



**Asia-Pacific
Economic Cooperation**

2014/SCSC/WKSP2/004

Session: 5.2

**Veterinary Drug Multi-residues in Chicken
Proficiency Testing Program in Thailand -
Experience Sharing**

Submitted by: Thailand



**Food Safety Cooperation Forum Partnership
Training Institute Network Proficiency Testing
Workshop
Beijing, China
10-11 September 2014**

APEC MYP (M CTI 02 12A)
Veterinary Drug Multi-residues in
Chicken
Proficiency Testing Program
Experience Sharing

@ Beijing
10th August 2014
Sujittra Phongvivat
AFRL for VDR,
BQCLP, DLD, THAILAND

Bureau of Quality Control of Livestock Products

BQCLP Building 1



BQCLP Building 2



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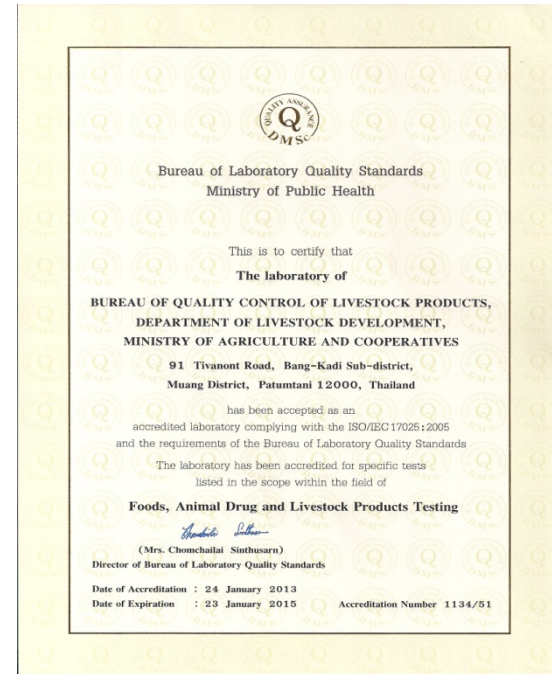
Sample reception & Result submission

Our lab code is APEC FSCF-MYP-007

- Receipt date: 25 Nov 2013
- Submission date: 4 Dec 2013

Test Methods for NFS, FQL and Sulfas:-

- were validated and ISO17025 accredited
- Performed target testing individually for
 - Nitrofurans metabolites (bound form) by LC-MS/MS
 - Fluoroquinolones by LC-MS/MS
 - Sulfonamides by HPLC-UV

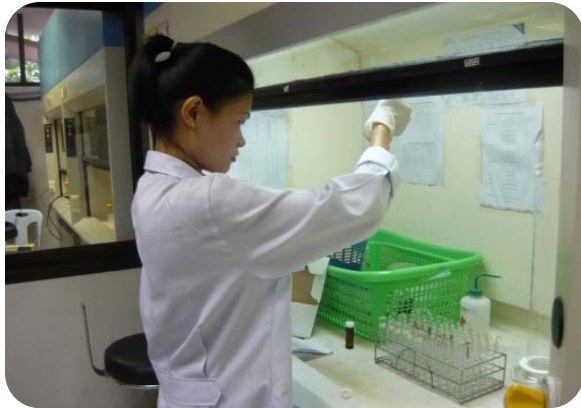


Sample Treatment & testing for 3 groups of VDR

- Follow instruction:- Add water to sample (3:1 g)
- To prepare chicken powder in vacuum packages.
Add H₂O into dried weight of sample to make fresh sample
- ✓ for Sulfa weigh 1.25 g sample + water 3.75 g = **5 g**
- ✓ for NFS weigh 0.25 g sample + water 0.75 g = **1 g**
- ✓ for FQL weigh 0.5 g sample + water 1.5 g = **2 g**
- Performed target testing individually for
 - Nitrofurans metabolites (bound form) by LC-MS/MS
 - Fluoroquinolones by LC-MS/MS
 - Sulfonamides by HPLC-UV

Scope

- Testing method
- Method validation
- APEC PT result
- Comments



NFS & Metabolite Residues

Parent Drug

Furazolidone

Furaltadone

Nitrofurantoin

Nitrofurazone

Metabolite

AOZ

AMOZ

AHD

SEM

Nitrofurans metabolites (Tissue bound residue)

- Standards

- AOZ (Fluka)
- AMOZ (Witega)
- AHD (Witega)
- SEM (Aldrich)

- Internal standards

- D4 AOZ (Witega)
- D5 AMOZ (Witega)

STANDARD SOURCES (The following standard can be substituted by the other brands if the purity of standards is equivalent)

3-amino-2-oxazolidinone (AOZ) (Sigma-Aldrich rare chemicals, S418498, WITEGA, Dr. Ehrenstorfer)

D⁴-3-amino-2-oxazolidinone hydrochloride salt (D⁴-AOZ) (BVL, Berlin, WITEGA)

1-aminohydantoin-2,4-dion (AHD) (WITEGA, Berlin)

3-amino-5-morpholinomethyl-1,3-oxazolidin-2-one (AMOZ). (WITEGA, Berlin)

D⁵-3-amino-5-morpholinomethyl-1,3-oxazolidin-2-one. (D⁵-AMOZ) (WITEGA, Berlin)

Semicarbazide hydrochloride (SEM) (Fluka, Aldrich S 220-1)

M. Validation for NFS metabolites

Table 14.1 CC_{α} , CC_{β} of 4 Nitrofurantol metabolites data from 2 method validations

	EU MRPL (ppb) in Poultry meat & Aquaculture products	CC_{α} (ppb)	CC_{β} (ppb)	CC_{α} (ppb)	CC_{β} (ppb)
		1 st validation (raw meat only)		2 nd validation (raw and processed meat, liver and egg)	
AHD	1.0	0.1	0.15	0.3	0.64
AOZ	1.0	0.04	0.06	0.18	0.27
SEM	1.0	0.15	0.25	0.31	0.97
AMOZ	1.0	0.02	0.03	0.19	0.3

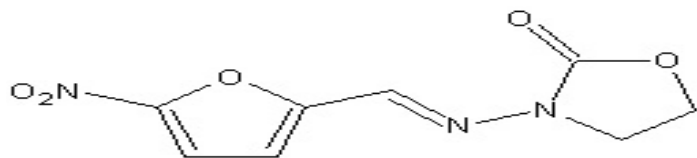
Remark: EU MRPL has been established against poultry meat and aquaculture products (16.9).

M validation for NFS metabolites

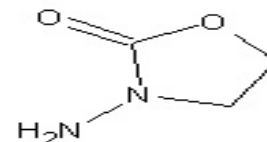
Table 15.1 Parameters validation summary

Parameters	1 st Validation (Oct. 2002; meat only)	2 nd (Feb.-March 2006; Raw and processed meat, liver and egg)
Concentration range (ng/g)		
- AOZ	0.2-0.4	0.1-2.0
- AMOZ	0.2-0.4	0.1-2.0
- AHD	0.4-0.8	0.1-2.0
- SEM	1.0-2.0	0.1-2.0
CC _α (ng/g)		
- AOZ	0.04	0.18
- AMOZ	0.02	0.19
- AHD	0.1	0.3
- SEM	0.15	0.31
CC _β (ng/g)		
- AOZ	0.06	0.27
- AMOZ	0.03	0.30
- AHD	0.15	0.64
- SEM	0.25	0.97
Accuracy (%Recovery)		
- AOZ	99.7-104.6%	109.8-113.9%
- AMOZ	93.0-94.5%	109.9-111.3%
- AHD	111.8-116.3%	100.1-126.8%
- SEM	88.2-93.4%	126.6-154.2%

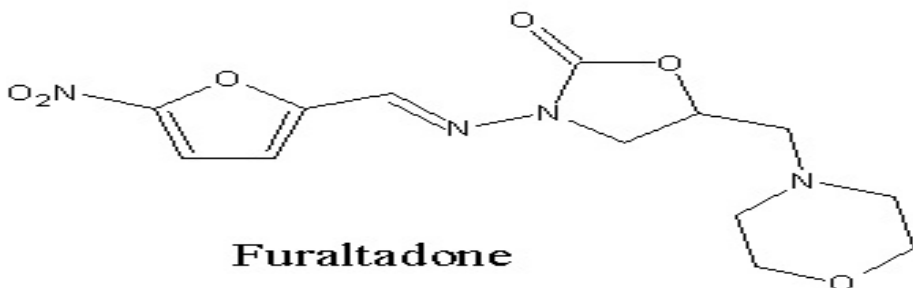
NFS structures & metabolites



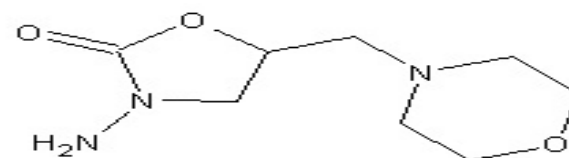
Furazolidone



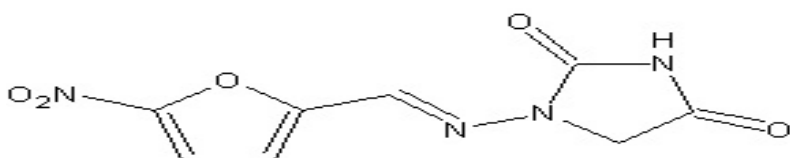
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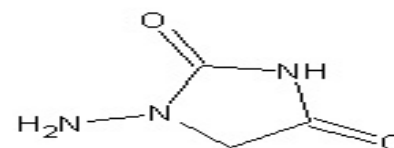
Furaltadone



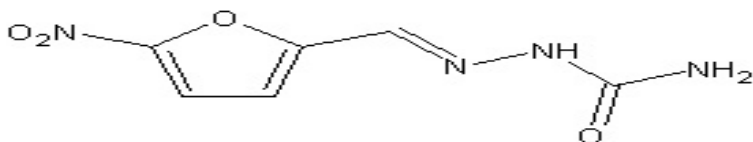
AMOZ



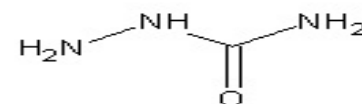
Nitrofurantoin



AHD

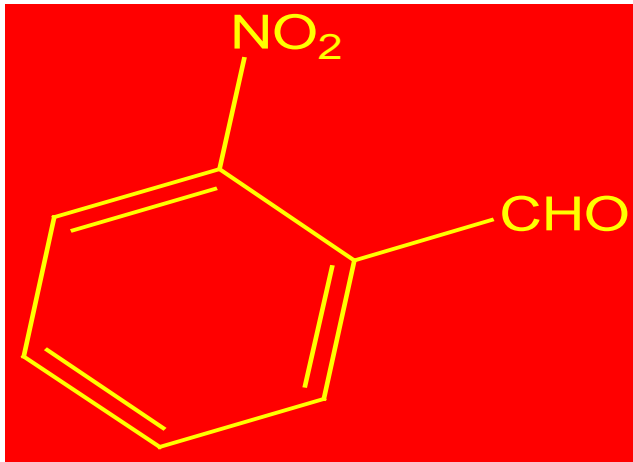


Nitrofurazone



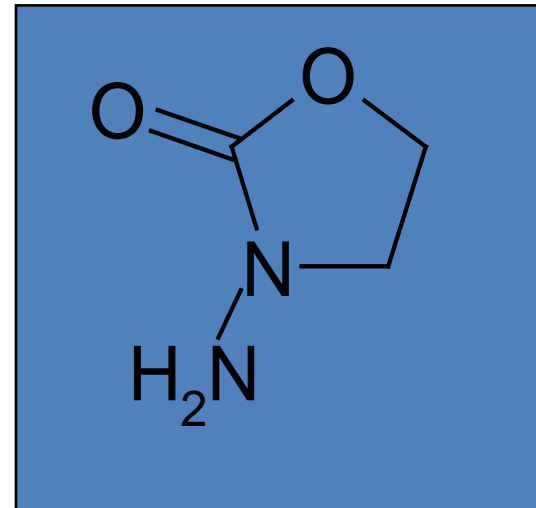
SEM

Detection of AOZ

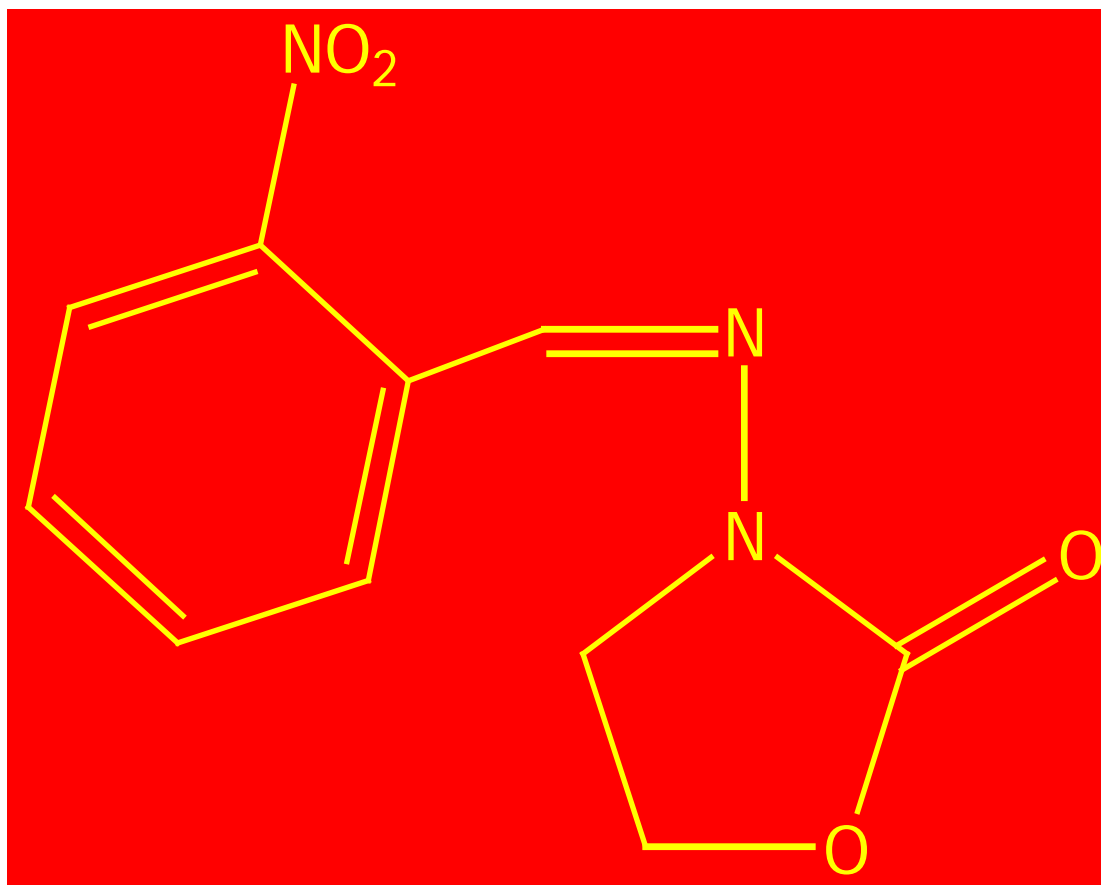


2-NBA

+



AOZ



NPAOZ

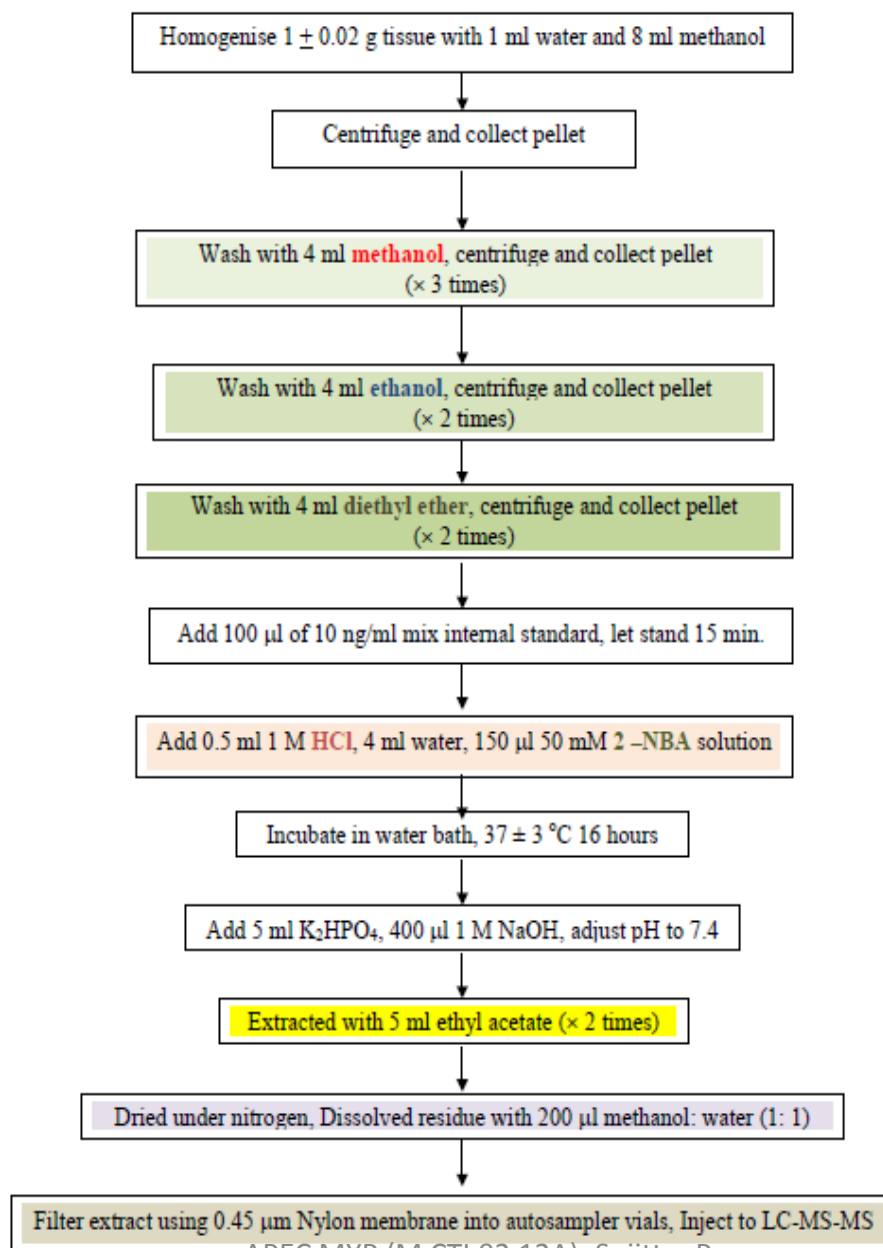
Nitrofuran Metabolites Analysis (Tissue Bound)

[McCracken, R. J. and Kennedy, D. G., 1997]

J. of Chromatography B, 691, 87-94.

1. Extraction & Wash free form metabolite
2. Cut side chain (marker residue) using HCl
3. Derivatisation with 2- NBA, at temp 37° C for 16-18 hours
4. Neutralisation to pH 7.4 (increase stability)
5. Extraction with Ethyl acetate
6. LC-MS/MS: Agilent + API 3000

Flow diagram of the Determination of Protein-Bound Nitrofurans Metabolite Residues (AOZ, AMOZ, SEM and AHD) in animal tissue by LC-MS-MS



NFS by LC-MS/MS

LC-MS/MS ANALYSIS

HPLC conditions

Columns: The following C 18 columns can be used:-

1. Merck LiChrospher 100 RP 18e, 5 μm , 4 x 125 mm
2. Symmetry C18, 5 μm , 3.9 x 150 mm
3. Zorbax SB- C18, 3.5 μm , 4.6 x 150 mm
4. Luna C 18 (2), 100 Å, 3.0 μm , 4.6 x 150 mm

Flow rate, split ratio ~1: 2 and run time:

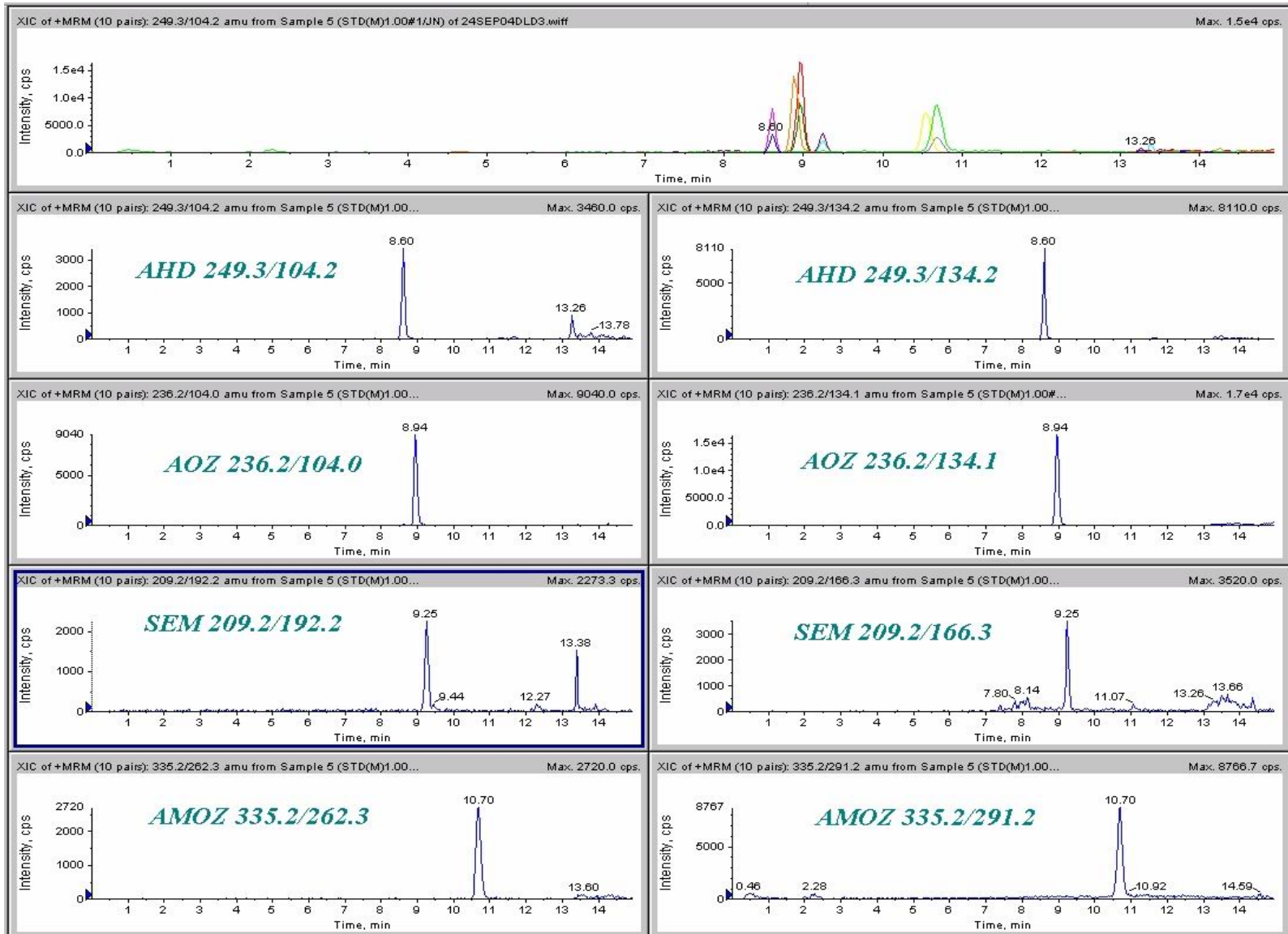
Column	Flow rate ($\mu\text{l}/\text{min}$)	Split to MS (μl)	Split to waste (μl)	Run time (min)
LiChrospher	800	260	540	12
Symmetry	800	260	540	15
Luna	800	260	540	15
Zorbax	800	260	540	15

LC gradient schedule: for Symmetry, Luna and Zorbax columns

Time (minute)	Mobile A (%)		Mobile B (%)
	0.5 mM Ammonium acetate: MeOH (80:20)		MeOH
0.00	95.0		5.0
5.00	60.0		40.0
8.00	60.0		40.0
9.00	5.0		95.0
12.00	5.0		95.0
13.00	95.0		5.0
15.00	95.0		5.0

HP1100 Autosampler setting:-

Injection Volume	25	μl
Draw Speed	200	$\mu\text{l}/\text{min}$
Eject Speed	200	$\mu\text{l}/\text{min}$
Needle Level	0.2	mm
Run Time	12.0 to 15.0	min

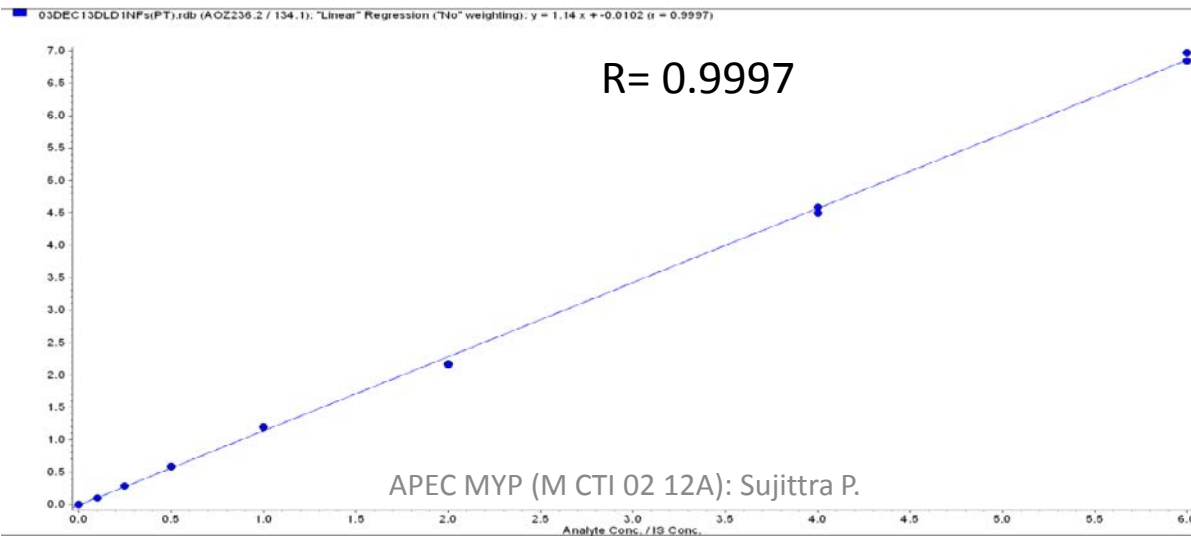
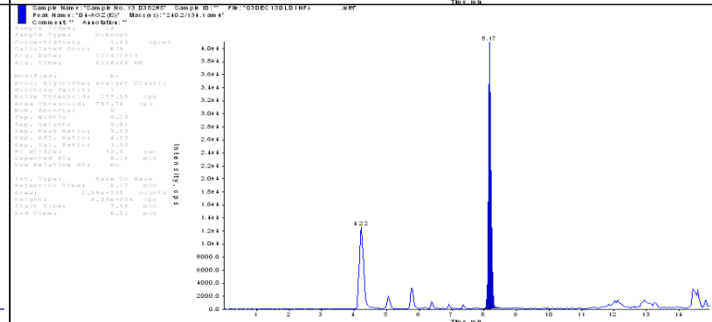
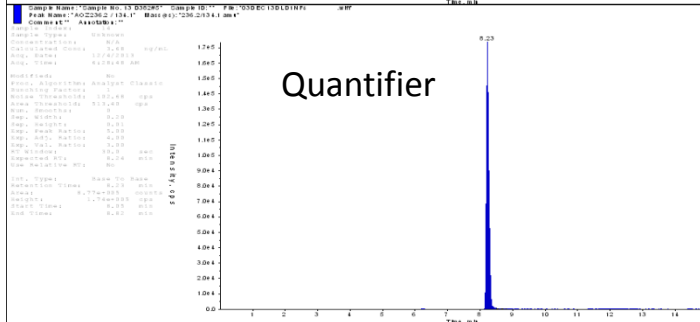
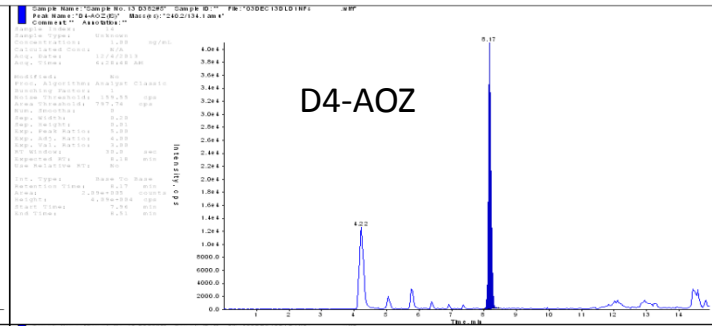
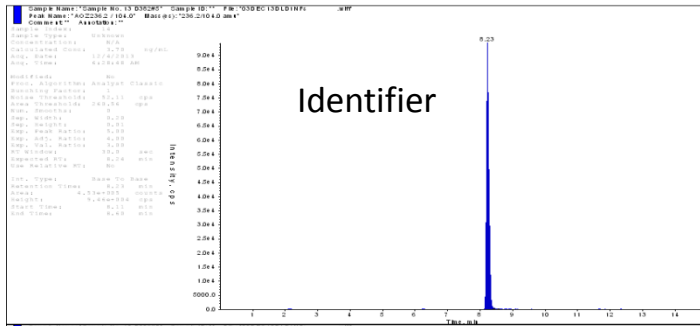


NFS by LC-MS/MS

Compound	Q1 Mass (amu)	Q3 Mass (amu)	Time (msec)	DP (volts)	FP (volts)	CE (volts)	CXP (volts)
AHD	249.3	104.2	300	36	150	33	6
AHD	249.3	134.2	300	36	150	18	8
AOZ	236.2	104.0	150	51	220	31	6
AOZ	236.2	134.1	150	51	220	19	8
SEM	209.2	192.2	150	41	180	15	10
SEM	209.2	166.3	150	41	180	15	10
AMAZ	335.2	262.3	150	46	210	23	18
AMAZ	335.2	291.2	150	46	210	17	18
D ⁺ -AMAZ	240.2	134.1	150	51	220	19	8
D ⁺ -AMAZ	340.2	296.0	150	46	210	17	18

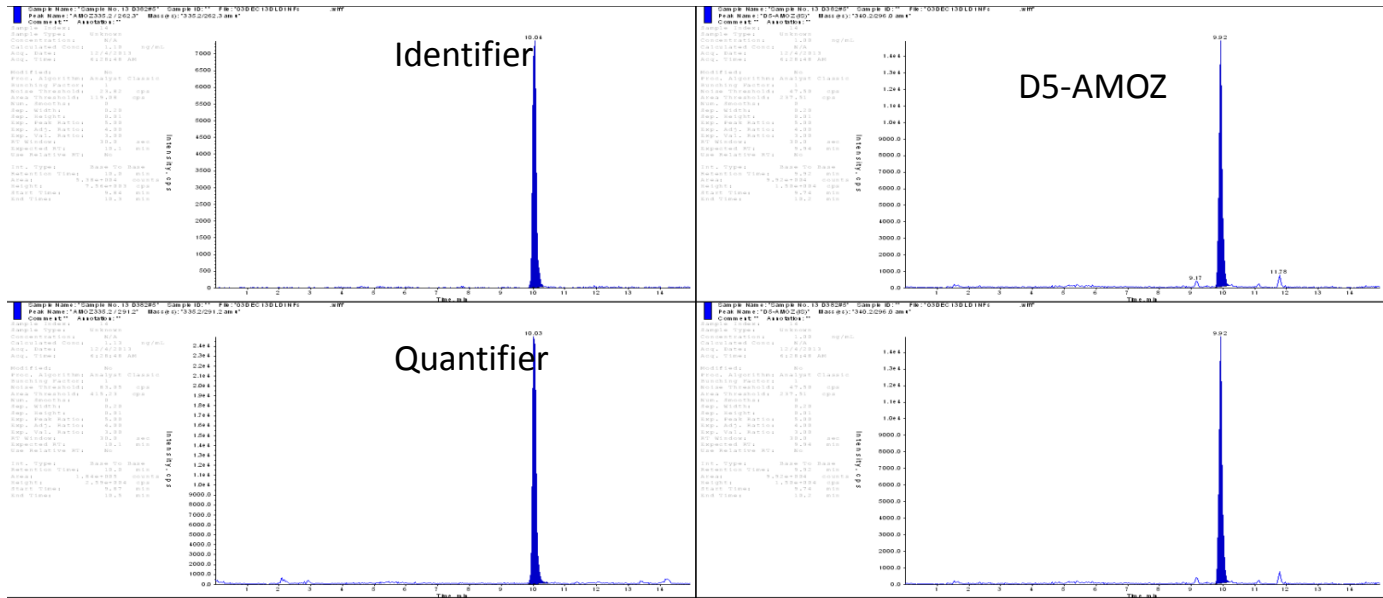
Result of sample no. 13-D382 (AOZ)

AOZ

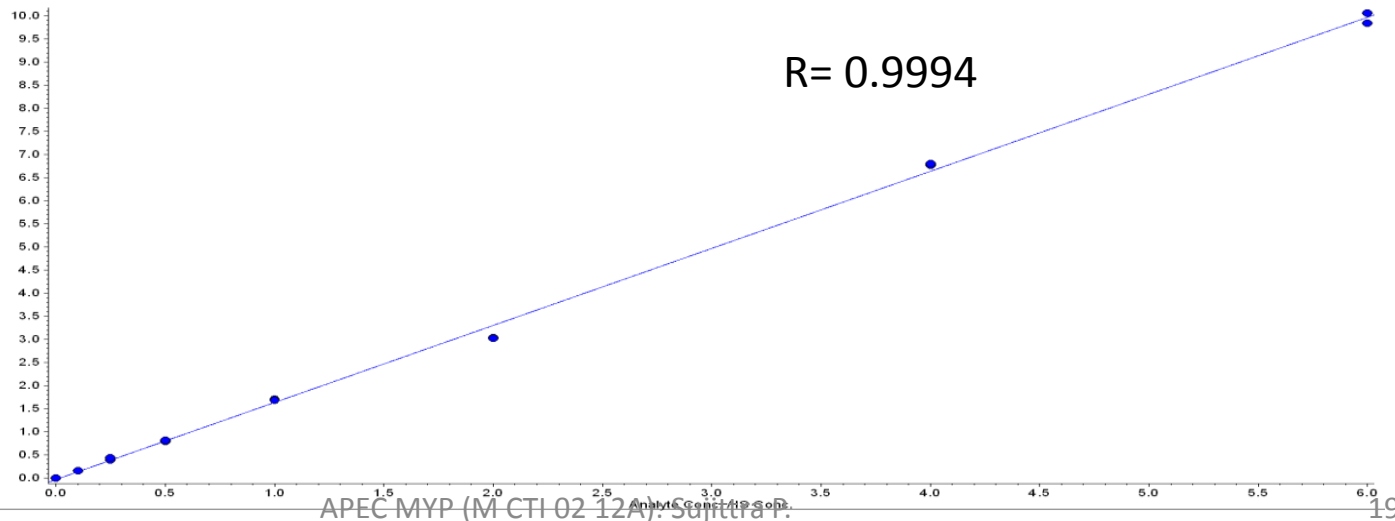


Result of sample no. 13-D382 (AMOZ)

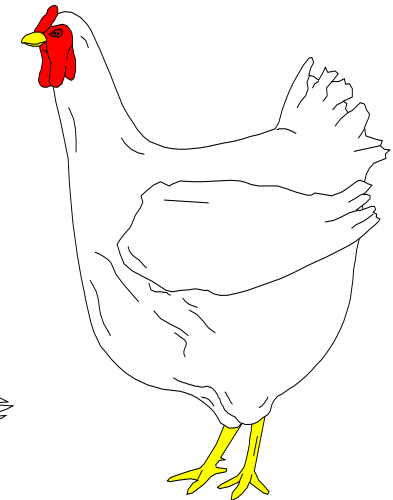
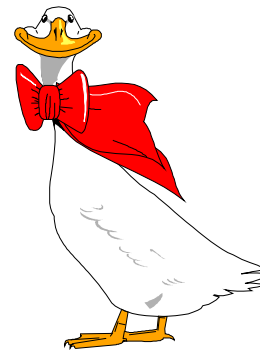
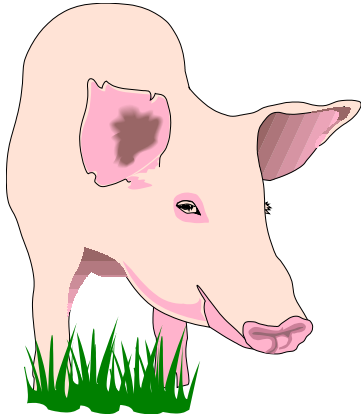
AMOZ



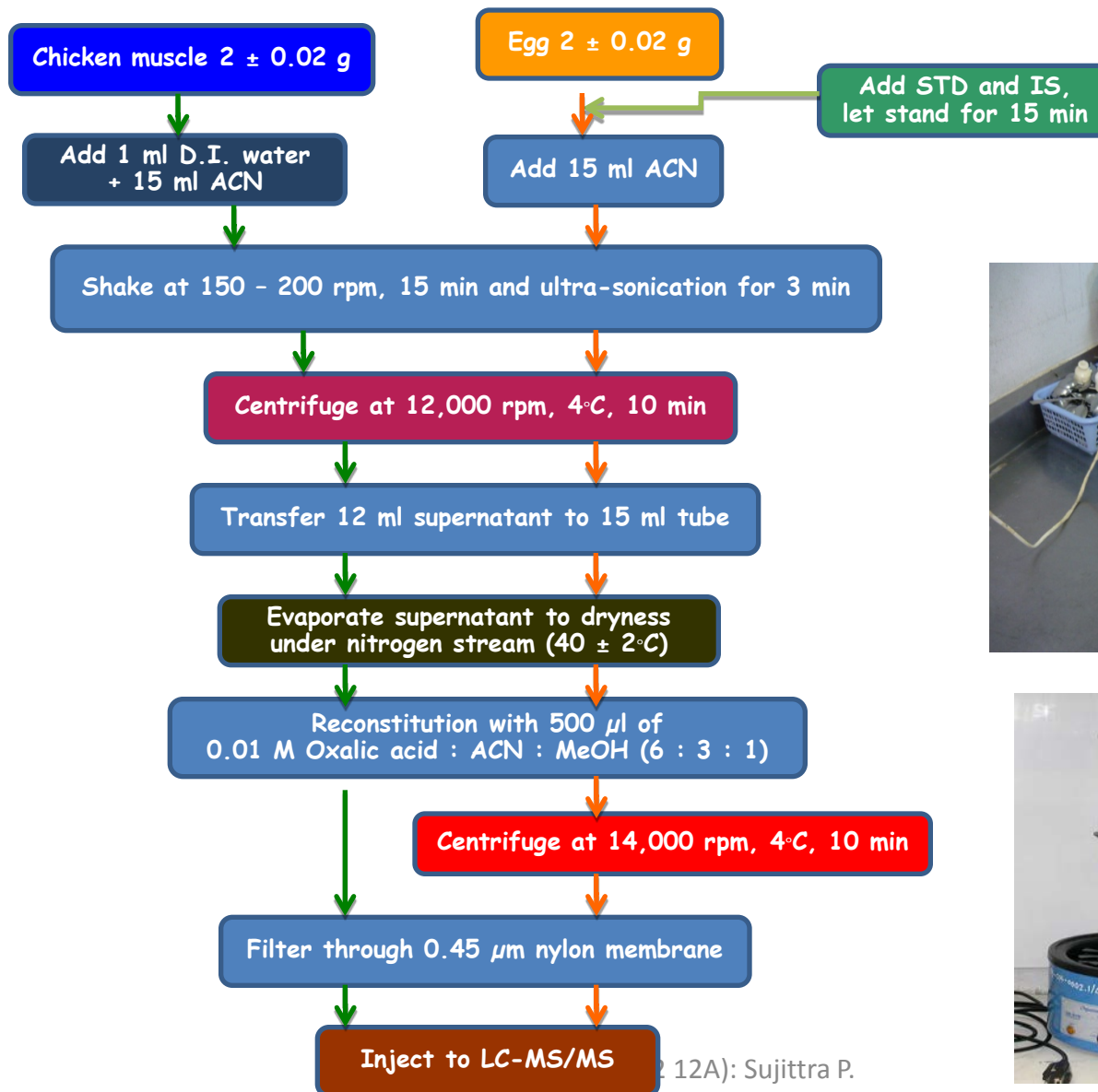
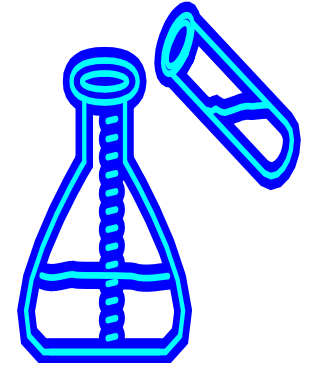
03DEC13DLD1NFs(PT).rd (AMOZ335.2 / 291.2): "Linear" Regression ("No" weighting): $y = 1.67x + -0.0328$ ($r = 0.9994$)



EFX an effective antibiotic



Sample Preparation



FQL by LC-MS/MS

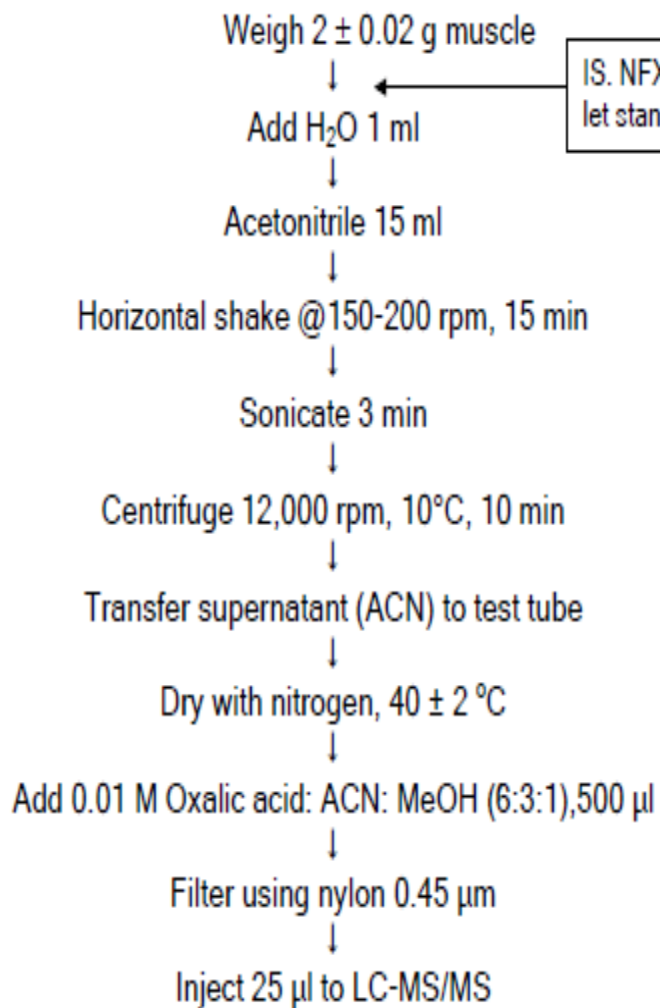
- HPLC: Agilent 1100
- Column: Zorbax C 18, 3.5 μm , 4.6 x 150 mm
- Mobile phase:
 - A: 0.1 mM Oxalic acid in 0.2 % Formic acid
 - B: Acetonitrile
- Flow rate: 0.9 ml/min
- TQD-MS: Applied Biosystem API 3000
 - Turbolonspray ESI+ mode



Fluoroquinolone in Muscle by LC-MS/MS

FLOW CHART

ENROFLOXACIN & CIPROFLOXACIN in Muscle by LC-MS/MS



IS. NFX 1000 ng/ml mix,
let stand for 15 min

HPLC conditions

Columns: ZORBAX SB-C18, 4.6 × 150 mm 3.5 µm

Flow rate: 0.9 ml/min

Injection Volume: 25 µl

Run time: 12 min.

Mobile phase: Line A: 0.1mM Oxalic Acid in 0.2%Formic Acid

Line B: Acetonitrile

MS/MS: API3000, ESI+

Table 1: LC gradient schedule

Step	Total Time (min)	Flow Rate (µl/min)	Mobile Phase A (%)	Mobile Phase B (%)
0	0.00	900	90.0	10.0
1	2.50	900	60.0	40.0
2	6.50	900	5.0	95.0
3	9.00	900	5.0	95.0
4	9.10	900	90.0	10.0
5	12.00	900	90.0	10.0

Max. Residue Limits for Sum of EFX & CFX residue in..

	CR (EU) 30/2010	Japan notification No. 645/2006
Muscle	100 µg/kg	50 µg/kg
Egg	No MRL (Prohibited for using in layers &)	No MRL
Honey	No MRL	No MRL

Decision Limit ($CC\alpha$) & Detection Capability ($CC\beta$)

Ref: 2002/657/EC

	Muscle		Egg	
	$CC\alpha$	$CC\beta$	$CC\alpha$	$CC\beta$
EFX	55	58	0.2	0.3
CFX	54	58	0.4	0.6

Residue conc. above $CC\alpha$ means non-compliant

MV Result: Method Accuracy & Precision

	Muscle				Egg			
	Conc. (µg/kg)	%rec	Repeatability	Reproducibility	Conc. (µg/kg)	%rec	repeatability	reproducibility
	25	95	5.9	2.2	0.5	90	11.9	4.2
EFX	50	101	4.7	1.2	1.0	91.3	7.9	3.9
	75	104	2.6	1.9	1.5	98.8	5.7	1.6
	25	93	6.0	5.8	1	92.5	11.5	0.8
CFX	50	98	4.9	0.6	2	92.8	7.2	1.2
	75	102	2.6	2.0	3	93	5.2	3.7

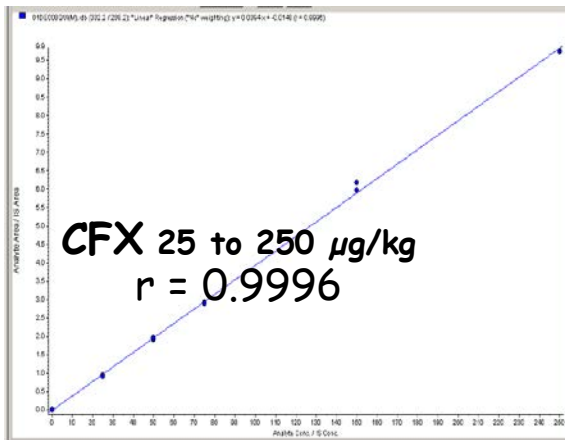
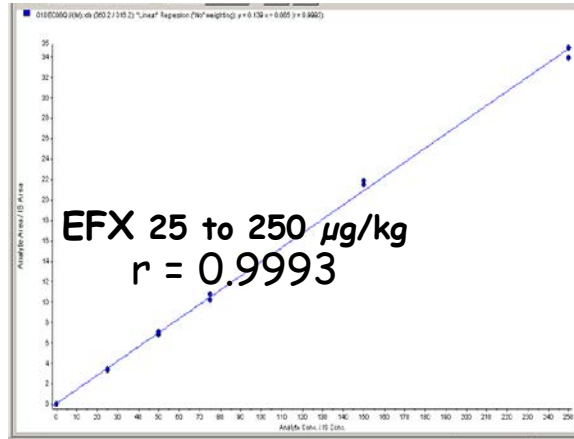
MV Results: Identification

Table: Transition ions of EFX, CFX for Identification and quantification.

Compound, Identification Point (IP)	Rt (min)	Quantification	Identification	Ion ratio (%)
EFX, IP = 4	5.0	360.2 > 245.2	360.2 > 316.2	57 ± 20%
CFX, IP = 4	4.8	332.2 > 245.2	332.2 > 288.2	30 ± 25%
NFX as IS	4.7	320.2 > 276.2		

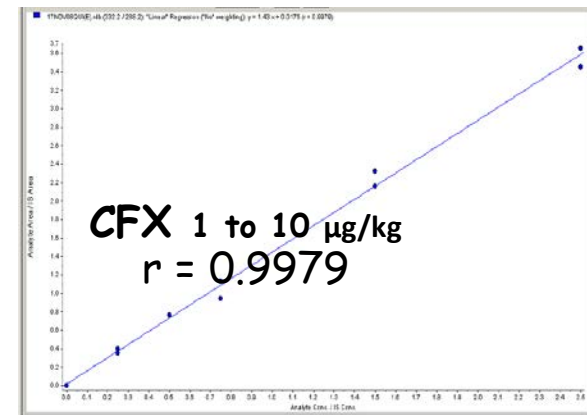
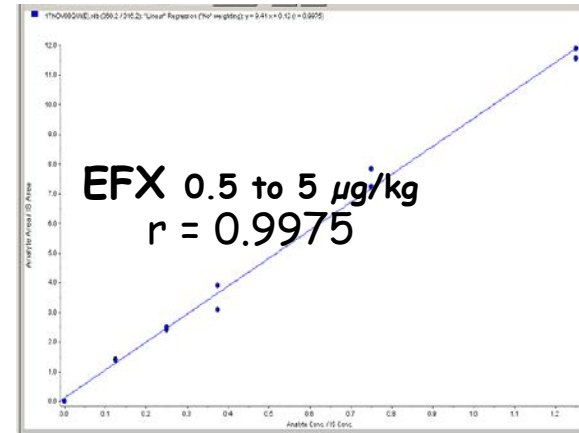
Linearity of calibration curve

Muscle



Working range 25 to 250 $\mu\text{g}/\text{kg}$ in muscle

Egg

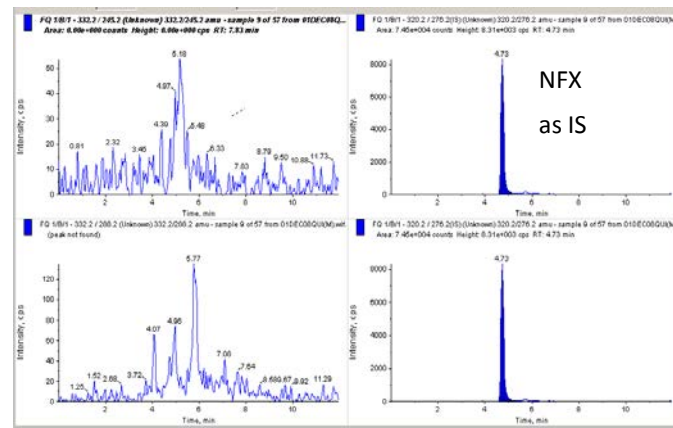
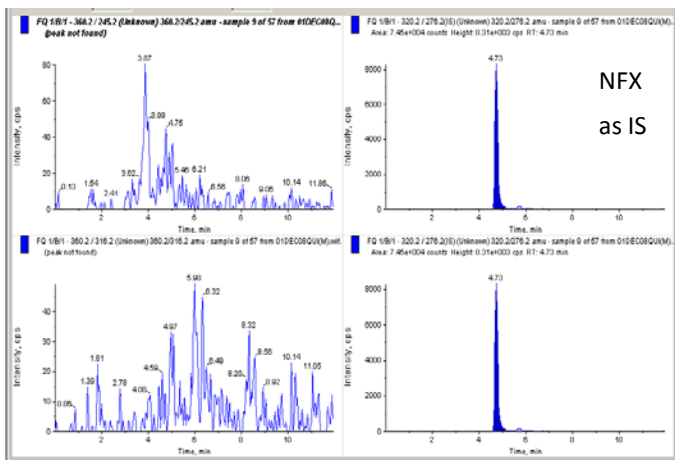


Working range 0.5 to 5 $\mu\text{g}/\text{kg}$ (for EFX)
and 1.0 to 10 $\mu\text{g}/\text{kg}$ (for CFX) in egg

Identification & Quantification

EFX 25 µg/kg

CFX 25 µg/kg



Blank

Identifier Ion

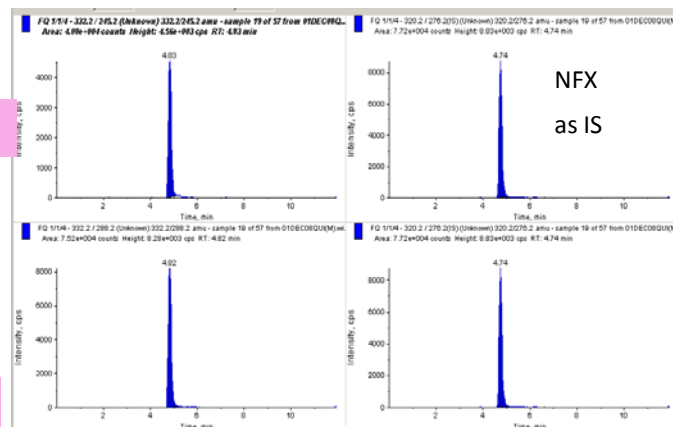
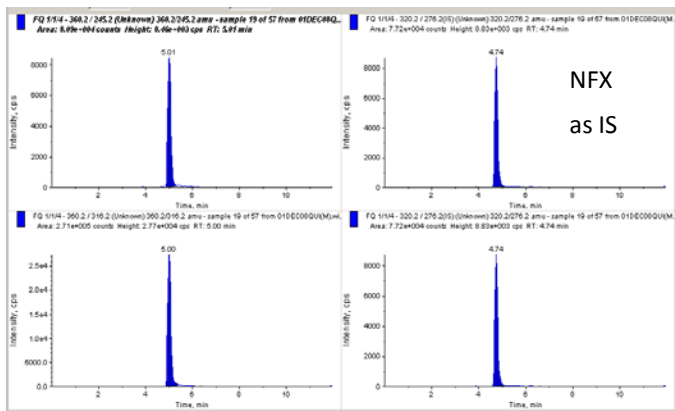
360.2>316.2

332.2>288.2

Quantifier Ion

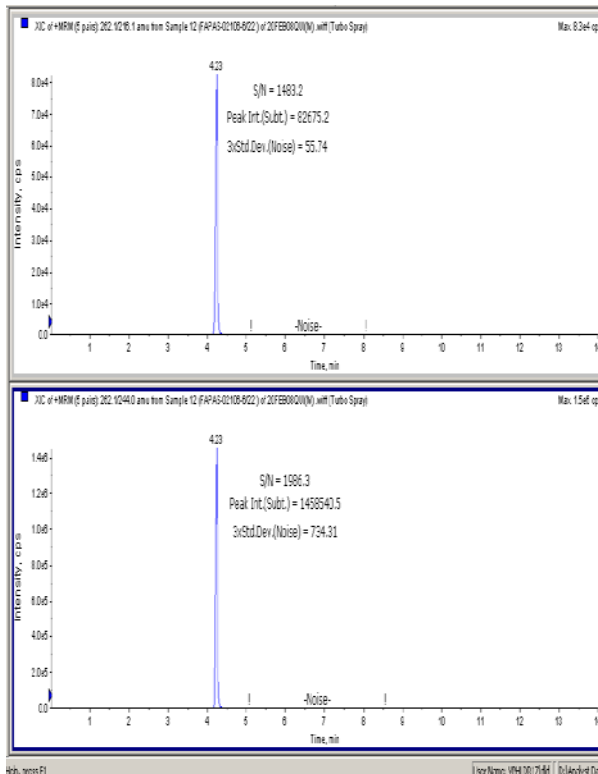
360.2>245.2

332.2>245.2



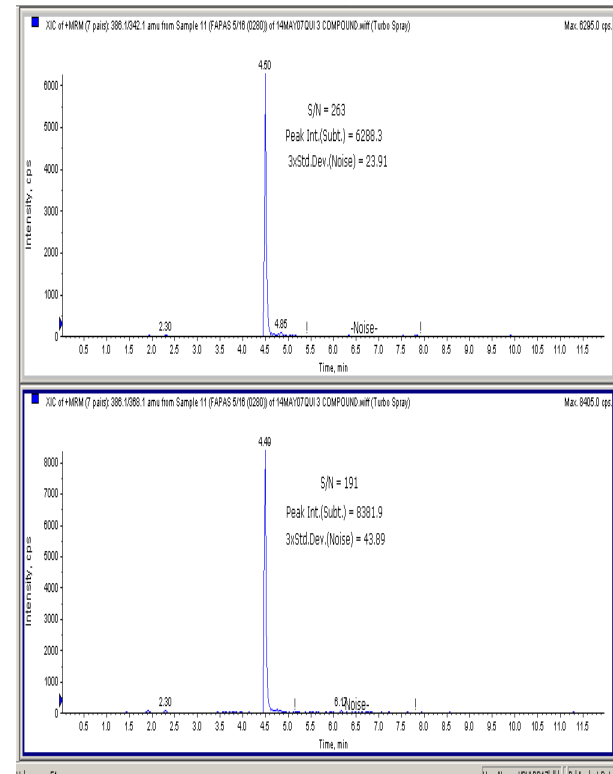
FAPAS PT result: Oxolinic acid & Sarafloxacin in chicken meat

Oxolinic acid in chicken muscle



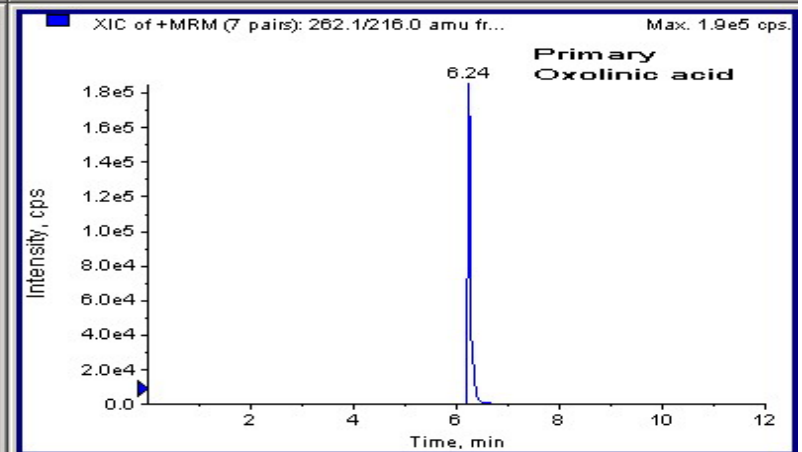
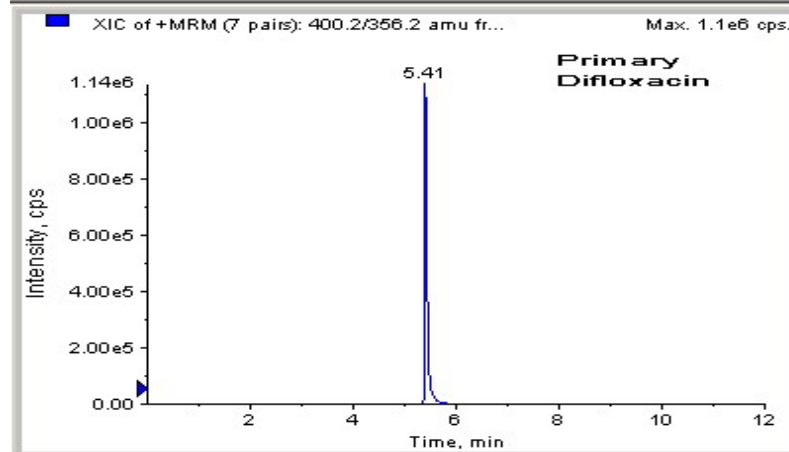
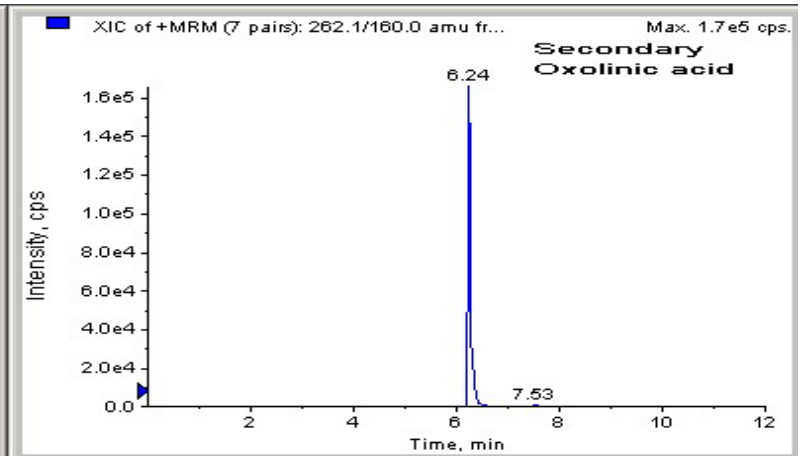
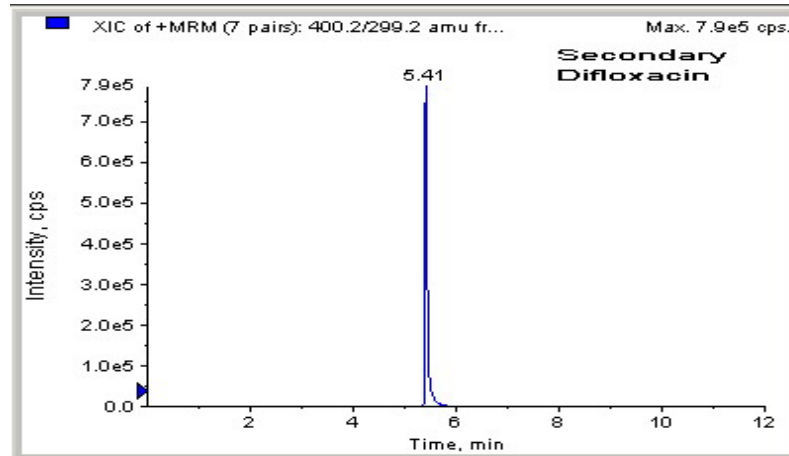
Z-score = -1.5

Sarafloxacin in chicken muscle



Z-score = -0.2

FAPAS PT result: Difloxacin & Oxolinic acid in rabbit muscle



Difloxacin
Z-score = -0.2

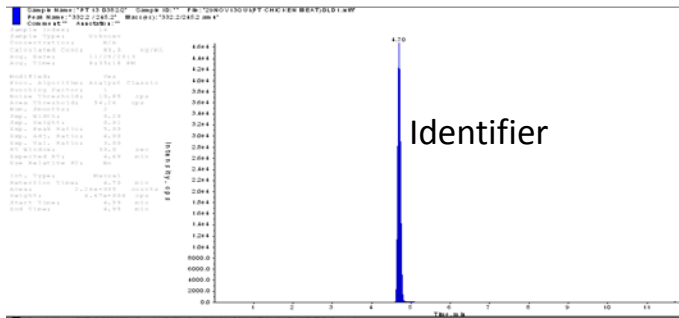
Oxolinic acid
Z-score = 0.9

Discussion

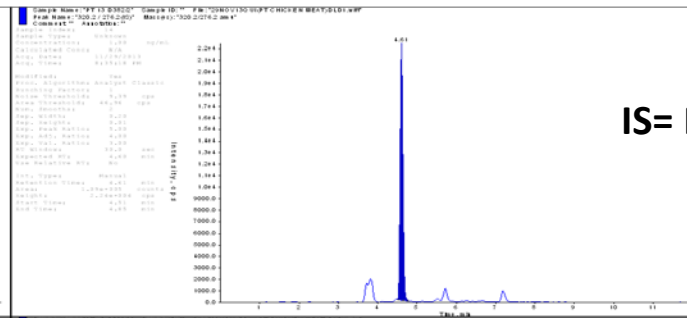
- The residue of EFX & CFX can be extracted from meat and egg with ACN.
- All parameters of method validity were evaluated and all results have shown within criteria and thus were acceptable.
- The method can be proven out of fitness for intended use.
- The method can be extended to other FQLs and Quinolone i.e. Sarafloxacin, Danofloxacin, Oxalic acid, and other matrices i.e. Rabbit muscle, Fish.

Result of sample no. 13-D382 (CFX)

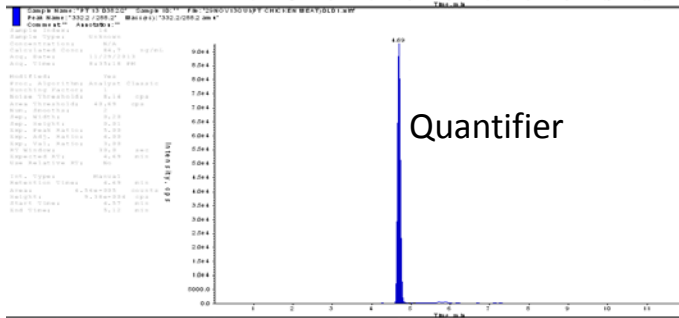
CFX



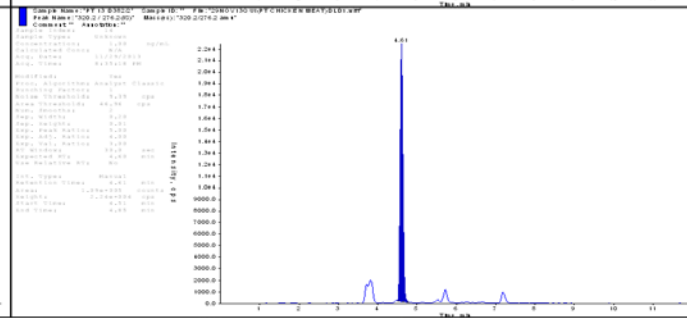
Identifier



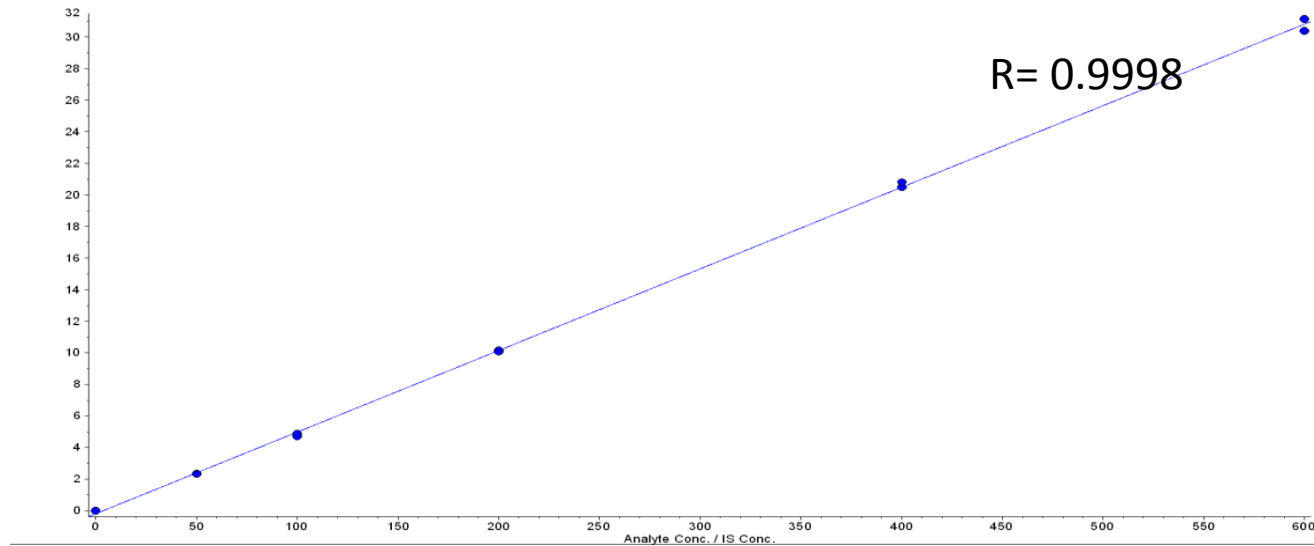
IS= NFX



Quantifier



29NOV13QU(PT CHICKEN MEAT)DLD1.rdb (332.2 / 288.2): "Linear" Regression ("No" weighting): $y = 0.0517x + -0.193$ ($r = 0.9998$)



Suphonamides in animal tissue by LC-UV

AGAL Method No. 7, Issue 1, June 1991,
Western Australian Regional Laboratory of A.G.A.L.

- Standards (SMX, SDM, SQ) sources: USP
- No IS was used
- Result calculation: corrected for recovery

Method Validation result (Sulphonamides in tissue by LC-UV)

Table 15.1: CC_{α} and CC_{β} of nine sulphonamides at MRL concentration 100 ng/g in chicken meat.

Compound	Mean X at 100 ppb	SD_{WR}	CC_{α}	CC_{β}	Linear
SDZ	80.04	9.90	116.24	132.48	0.9998
SPY	86.71	8.88	114.49	128.97	0.9999
SMR	93.59	5.52	109.05	118.09	0.9996
SMZ	90.94	8.82	113.51	127.02	0.9996
SMN	92.72	6.45	110.58	121.15	0.9998
SCP	83.71	7.24	111.88	123.75	0.9993
SMX	94.06	6.19	110.15	120.29	0.9997
SDM	92.47	6.66	110.93	121.86	0.9996
SQ	89.66	9.10	114.92	129.84	0.9995

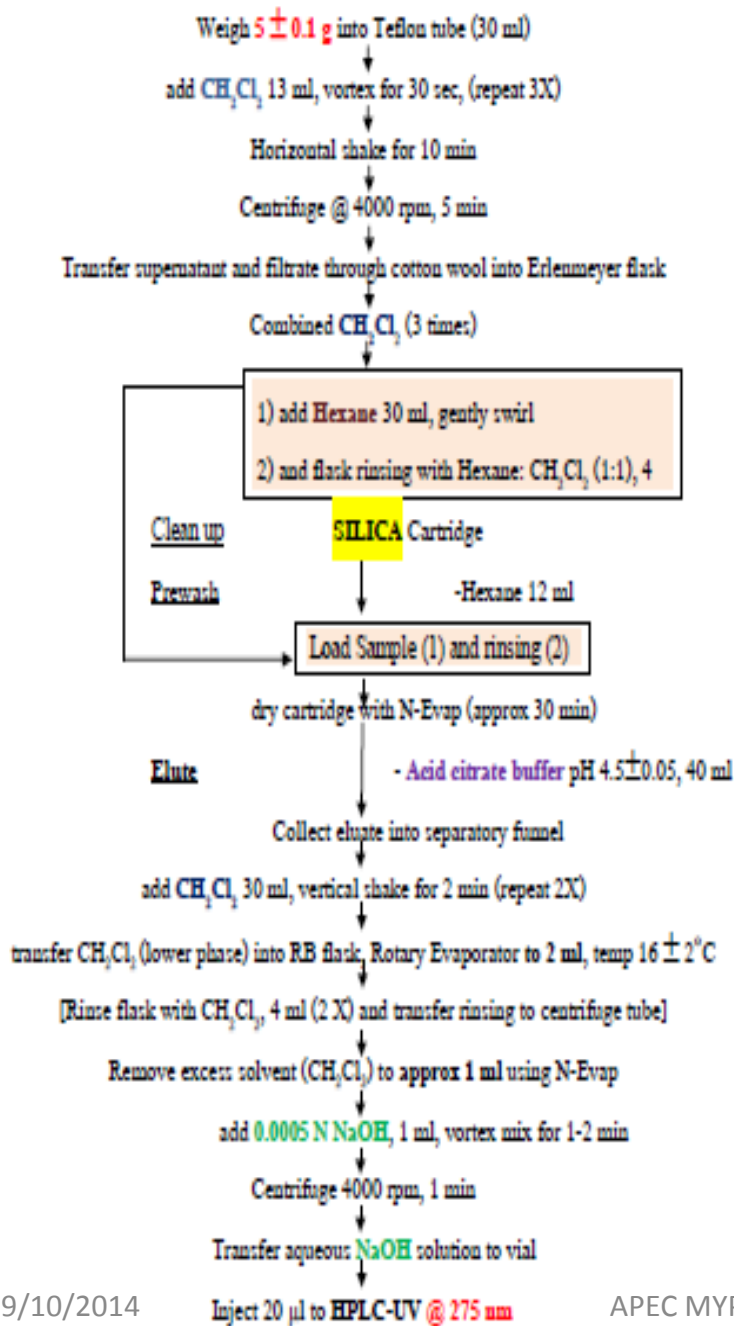
Method Validation result

(Sulphonamides in tissue by LC-UV)

Table 15.2 (cont.): % Recovery, Repeatability, Reproducibility of spiked samples at 50, 100 and 200 ng/gm in Chicken meat (n = 21, at each level), M.D.L. and M.Q.L.

Compound	M.D.L.	M.Q.L.	ng/gm	% Recovery	Repeatability		Reproducibility	
					S.D.	%	S.D.	%
SMZ	11.5	34.4	50	86.18	3.77	8.74	4.54	10.54
			100	90.94	6.86	7.54	8.24	9.06
			200	94.85	14.20	7.49	14.80	7.80
			Av.	90.66				
SMN	6.95	20.9	50	91.40	2.12	4.65	2.75	6.01
			100	92.72	6.45	6.96	6.45	6.96
			200	91.51	14.21	7.76	14.42	7.88
			Av.	91.88				
SCP	17.7	53.1	50	82.78	7.00	16.92	7.00	16.92
			100	83.71	6.05	7.23	7.24	8.65
			200	84.14	12.91	7.67	13.52	8.04
			Av.	83.54				
SMX	7.74	23.2	50	88.58	2.71	6.13	3.06	6.91
			100	94.06	5.20	5.53	6.19	6.58
			200	96.26	11.18	5.81	12.06	6.26
			Av.	92.97				
SDM	8.17	24.5	50	94.24	2.82	5.97	3.23	6.86
			100	92.47	6.39	6.91	6.66	7.21
			200	96.50	15.02	7.78	15.02	7.78
			Av.	94.40				
SQ	13.7	41	50	86.26	5.37	12.46	5.40	12.52
			100	89.66	9.10	10.15	9.10	10.15
			200	94.28	17.66	9.36	17.66	9.36
			Av.	90.07				

SULPHONAMIDES in animal tissues by HPLC



Shimadzu series 10 Avp

SYSTEM CONTROLLER: SCL-10Avp

PUMP: LC -10ADvp with Degasser DGU-14AM

COLUMN OVEN: CTO-10ASvp

INJECTOR: Autosampler SIL 10ADvp

DETECTOR: UV-VIS SPD - 10Avp

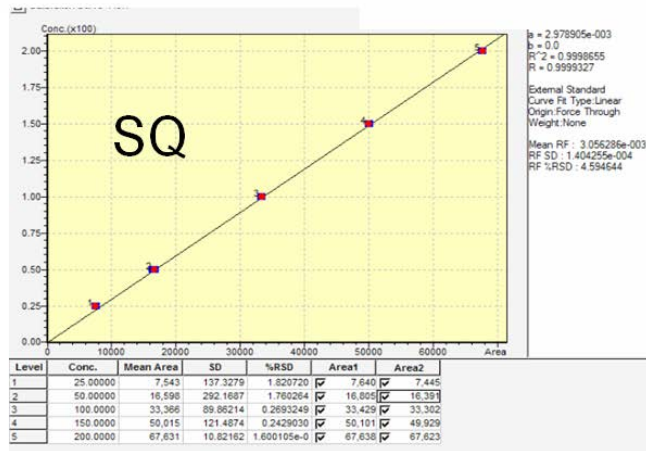
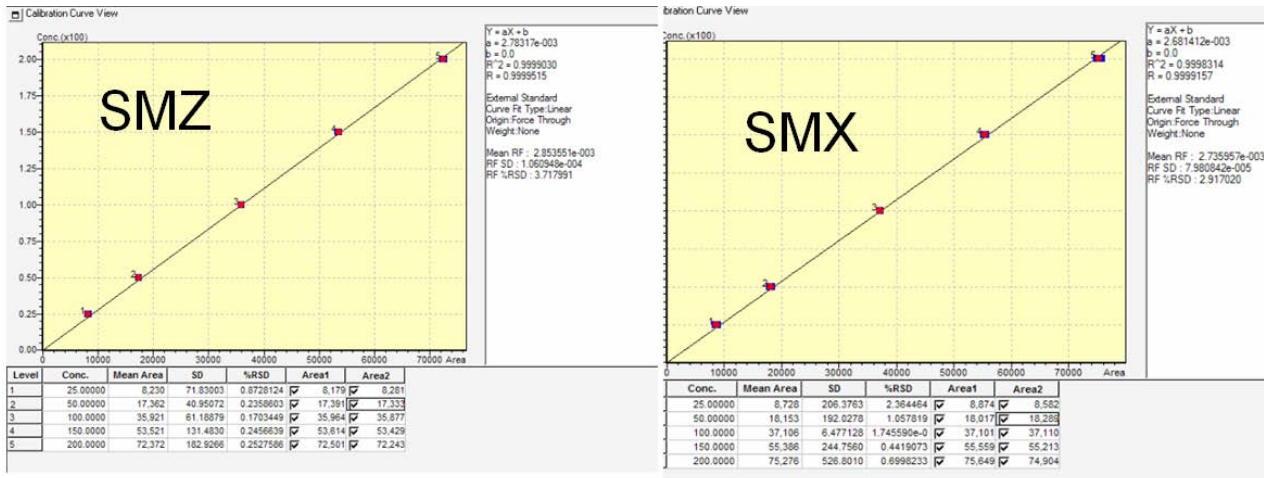
Symmetry C18 3.9×150 mm (5μ) WAT 046980.

Guard column: Symmetry C18 2.1×10 mm, WAT 054225.

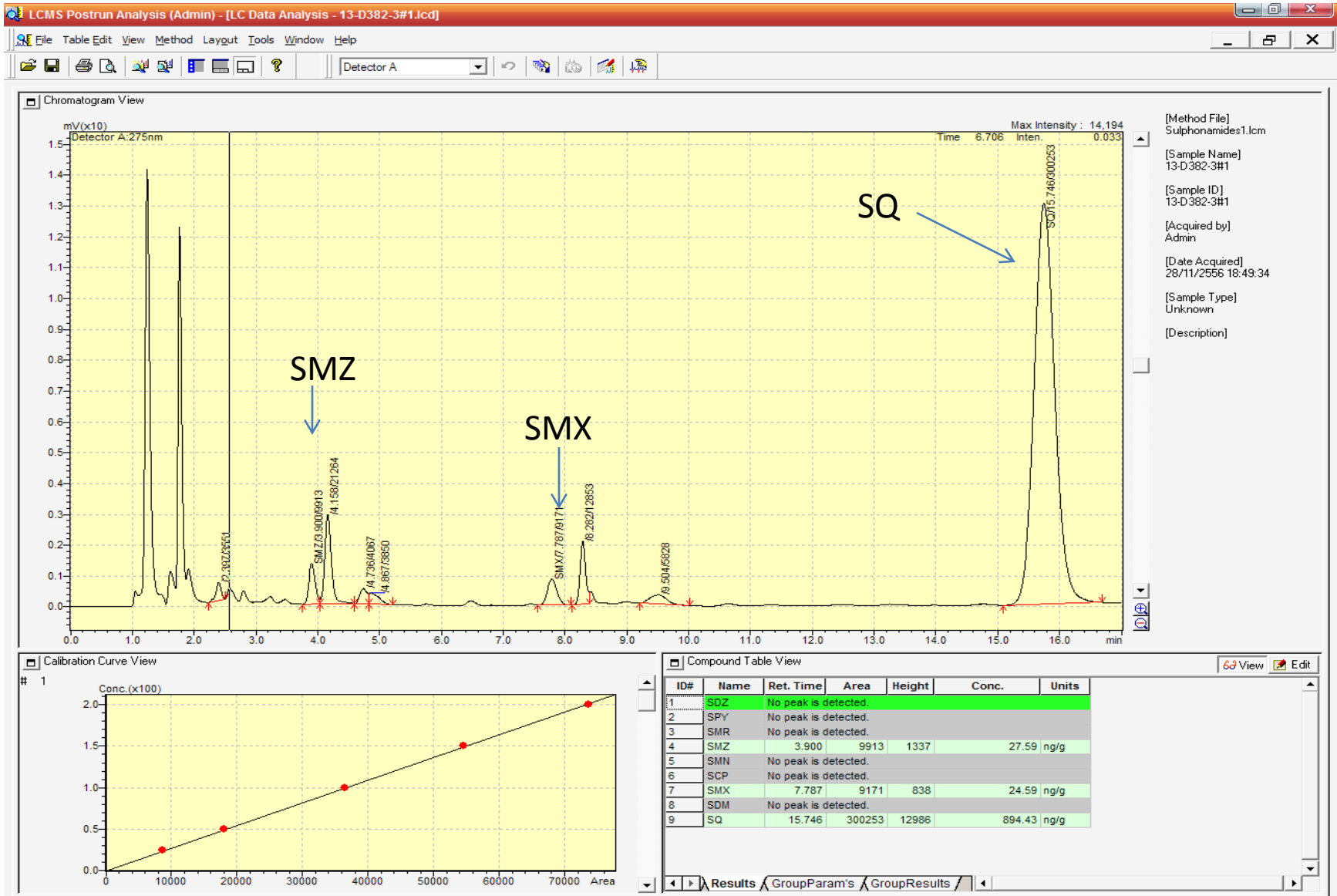
HPLC OPERATING CONDITION:-

COLUMN OVEN Temperature	35 °C
FLOW RATE	1.0 ml/min
INJECTION VOLUME	20 μl
UV WAVELENGTH	275 nm
MOBILE PHASE	Acetonitrile/Water/Acetic acid (190:800:10)

Calibration curve of SMZ, SMX and SQ



Chromatogram of sample no. D 382



APEC MYP (M CTI 02 12A) PT result

analyte	Sample no.	Result 1 (ug/kg)	Result 2 (ug/kg)	Average (ug/kg)	% recovery	
AOZ	13-C594	2.18	2.17	2.18		
	13-D382	3.68	3.43	3.56		
AMOX	13-C594	0.460	0.510	0.485		
	13-D382	1.13	1.04	1.08		
SMX	13-C594	167	161	164	83.7	
	13-D382	38.5	39.4	39.0	63.8	
SMZ	13-C594	139	136	138	86.5	
	13-D382	41.1	36.6	38.8	67.2	
SQ	13-C594	230	220	225	95.2	
	13-D382	910	998	954	95.2	
CFX	13-C594	411	437	424		
	13-D382	84.7	80.1	82.4		

The results of sulfa residues were corrected for recovery

Comments

- Instruction for participants & PT scheme for VDR

Comments:

- The instruction requested the testing should be performed as soon as samples were received. ** It was not clearly stated the due date for result submission.
- But the due date (within 10 days after samples reception) was stated in email not in the Instructions for participants. **Ten days were too short.** One month for analysis would be more appropriate.
- For **Nitrofurans**: not specify which form of residue should be tested.
- The PT for veterinary drug residues should be focused as well on **Identification** of the residues rather than focused on Quantification only

Thank YOU very much

1. APEC sponsorship & APEC FSCF PTIN coordinator

2. AFRL for VDR analysts team



3. ALL of YOU