Rapid Screening Methods for THE Feed Additive Zilpaterol

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Introduction

- Zilpaterol is a β-adrenergic agonist feed additive approved in the United States to increase weight gain and improve feed efficiency in cattle.
- Countries which ban β-adrenergic agonist feed additives can reject imported beef products that contain zilpaterol.
- Efficient, portable, and user friendly screening assays towards zilpaterol are needed.
- Our goal was to determine levels of zilpaterol exposure in animals that will result in its detection using qualitative and quantitative assays.

Instrumental Analysis

~ $450,000

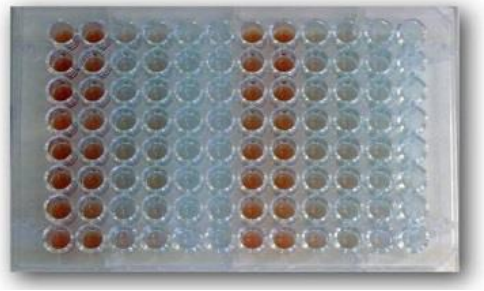
~ $250,000

Shipping & Installation

- Cost
- Space
- Training
- Throughput

Screening Assays

- Enzyme Linked Immunosorbent Assay (ELISA)



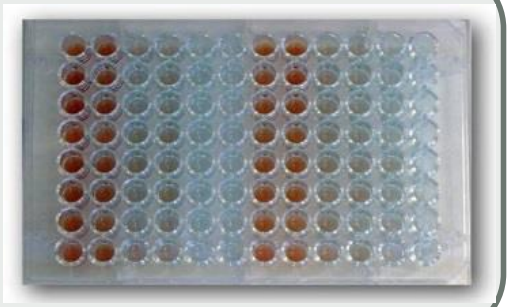
- Lateral Flow Immunoassay

- Microbial Inhibition Assay

- Initial investment
  - ~ $1-5/test
- Turn around time
  - Min to hours
- Operator training
  - Minimal
- Field deployable
- Parallel Assay

Screening Assays (2)

- Enzyme Linked Immunosorbent Assay (ELISA)



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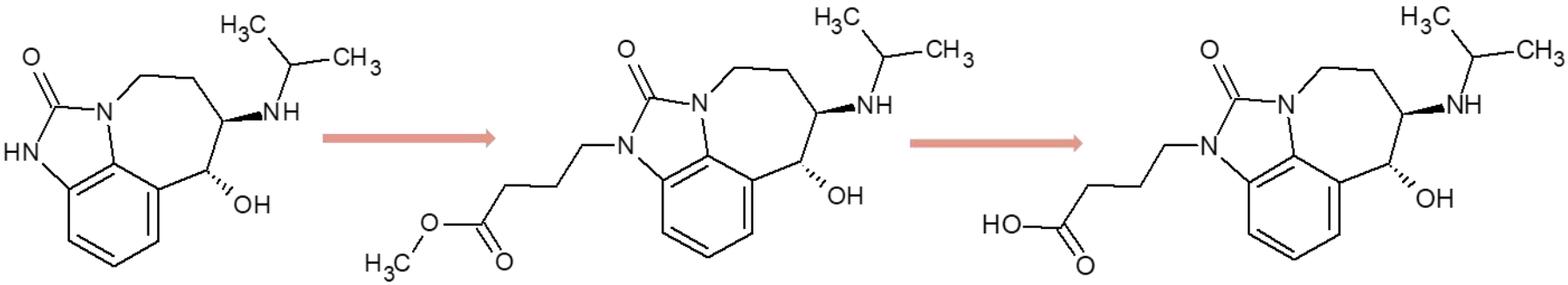
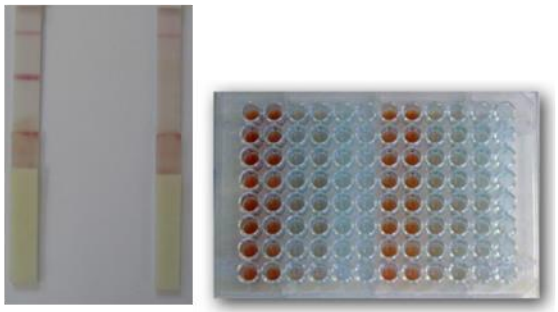
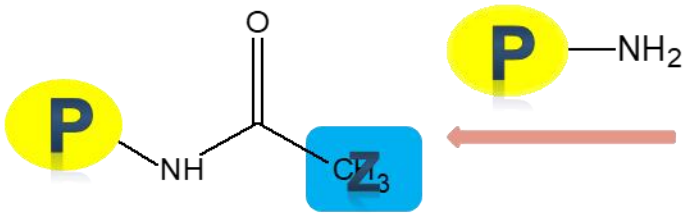
Zilpaterol Immunoreagent Generation



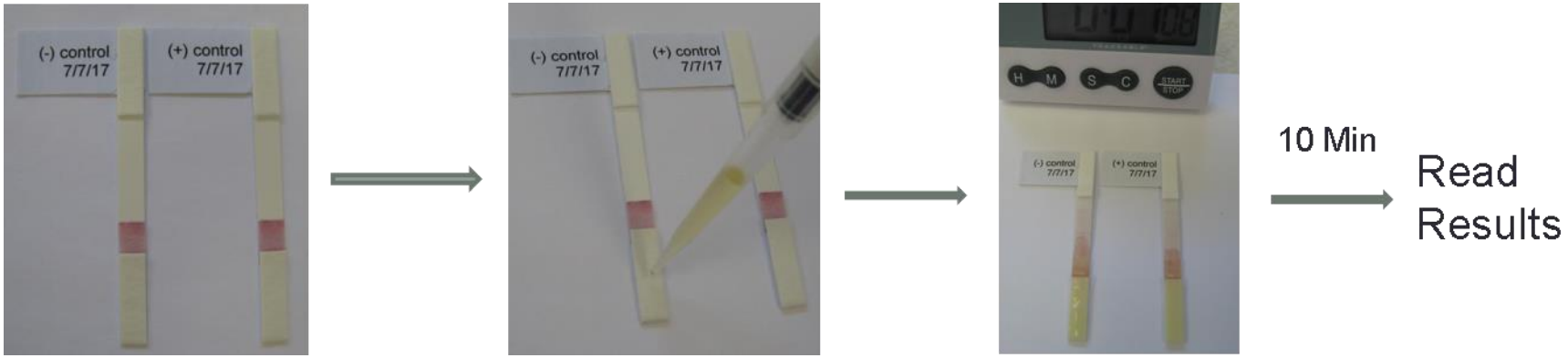


Zilpaterol-BSA

Zilpaterol-KLH

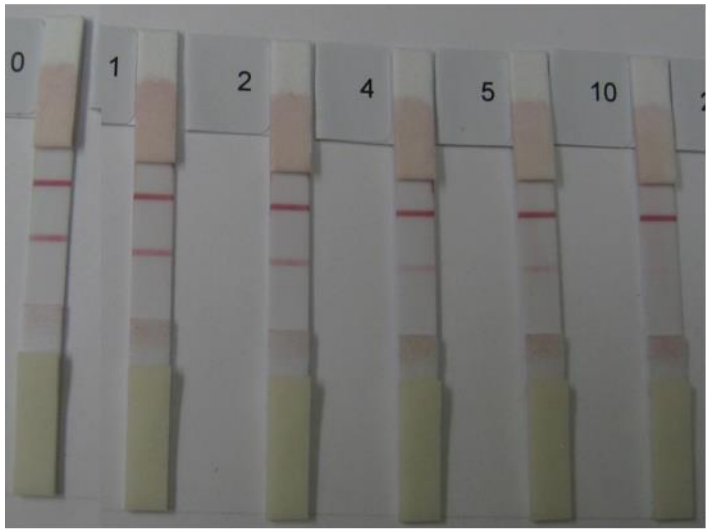


Lateral Flow Assay Procedure



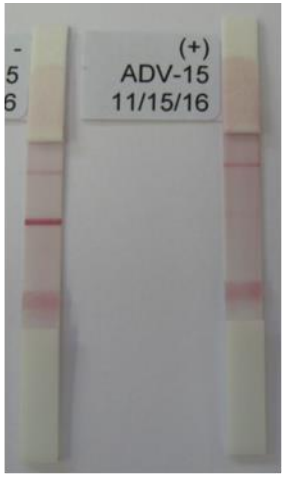
- No pretreatment for cattle and sheep urine
- Horse urine was diluted 1:3 (v/v) with water.
- Feed was added 1:4 (w/v) with water
- Tissues were either diluted with water or extracted with ethyl acetate followed by reconstitution in PBS.

Lateral Flow Assay Results



Sheep Urine

Cattle Feed



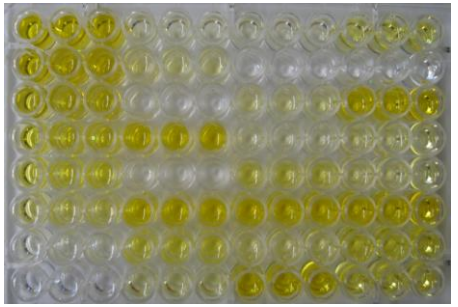
Sheep Muscle

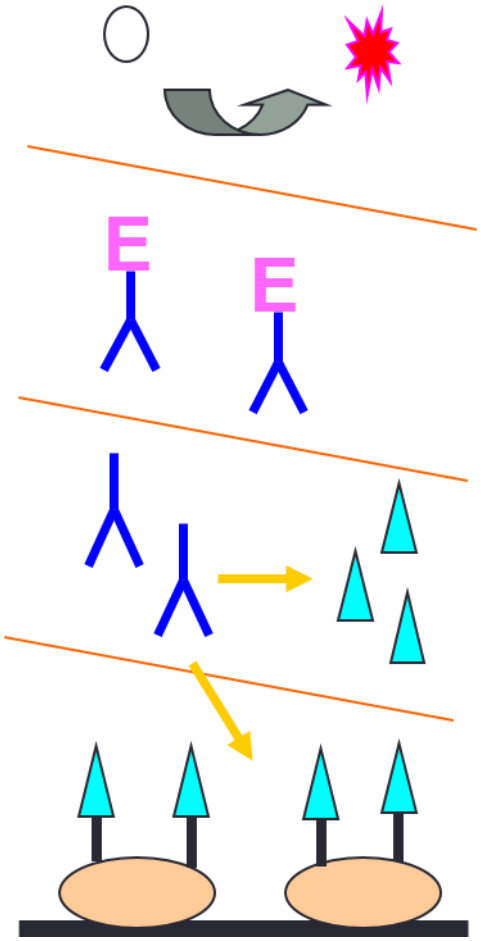
	90/95
Cattle Urine	1.7 ng/mL
Sheep Urine	1.7 ng/mL
Horse Urine	8.8 ng/mL
Sheep Muscle	2.4 ng/g
Cattle Feed	23.2 ng/g

Cow Urine

Horse Urine

ELISA Procedure





Read color intensity	TMB 450nm
Enzyme-Ab	Rabbit anti-mouse-HRP
Competition	Zilpaterol 0-100 ng/mL, Urine dilution matrix match 1^0 Ab
Hapten-conjugate	Zilpaterol-butyrate-BSA

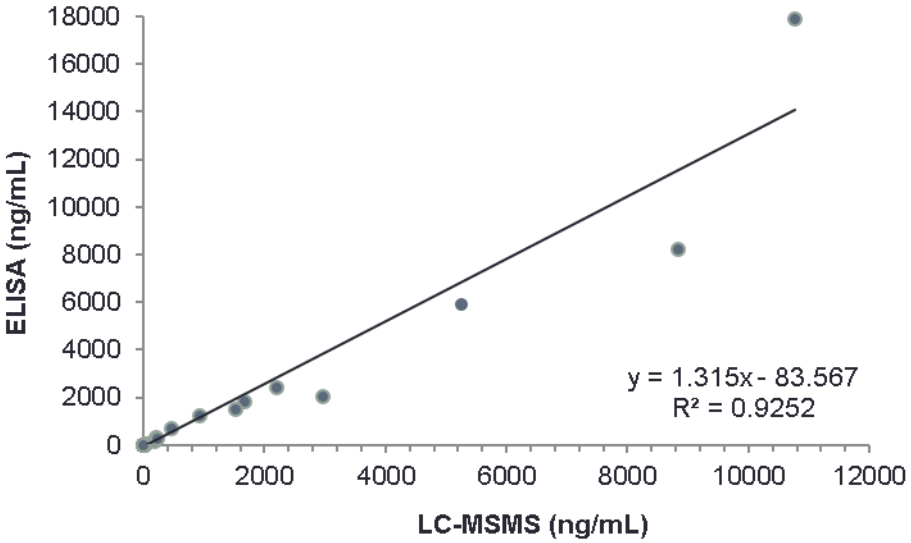
Horse Protocol

- Horses (n = 3) were privately owned
  - 479 kg, 3-year old filly (Hannah)
  - 462 kg, 5-year old mare (Foxy)
  - 469 kg, 4-year old gelding (Hollywood)
- 7-day acclimation period
  - Ad libitum access to hay and water
  - Daily 0.23 kg supplement of cracked corn in morning
  - Housed indoors with daily morning and afternoon exercise of variable length
- 160 µg/kg body wt at day 0, hour 0 followed by 43 µg/kg body wt. (25% of initial dose) on day 1 for 2 horses
- urine samples collected daily

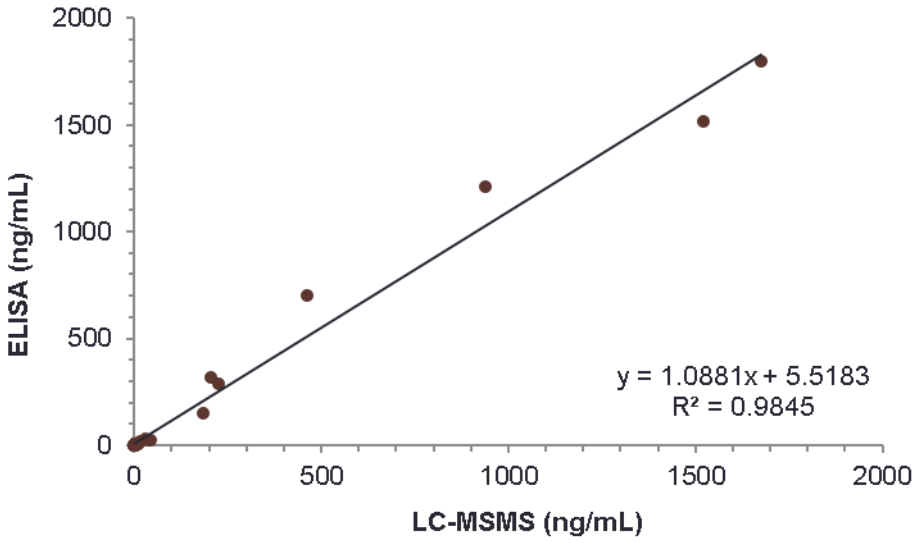
Application to Horse Urine

Withdrawal	Hollywood		Hannah		Foxy	
Day	MS (ng/mL)	LFA	MS (ng/mL)	LFA	MS (ng/mL)	LFA
0	10771	+	8829	+	5267	+
1	462	+	1677	+	1520	+
2	2197	+	938	+	2960	+
3	184	+	204	+	226	+
4	31	+	15.9	+	37.4	+
5	10.7	+	13.9	+	44.6	+
6	6.3	-	9.9	+	10.2	~
7	2.5	-	6.0	~	5.8	+
8	2.7	-	4.3	-	4.2	+
9	2.1	-	3.2	-	3.8	-

ELISA and LC-MSMS results

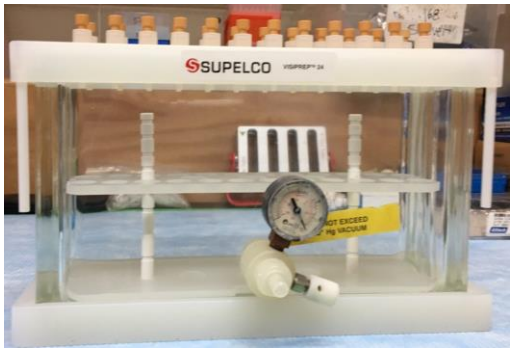


P = 0.328



P = 0.073

LC-MSMS



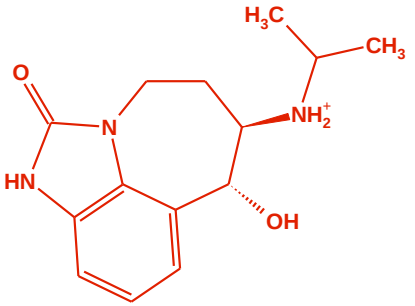
Sample Treatment

Varian Bond Elute Certify

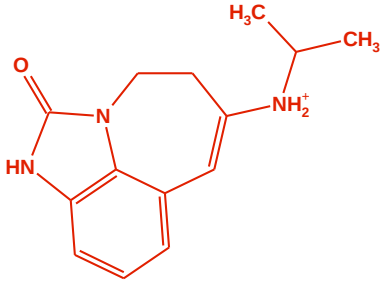
Precondition	Wash	Elute
MeOH	Phosphate Buffer	CH_2Cl_2/isopropanol/
H_2O	1M HAc	NH_4OH
Phosphate Buffer	MeOH	

LC-MSMS (2)

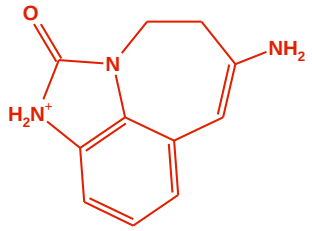
- Waters Acquity Triple Quadrupole MS
- Column: UPLC BEH C_{18}
- MRM, electrospray positive mode
- Matrix matched curves



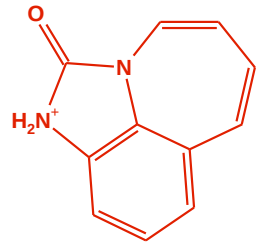
Monoisotopic Mass = 262.155003 Da



Monoisotopic Mass = 244.144439 Da

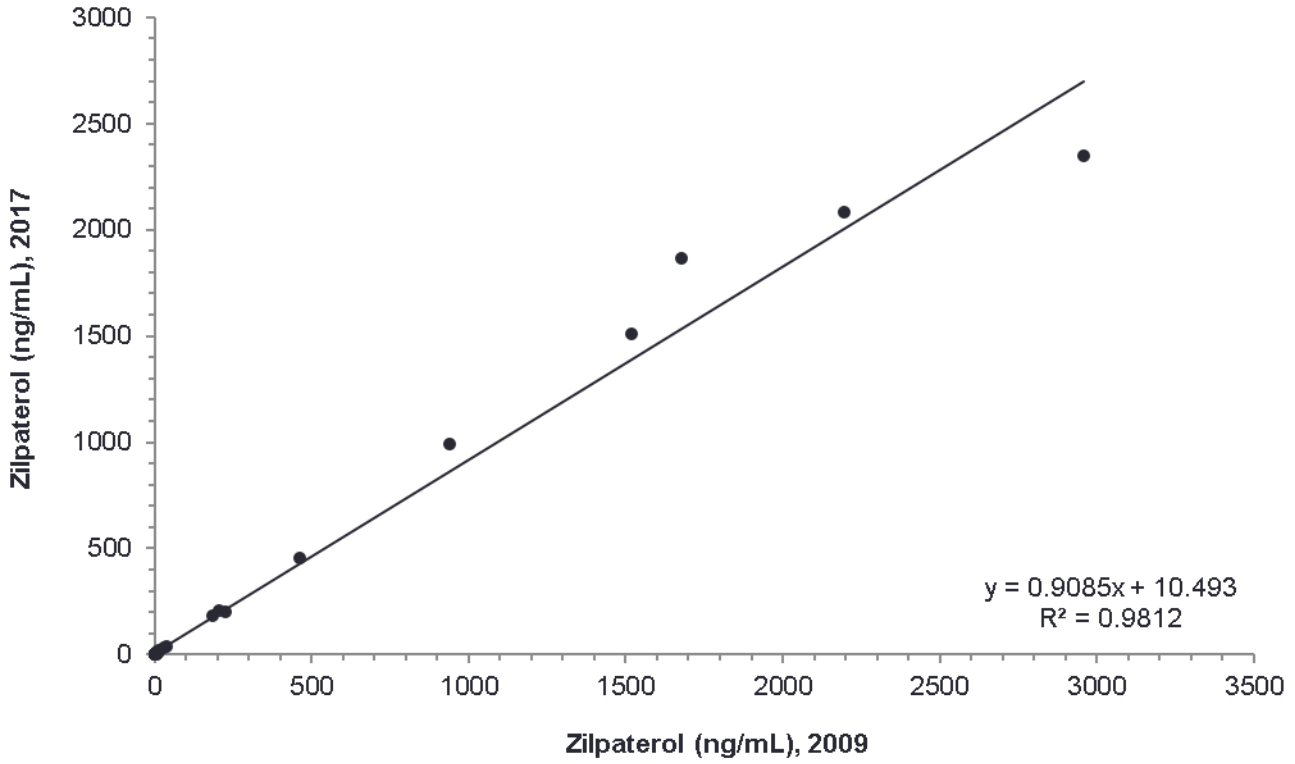


Monoisotopic Mass = 202.097488 Da



Monoisotopic Mass = 185.070939 Da

Zilpaterol Stability



P = 0.445

Sheep Protocol - I

- Sheep were fed 6 mg/kg for 10 days
- Two zilpaterol-treated ewes each were euthanized on withdrawal days 0, 2, 5, and 9
- Two control animals were euthanized on withdrawal day 0
- Urine collection
  - treatment days 1, 3, 5, and 8
  - withdrawal days 0, 1, 2, 5, 7, and 9 (or until slaughter)
- Tissues - at slaughter

Sheep Results (Protocol - I)

Withdrawal	Urine		Muscle	
Day	LC-MSMS (ng/mL)	LFA	LC-MSMS (ng/g)	LFA
0	591	+	12	+
	718	+	14	+
2	95.2	+	1.3	~
	42.1	+	0.5	-
5	1.7	+	<0.25	-
	<0.25	-	<0.25	-

Sheep Protocol-II

- Sheep were fed trace levels of zilpaterol HCl in a finishing diet
  - Low Dose 0.0075 mg/kg (1000x less than label concentration)
  - Med Dose 0.075 mg/kg (100x less than label concentration)
  - High Dose 0.75 mg/kg (10x less than label concentration)
- 12 day feeding period, 4 trials, 1 animal/treatment/trial
- Urine collected on treatment days 0, 2, 4, 6, 8, 10, 12
  - LFA – on site
  - ELISA – conclusion of each trial
  - LC-MSMS – ongoing
- Kidney, muscle, liver, lung collected on T_{12}/W_0 or W_3
  - LFA, ELISA – conclusion of trial
  - LC-MSMS – ongoing

Sheep Tissues Results

Control, low dose, high dose 3 day withdrawal tested negative by ASAP, DESI-like, ELISA and lateral flow assay for all tissues.

Tissue	Med (ng/g)				High (ng/g)			
	ASAP	DESI	ELISA	LFA, +	ASAP	DESI	ELISA	LFA, +
Kidney	0.8 (1)	0	0.7	0	3.0 (3)	6.2 (1)	4.1	4
Liver	0	0	0.4 (1)	0	4.0	2.8 (1)	3.8	4
Lung	0	0	0.3 (1)	0	2.6 (1)	0	2.3	4
Muscle	0	0	0	0	8.5 (3)	5.2 (2)	2.2	2

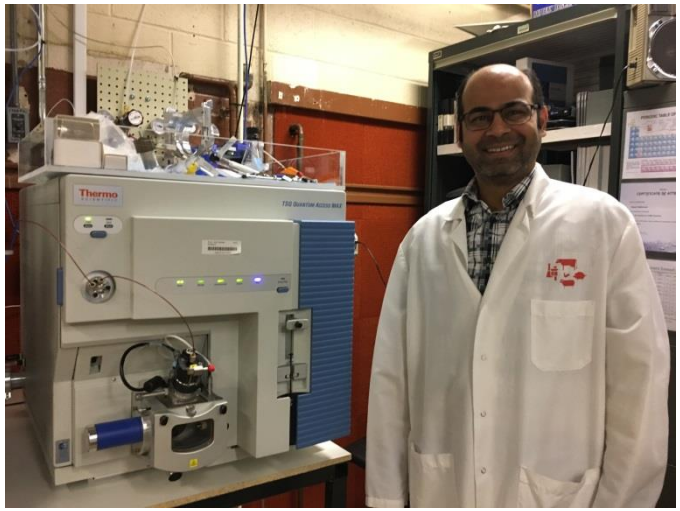
N=4 with the exception of those in parenthesis.

Sheep Urine Results

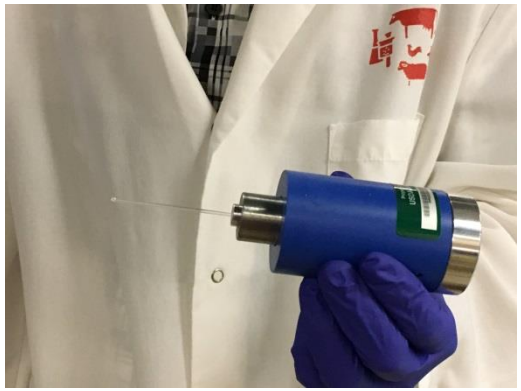
Day	Low		Med		High		High-3	
	ELISA ng/mL	LFA, +	ELISA ng/mL	LFA, +	ELISA ng/mL	LFA, +	ELISA ng/mL	LFA, +
T0	0	1	5.8	1	10.4	2	4.1	1
T2	4.6	3	20.6	3	318	4	188	4
T4	21.3	3	23.7	4	267	4	220	4
T6	4.7	2	18.3	4	311	4	263	4
T8	3.8	2	26.9	4	352	4	260	4
T10	3.8	2	28.8	4	345	4	213	4
T12/W0	2.6	1	16.3	4	267	4	244	4
W1							32.6	4
W2							5.5	2
W3							1.3	1

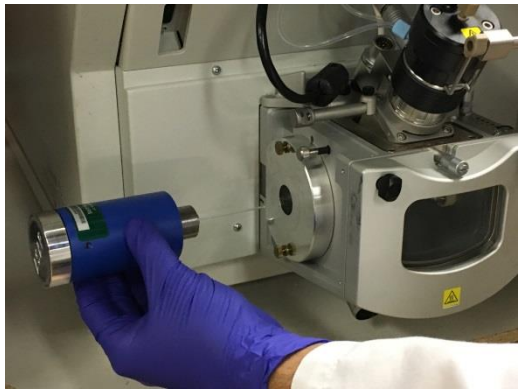
Instrumental Analysis (2)

Atmospheric
Solids Analysis
Probe (ASAP®)

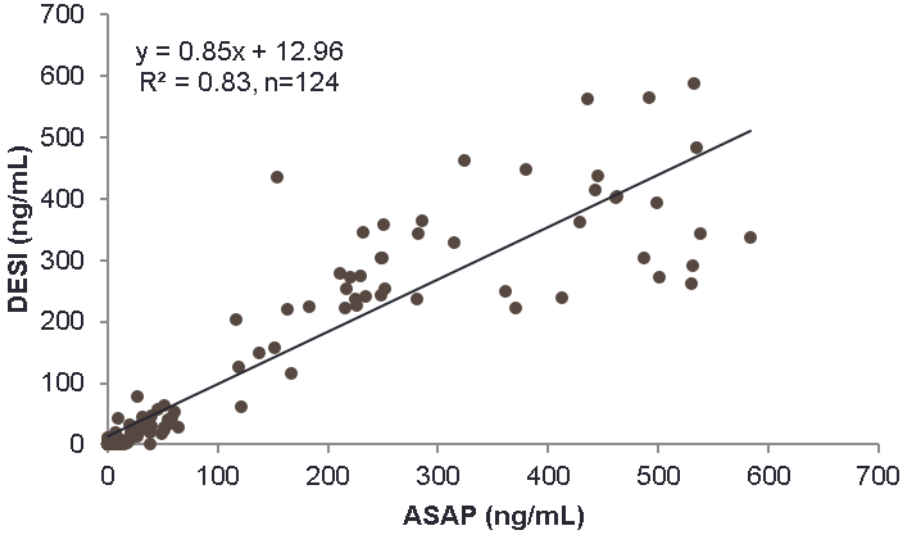


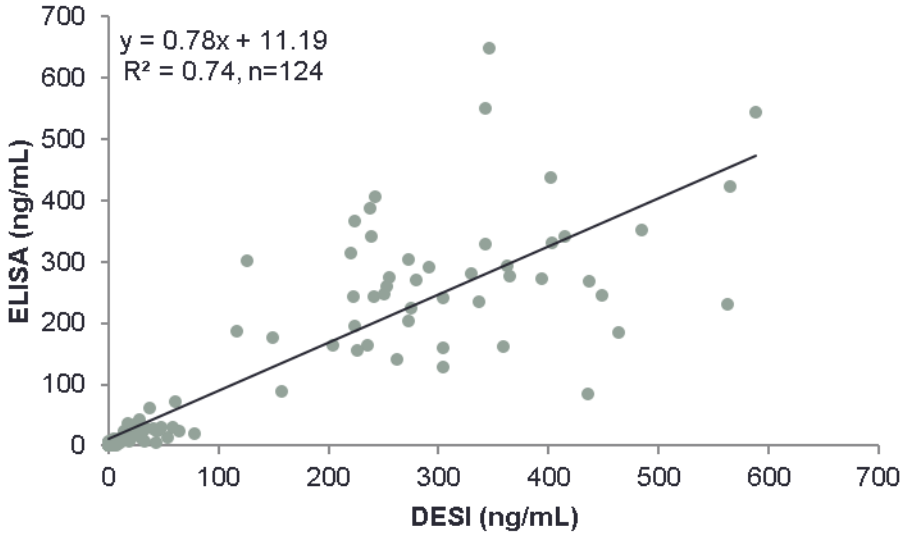
Desorption
Electrospray
Ionization
(DESI)-like

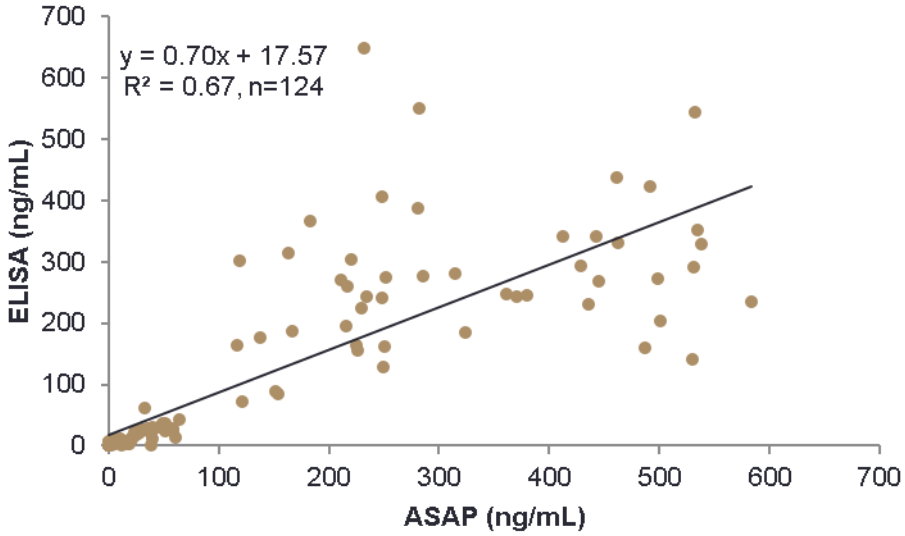




ELISA vs ASAP vs DESI-like







Conclusion

- Zilpaterol lateral flow assay has been developed and used to detect urine samples on-site with capability to run large numbers of samples in parallel.
- Assay time for lateral flow assay is near real time 10 min.
- Zilpaterol ELISA results correlate well with MS results
- Zilpaterol is minimally degraded when stored at -20^{o} C for prolonged period of time (~ 10 years).

Acknowledgements

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Thank you