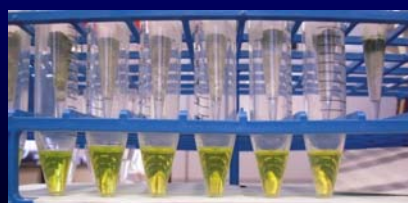
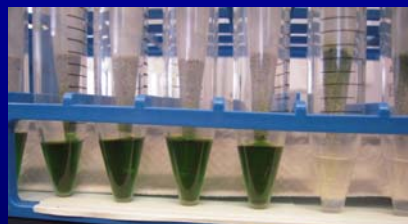
Currently with 7-plate bioassay, but we propose to use UPLC-MS/MS at least for lab-based screening and antibiotic identifications

New Method for Aminoglycosides in Bovine Kidney, Liver and Muscle

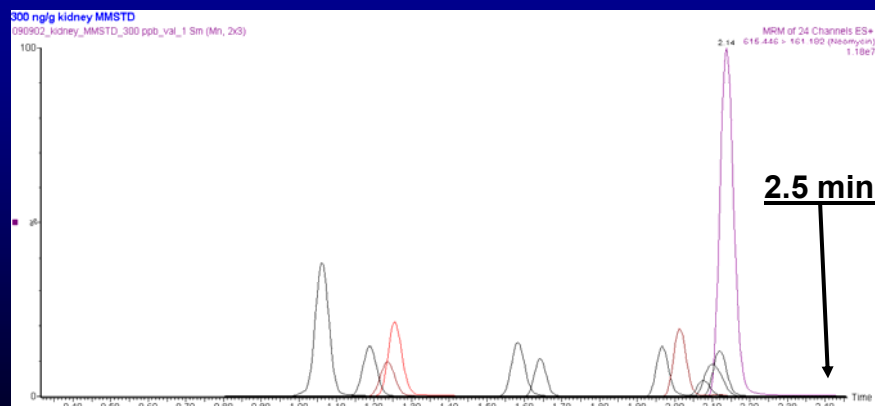
- Mix a 2.0 g sample with 10 mM NH₄OAc / 0.4% EDTA / 1% NaCl / 2% TCA buffer in stomacher.
- Centrifuge and decant supernatant.
- Adjust pH to 6.5 with NaOH and/or HCl solutions.
- Sample clean-up / analyte recovery performed using disposable pipette extraction (loose 50 mg WCX sorbent in 5 mL tip) on manifold extractor apparatus.
- Elute the analytes using 1.0 mL of 10% formic acid in water

Disposable Pipette Extraction (DPX)

Patented in 2003 by William Brewer, University of South Carolina



UPLC-MS/MS Chromatographic Profile of 12 Aminoglycosides



Results from Validation Study for Aminoglycosides in Bovine Kidney

Analyte	%Recovery (n=6 at each level)				%RSD			
	0.5x	1x	2x	Overall	0.5x	1x	2x	Overall
Neomycin	93	96	89	93	8	8	9	8
Spectinomycin	94	93	90	92	8	5	8	7
Dihydrostreptomycin	117	106	110	111	11	8	7	9
Streptomycin	95	89	98	94	4	7	10	7
Amikacin	95	82	85	87	22	13	3	13
Apramycin	112	104	96	104	24	15	13	17
Gentamicin c1	102	87	84	91	19	10	15	15
Gentamicin c1a	72	92	103	89	30	28	7	22
Gentamicin c2+c2a	103	89	96	96	19	11	10	13
Gentamicin (sum)	96	89	93	92	8	10	8	8
Hygromycin B	89	60	59	69	9	22	12	14
Kanamycin	94	90	89	91	14	11	7	11

Results from Validation Study for Aminoglycosides in Bovine Liver

Analyte	%Recovery (n=6 at each level)				%RSD			
	0.5x	1x	2x	Overall	0.5x	1x	2x	Overall
Neomycin	93	103	96	97	3	8	6	7
Spectinomycin	76	77	83	79	9	9	4	8
Dihydrostreptomycin	91	103	103	99	6	8	8	9
Streptomycin	77	70	76	74	7	10	7	8
Amikacin	87	89	97	91	12	11	11	11
Apramycin	95	98	99	97	17	17	6	13
Gentamicin c1	101	101	101	101	13	12	8	10
Gentamicin c1a	110	110	106	109	27	33	17	25
Gentamicin c2+c2a	99	118	97	105	29	14	8	19
Gentamicin (sum)	102	110	100	104	17	14	7	13
Hygromycin B	69	56	64	63	24	16	15	19
Kanamycin	63	89	97	83	23	12	12	22

Results from Validation Study for Aminoglycosides in Bovine Muscle

Analyte	%Recovery (n=6 at each level) %RSD							
	0.5x	1x	2x	Overall	0.5x	1x	2x	Overall
Neomycin	89	98	102	96	3	7	12	10
Spectinomycin	76	80	88	82	15	14	14	14
Dihydrostreptomycin	100	101	100	100	5	3	13	7
Streptomycin	93	94	91	93	12	14	8	11
Amikacin	85	80	84	83	15	24	10	16
Apramycin	74	89	98	87	31	18	17	23
Gentamicin c1	111	105	96	104	36	20	12	24
Gentamicin c1a	133	106	120	120	33	26	25	28
Gentamicin c2+c2a	86	97	89	91	22	8	11	14
Gentamicin (sum)	101	101	97	100	13	9	11	10
Hygromycin B	55	59	66	60	41	24	27	29
Kanamycin	76	80	96	84	17	18	9	17

Analysis of Incurred Samples

Spectinomycin concentrations ($\mu\text{g/g}$) normalized to the IS and identified by UPLC-MS/MS (kidney results confirmed using HPLC-MS/MS).

Sample	Kidney	Liver	Muscle
#1	1.7	0.082	0.013
#8	0.45	0.049	<0.005 (not identified)
#18	0.25	0.056	not indicated

Spectinomycin was determined and identified in the monohydrate form in the incurred samples.

Analysis of Incurred Samples

Dihydrostreptomycin concentrations ($\mu\text{g/g}$) normalized to the IS and identified by UPLC-MS/MS (kidney results confirmed using HPLC-MS/MS).

Sample	Kidney	Liver	Muscle
#1	0.030	not indicated	not indicated
#2	0.15	0.010	not indicated
#4	0.93	0.064	0.005 (not identified)
#6	0.32	0.038	0.006 (not identified)
#8	0.019	not indicated	not indicated
#13	0.095	not indicated	not indicated
#19	0.028	not indicated	0.005 (not identified)

Neomycin concentrations ($\mu\text{g/g}$) ...

Sample	Kidney	Liver	Muscle
#1	0.010	0.006	<0.005
#2	0.007	not indicated	not indicated
#3	<0.005	not indicated	not indicated
#4	1.5	0.009	0.009
#5	<0.005	not indicated	not indicated
#10	2.1	0.005	0.013
#11	0.75	not indicated	<0.005
#12	0.007	not indicated	not indicated
#13	12	0.12	0.013
#14	4.4	0.19	0.008
#15	13	0.48	not indicated
#16	0.043	<0.005	not indicated
#18	0.24	0.008	<0.005 (not identified)
#19	2.1	<0.005	<0.005
#20	0.007	0.012	not indicated

Analysis of Incurred Samples

Gentamicin (sum) estimated concentrations ($\mu\text{g/g}$) normalized to the IS and identified by UPLC-MS/MS (kidney results confirmed using HPLC-MS/MS).

Sample	Kidney	Liver	Muscle
#1	16*	0.40*	0.032
#2	0.019	not indicated	not indicated
#3	5.8*	1.5*	0.009
#4	0.007	not indicated	not indicated
#8	27*	1.8*	0.038
#9	12*	not indicated	not indicated
#10	0.017	0.22	not indicated

*Calculated estimated concentration exceeded the calibration range, which went to 0.3 $\mu\text{g/g}$.

Analysis of Incurred Samples

Gentamicin average peak area ratios

	C1	C1 _a	C2 + C2 _a
Stds	22%	32%	46%
Kidney	34%	15%	51%
Liver	18%	36%	46%
Muscle	18%	26%	56%

Calculated conc'ns depend on the individual gentamicin components.

However, the presence of the different gentamicin components in the same sample helps greatly for qualitative analysis purposes!

Comparison of Methods for Aminoglycosides in Tissues

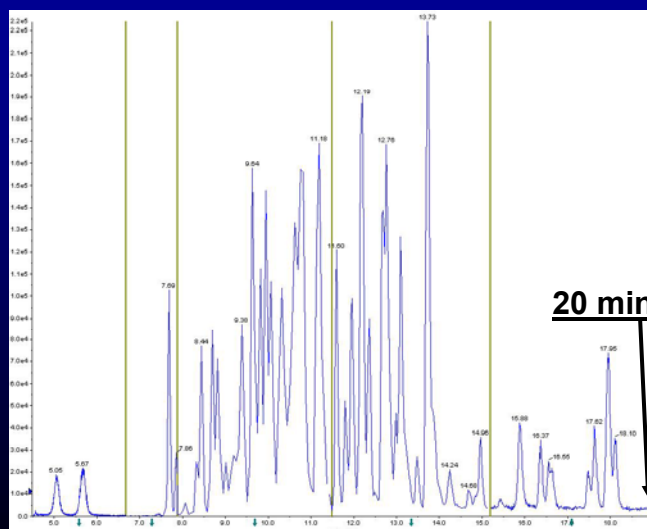
<u>Old Method</u>	<u>New Method</u>
Phosphate Buffer	Acetate Buffer
SPE	DPX
HPLC (x min)	UPLC (3 min)
Qualitative only	Quantitative, too
18 samples/day	40 samples/day

General Sample Prep for Vet. Drugs

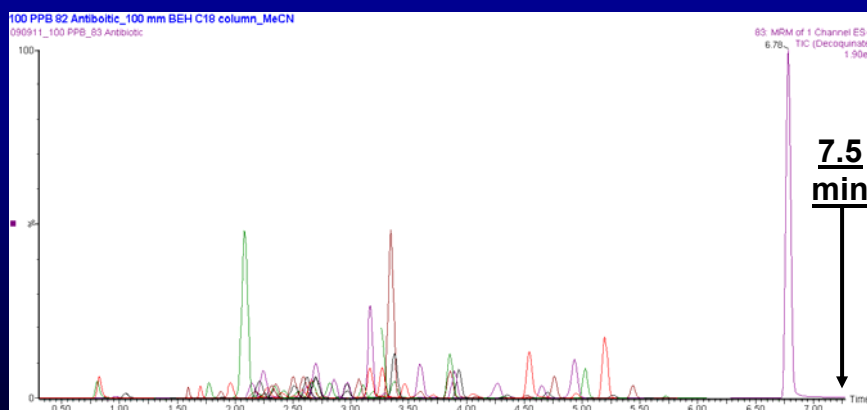
extraction	1 mL or 1 g sample in a 15 mL tube
	add internal standards (PENV, CEFD, ¹³ C-SMZ)
	add 4 mL of 4/1 (v/v) acetonitrile/water
	vortex briefly, shake for 5 min centrifuge for 5 min at 3700 rcf
clean-up	supernatant + 250 mg C18 sorbent mix for 30 s, centrifuge for 1 min
	evaporate 2.5 mL supernatant to 0.5 mL
	filter extract with the Mini-UniPrep™
	(RP) LC-MS/MS analysis

K. Mastovska, A.R. Lightfield, *J. Chromatogr. A* 1202 (2008) 118-123.

HPLC-MS/MS Chromatographic Profile of 121 Veterinary Drugs



UPLC-MS/MS Chromatographic Profile of 82 Antibiotics



Mobile Phase: A – 95% water / 5% MeCN / 0.1% formic acid
 B – 100% MeCN / 0.1% formic acid

Antibiotics and Other Vet. Drugs in our UPLC-MS/MS Methods

Aminoglycosides (11+3)

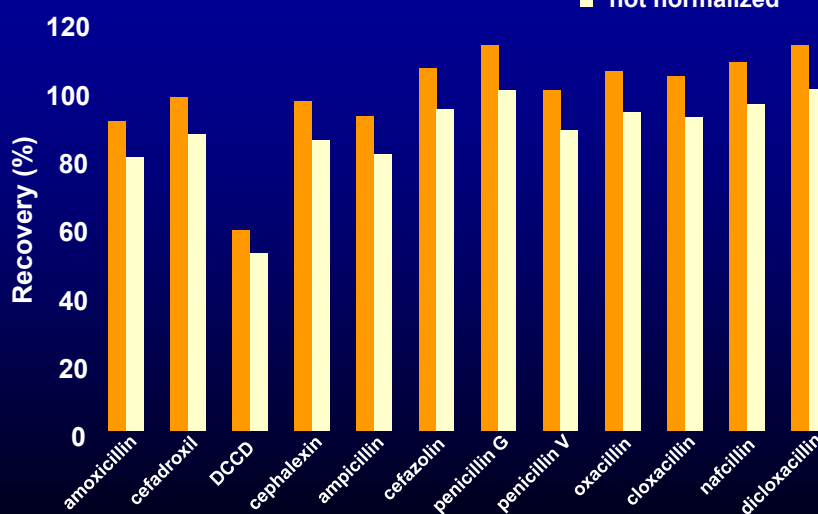
Tetracyclines (5)	Steroids (8)
Quinolones (18)	β -Agonists (11)
Nitrofurans (4+4)	Thyreostats (6)
Sulfonamides (27)	Anthelmintics (40)
Miscellaneous (9)	Tranquilizers (11)
Macrolides (12)	Coccidiostats (15)
β -Lactams (15+2)	Corticosteroids (11)
Phenicols (4)	NSAIDs (12)

228 Vet. Drugs (107 Antibiotics) in the Method

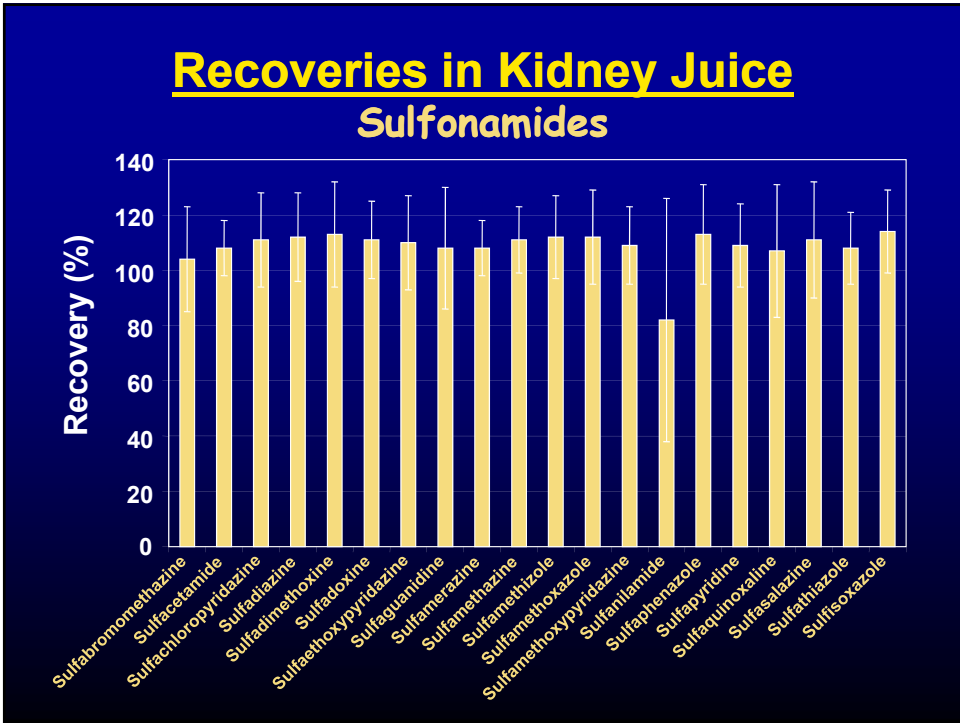
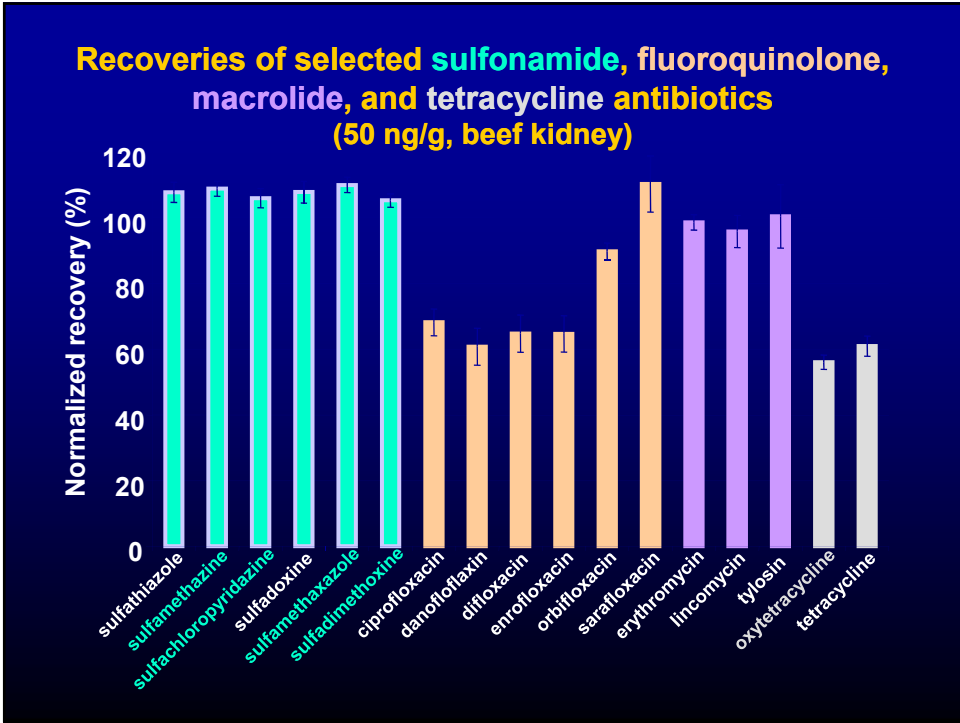
Recoveries of β -Lactam Antibiotics

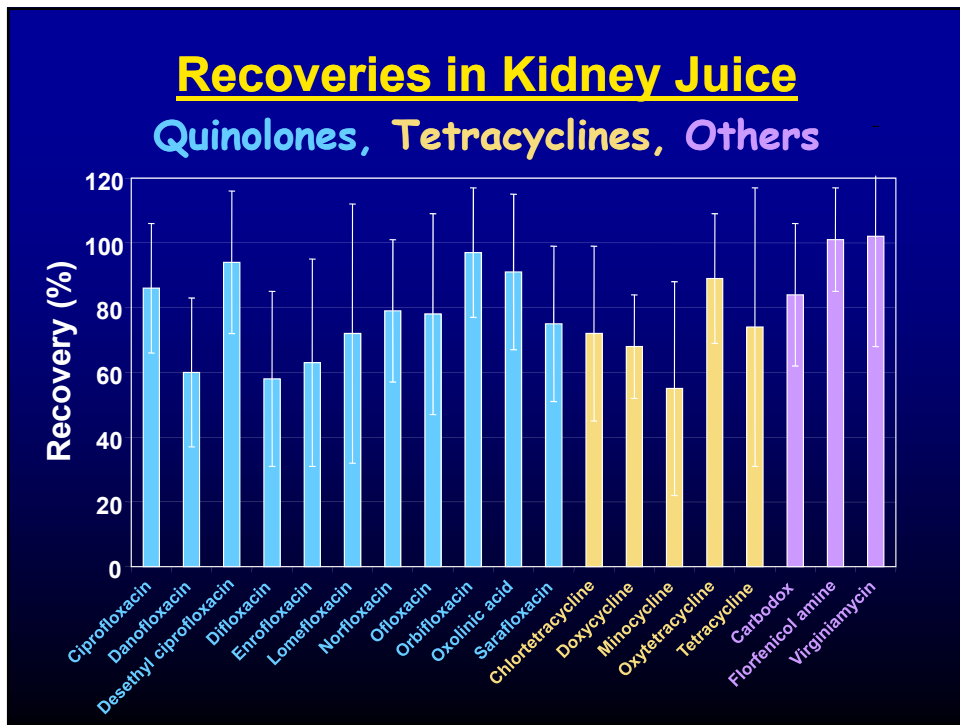
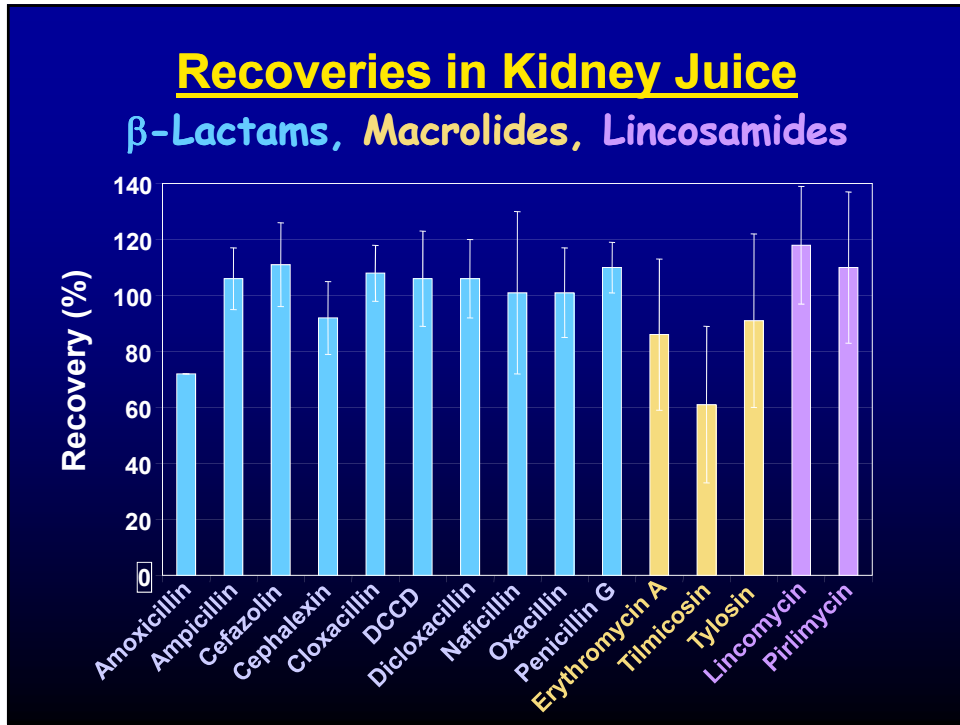
(50 ng/g, beef kidney)

■ normalized to Pen V
■ not normalized



DCCD = desferoylcefthiofur cysteine disulfide





Results of LC-MS/MS Analysis for 121 Veterinary Drugs in Kidney Juice

spiked at 10, 25, 50, and 100 ng/mL
n = 16 (1 spike each day for 16 days)

	Average recovery (%)	RSD (%)	Number of analytes
Quantitative? Screening?	80 - 120	10 - 20	37
	70 - 120	21 - 30	52
	50 - 69	20 - 30	9
	55 - 130	31 - 42	17
	20 - 35	30 - 45	6

Feasibility Study: Vet. Drug Residues in Bovine Kidney and Kidney Juice

- Analysis of 121 drug residues, including 65 antibiotics
- Two sample preparation and LC-MS/MS methods
 - > aminoglycosides (ion-pairing LC)
 - > other veterinary drugs (reversed-phase LC)
- Screened / analyzed 235 kidney and kidney juice samples collected from a processing plant

Note: It is possible to use a single LC-MS system (instrument and mobile phase) using a dual-column approach, employing reversed phase and aqueous normal phase retention mechanisms.

K. Mastovska, A.R. Lightfield, *Am. Lab.* 6-7 (2008) 37-40.

Drug Residues Found in the 235 Kidney Juice Samples

<u>Antibiotics – with US tolerance</u>	<u>Number</u>
• Dihydrostreptomycin - 6-14,000 ng/mL	7
• Streptomycin - 75 ng/mL	1
• DCCD - 2-28 ng/mL	10
• Floramfenicol amine - 33 ng/mL	1
• Oxytetracycline - 3-57 ng/mL	13
• Penicillin G - 2-3 ng/mL	3
• Pirlimycin - 2-29 ng/mL	5
• Sulfamethazine - 2-140 ng/mL	2

M.J. Schneider, K. Mastovska, S.J. Lehotay, A.R. Lightfield, B. Kinsella, C.E. Shultz, Anal. Chim. Acta, 637 (2009) 290-297.

Acknowledgments

- ◆ Limei Yun
- ◆ Teagasc, Ireland
- ◆ USDA-FSIS Midwestern Laboratory
- ◆ DPX Labs

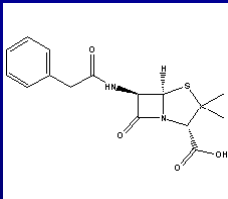


The image is a collage on a dark blue background. At the top, a yellow-bordered box contains four items: two grey gas cylinders, a grey tank, a yellow gas mask, and a blue gas canister. Below this box is a white plus sign. To the left is a photograph of a green leaf with a dark berry, with a black arrow pointing to the berry. To the right of this is another white plus sign, followed by a photograph of hands holding dark soil. To the right of the soil is a white exclamation mark. At the bottom, the text "Tanks Berry Mulch!" is written in yellow.

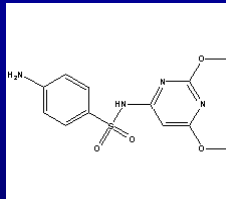
Tanks Berry Mulch!

Major Classes of Antibiotics

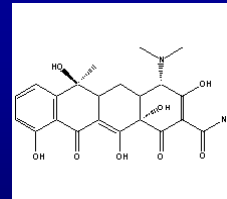
β -Lactams (17)



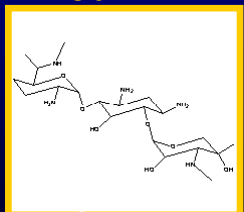
Sulfonamides (27)



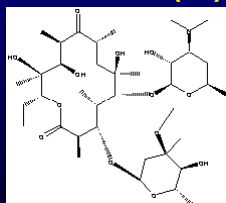
Tetracyclines (5)



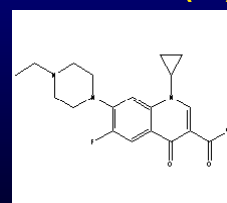
Aminoglycosides (11)



Macrolides (12)



Quinolones (18)



- Currently, **214 vet. drugs (including 94 antibiotics)** are on our list for multiclass, multi-residue analysis to meet monitoring needs

Conclusions

- Aminoglycosides don't fit into the multiclass, multi-residue scheme, but most other classes do.
- The new method for aminoglycosides gives better results faster and easier than the current method.
- UPLC better than halves the analysis time vs. HPLC for the 100+ veterinary drug analytes.
- ☐ The MS/MS conditions for ≈ 200 analytes have been set, but UPLC method optimization is in progress.
- ☐ The sample prep method will be tested using UPLC-MS/MS for recoveries, reproducibilities, detection limits, and ruggedness in the different matrices.