Enantiomeric Separation of Clenbuterol as Analytical Strategy to Distinguish Abuse from Meat Contamination

CHINADA-WADA, October 18-19th 2017, Saskia S. Sterk

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Outline

- Introduction (very short)
- Hypothesis
- WADA project part 1
    - Methods and validation
- Proof of Principle part 1
- WADA project part 2
- Conclusion

- Outlook

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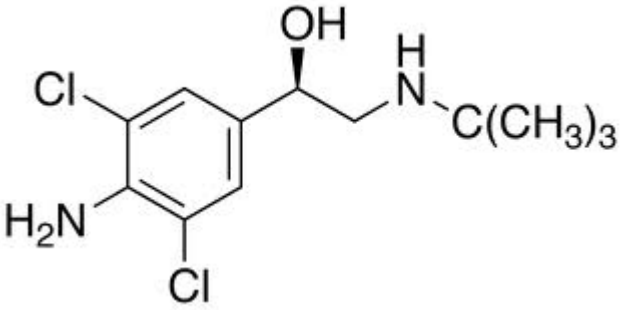
Introduction

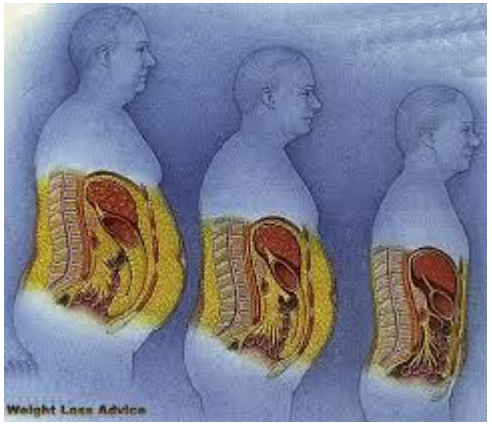


- Beta-2-agonist

- Therapeutically clenbuterol is mainly used in reversible airway obstructions such as bronchial asthma and pulmonary disease.

- Illegal use in animal husbandry as repartioning agent producing lean muscle meat

- Illegal use in sports



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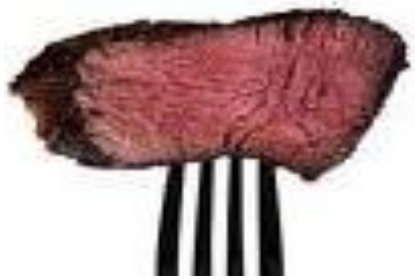
Introduction

- Several Clenbuterol cases

- 2010 positive Clenbuterol cases, athletes claimed meat as source



- 2011 warning on Clenbuterol contaminated meat from China and Mexico
- Need for discrimination abuse versus meat contamination

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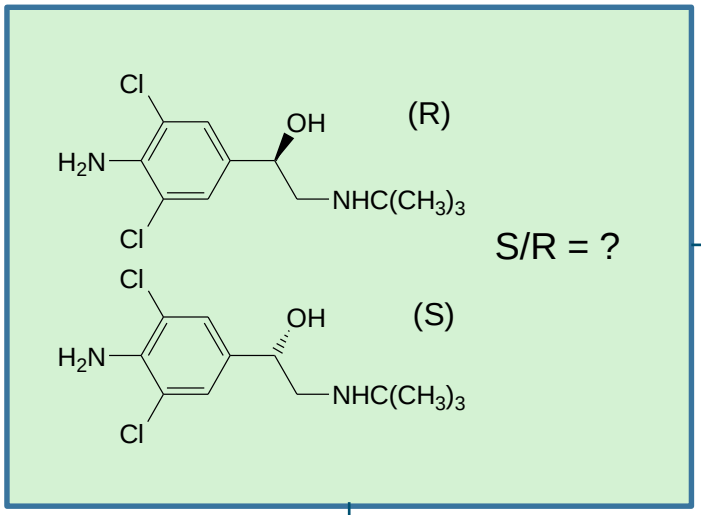
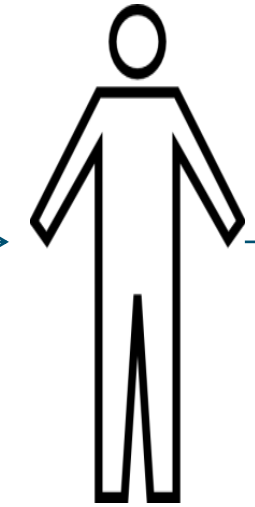
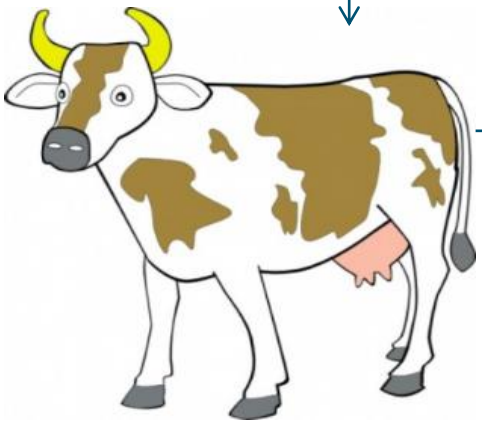
Hypothesis

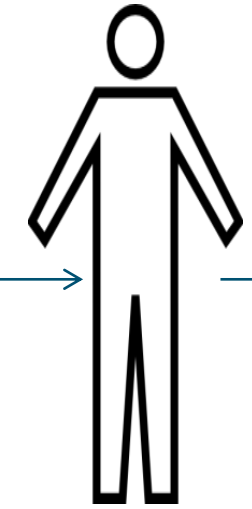




S/R = ?

S/R = ?





S/R = ?

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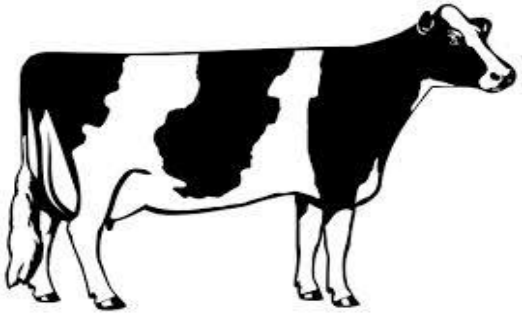
Methods and validation

Animal Experiment

14 Days

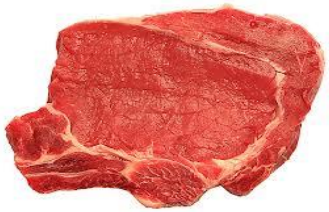
Control

Low dose



High dose

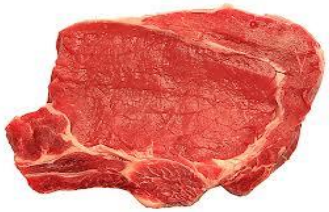
Milk



Milk

+

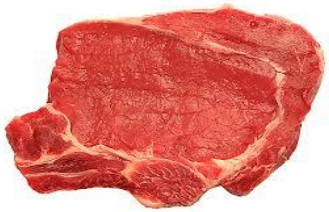
1.6 microgr/kg bw/d in 2 doses



Milk

+

3.2 microgrg/kg bw/d in 2 doses



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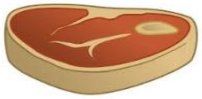
Methods and Validation

UHPLC-MS/MS for meat and bovine urine



1 gram meat

Extraction with acetonitril

2 ml urine

Extraction and de-glucuronidation with buffer

SPE with Stata-XC

Reconstitute in MeOH

LC-MS/MS
Chiral column
Astec Chirobiotic V2
Waters Xevo TQS

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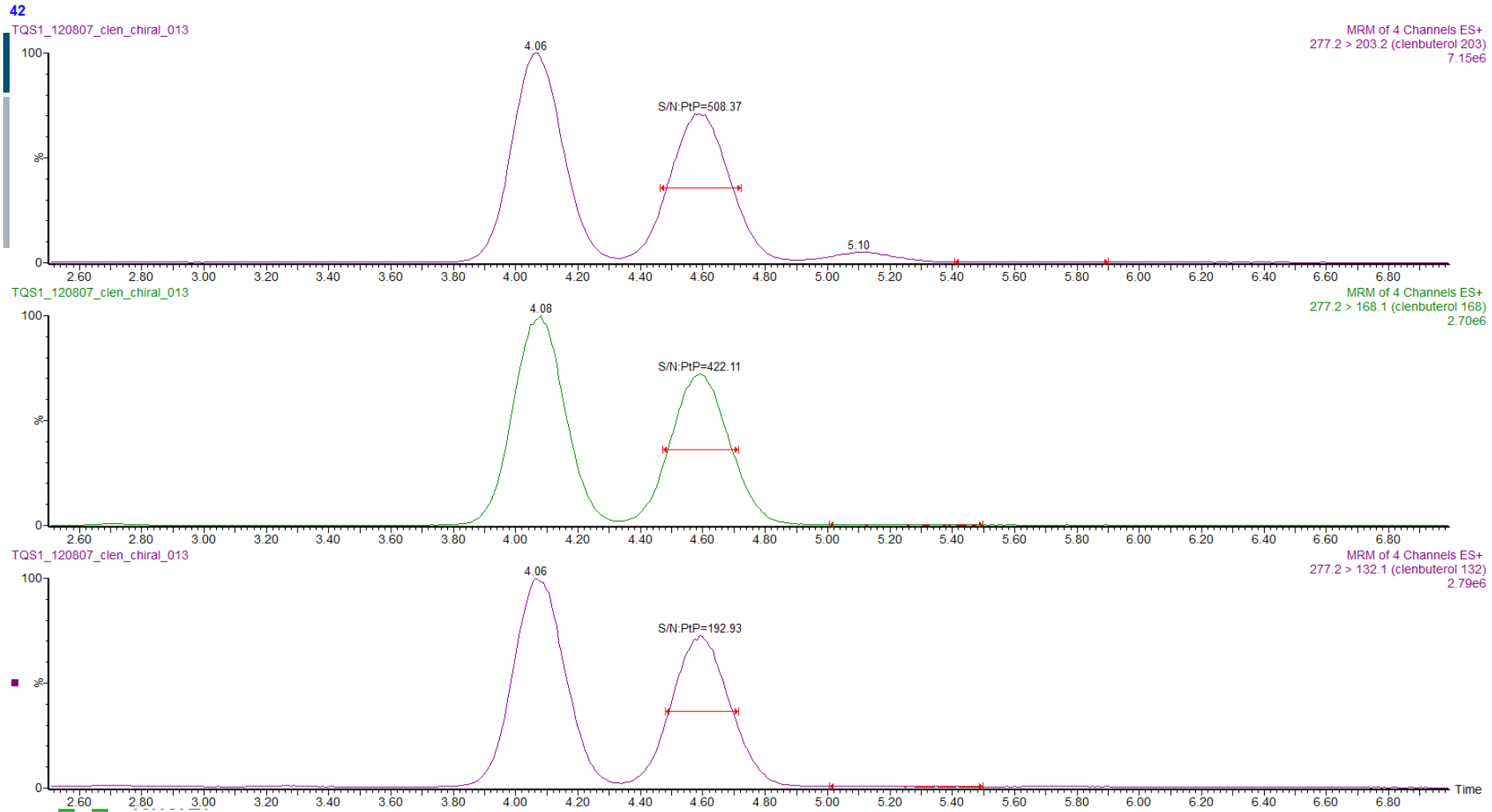
Methods and Validation

UHPLC-MS/MS for meat and bovine urine



Incurred meat +/- 0.5 microgram/kg clenbuterol

Methods and validation

SFC-MS/MS for human urine

Freie Universität Berlin

Methods and validation

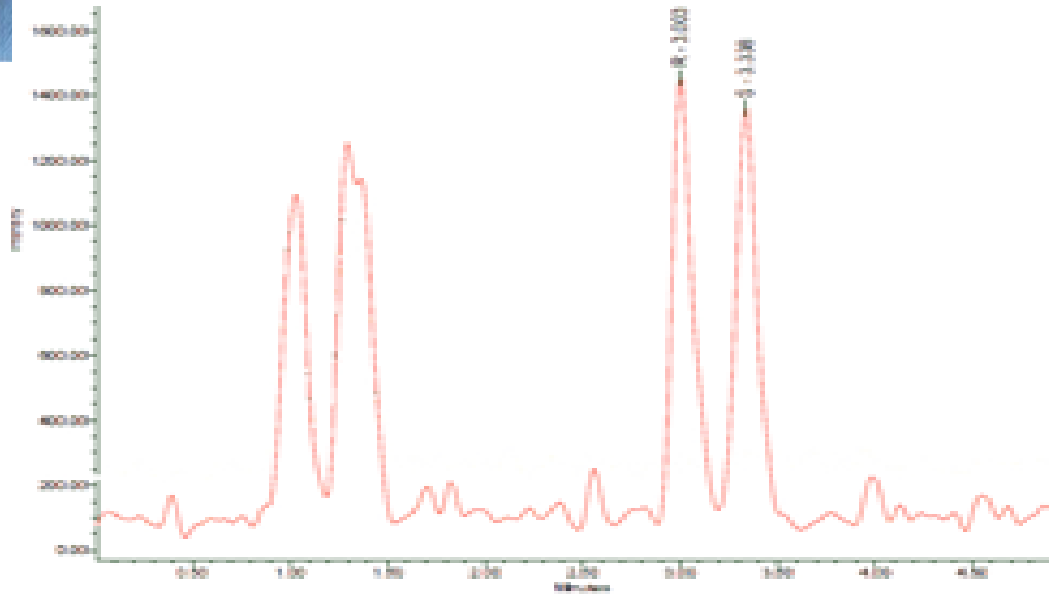
SFC-MS/MS for human urine

3 ml urine

Extrelut NT-3
Extraction 2 x 5
ml TBME

Reconstitution
in MeOH

UPC2-MS/MS
Chiral column
Astec Chirobiotic V2
Waters Acquity TQD



200 pg/ml spike in human urine with SFC-MS/MS

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Methods and Validation

	Bovine meat		Bovine urine		Human urine		
	S(+)	R(-)	S(+)	R(-)	S(+)	R(-)	
Accuracy	97.1%	99.5%	99.2%	97.7%	97.4%	94.1%	
CCalfa ng/ml	0.02	0.02	0.01	0.02	3.9	3.5	LOD pg/ml
CCbeta ng/ml	0.05	0.05	0.03	0.04	14.5	13.2	LOQ pg/ml
Reproducibility	6.3%	3.3%	2%	3.6%	22.6%		
Repeatability	3%	2.8%	1.5%	2.2%	8.6%		
Measurement Uncertainty	11.2%	8.8%	6.6%	9.3%	-	-	

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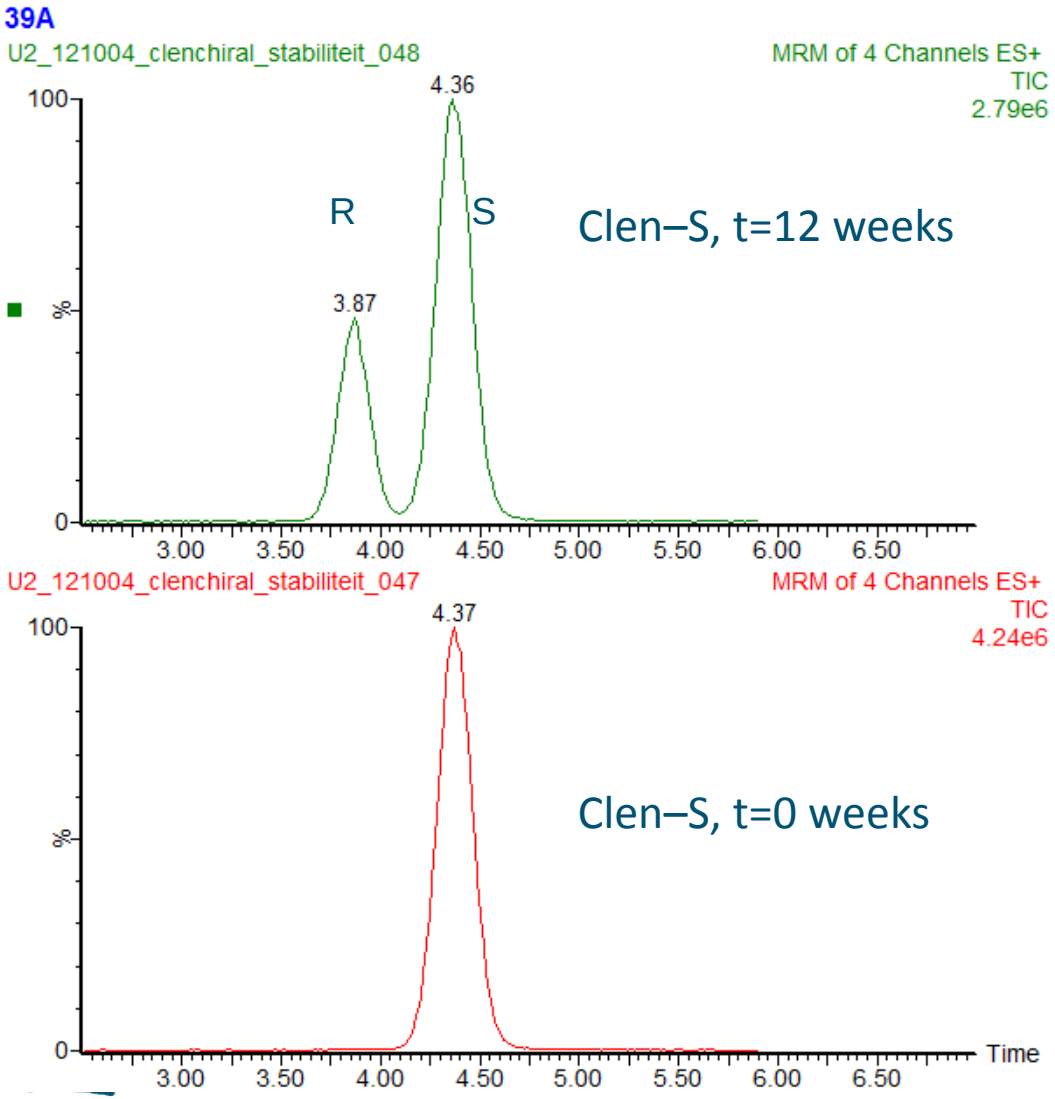
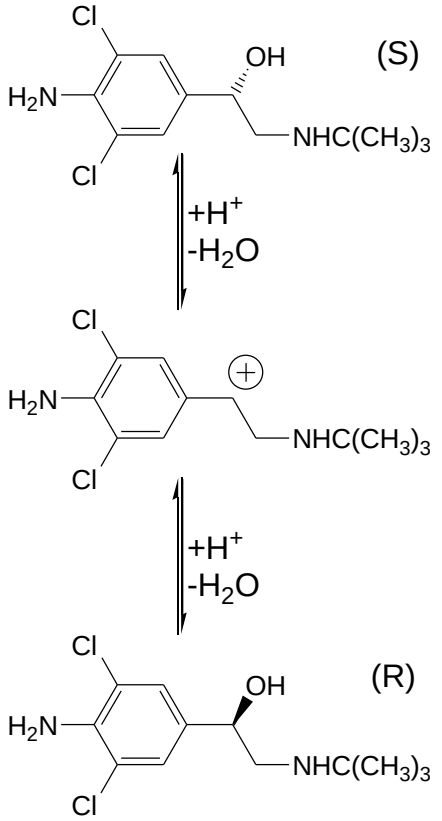
Stability Studies, acetic pH 1, 37°C





starts after 7 days at 37°C

stable at all other tested conditions

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Proof of Principle

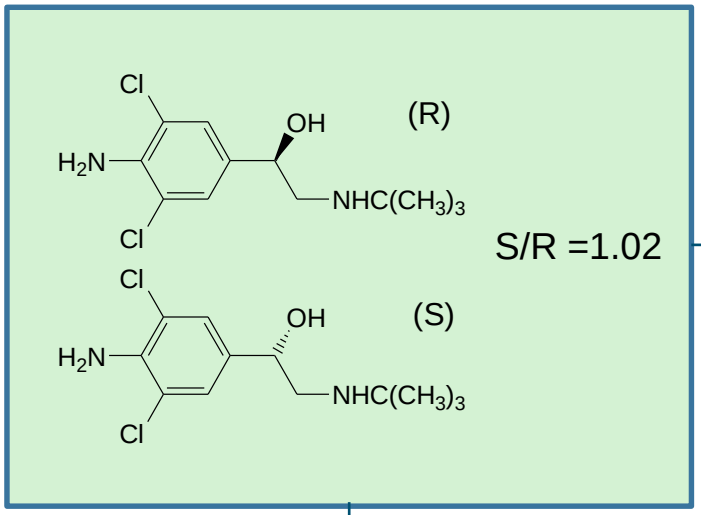
S/R Ratio in Preparations

- S/R Ratio Ventipulmin Syrup 1.04
- S/R Ratio Ventipulmin Injection 1.03

- S/R Ratio Spiropent tablets 1.02

- Mean S/R preparations 1.02+/-0.02

Hypothesis



S/R =1.02

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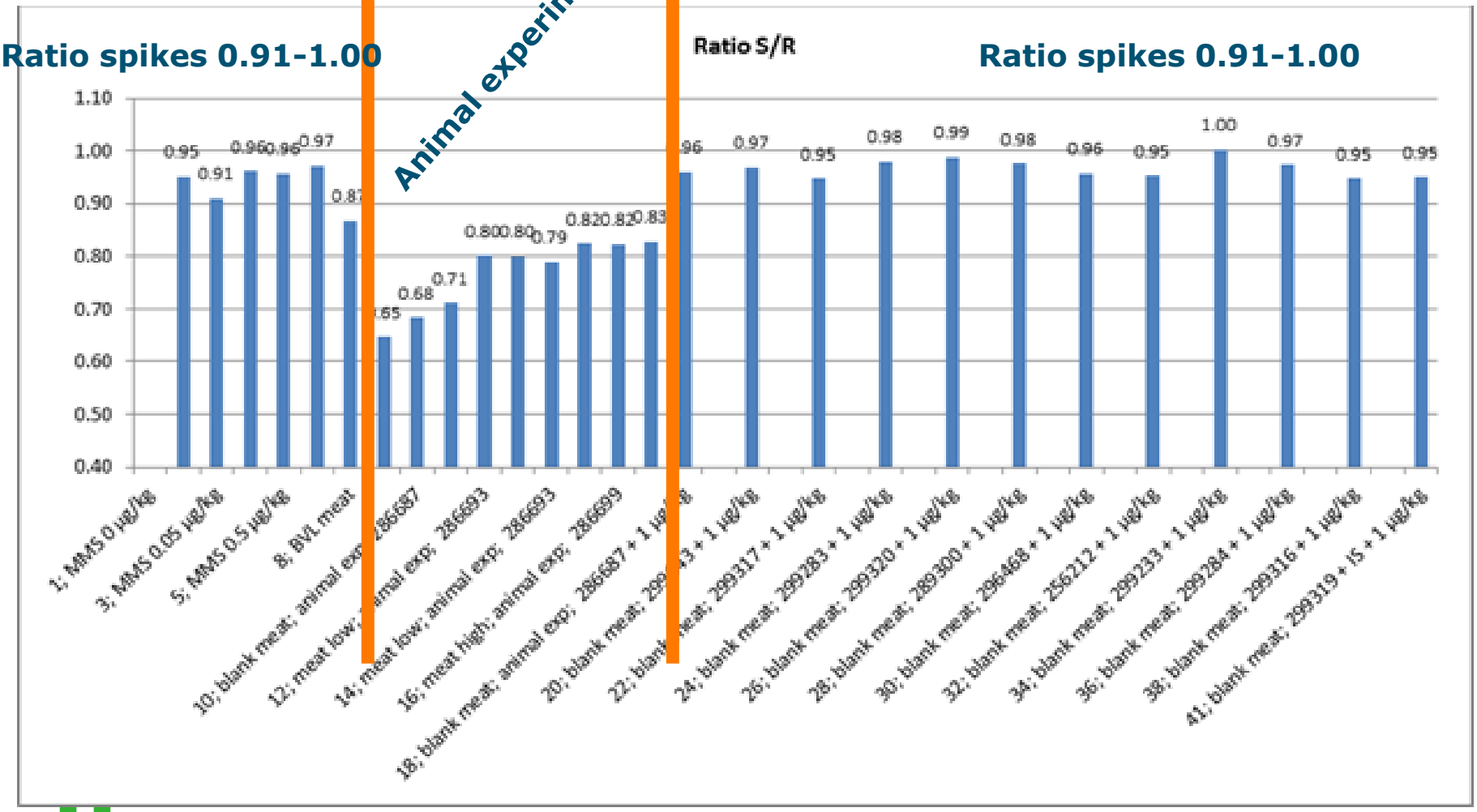
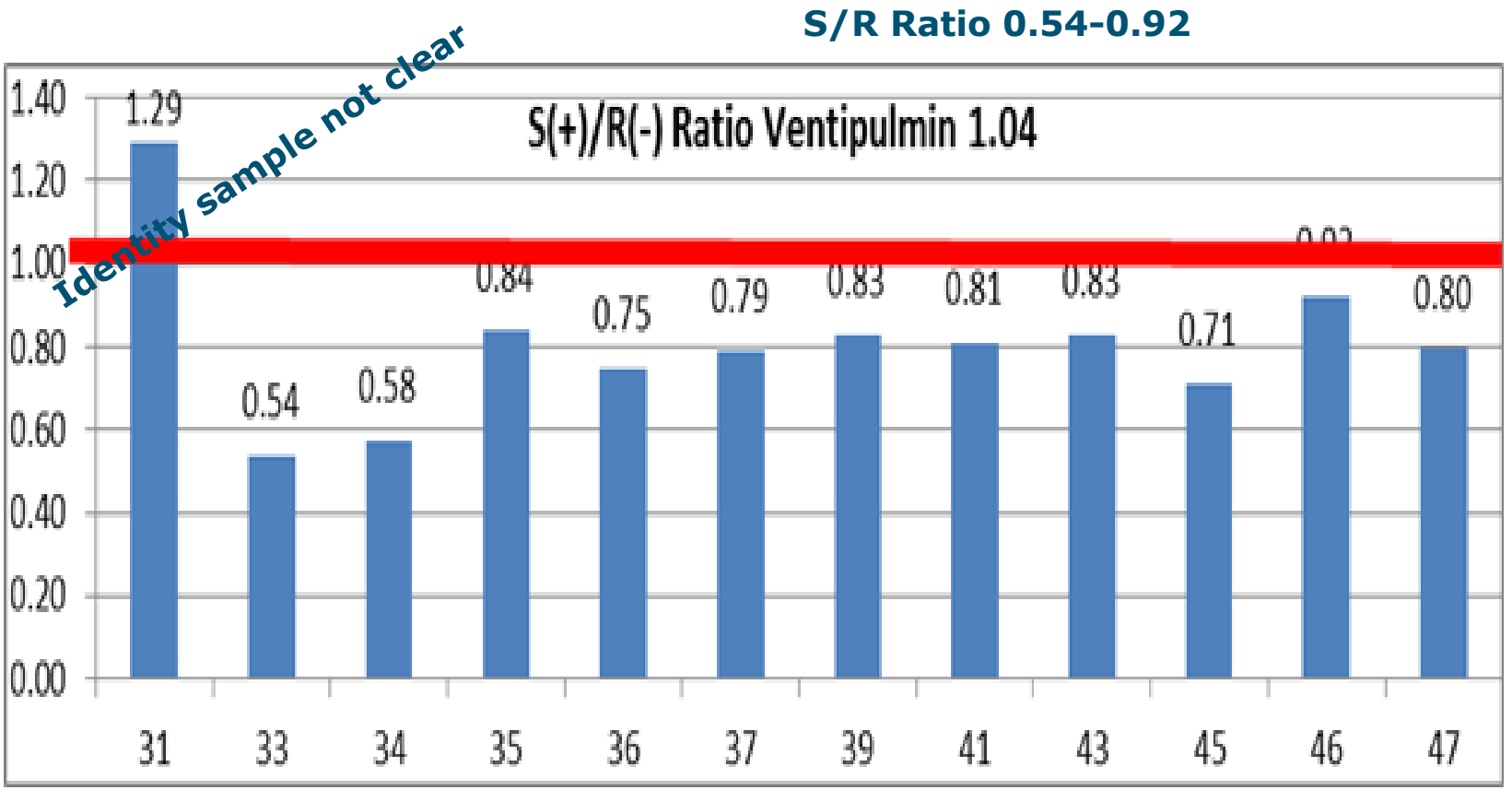
Proof of Principle

S/R Ratio in raw meat



Proof of Principle

S/R Ratio in prepared meat



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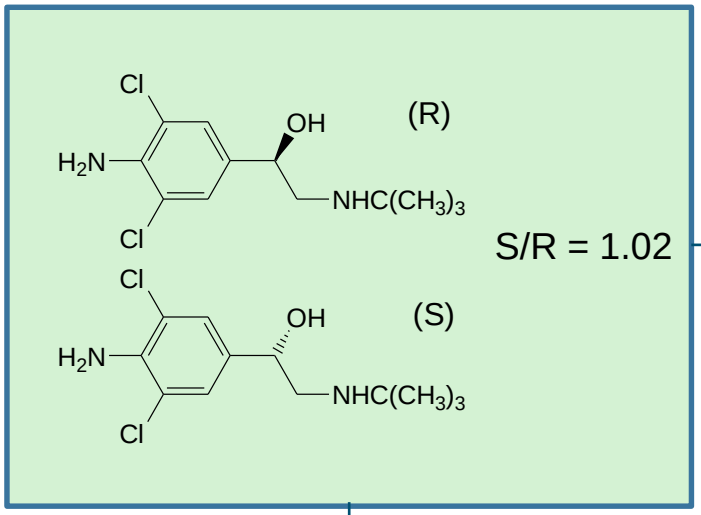
Hypothesis



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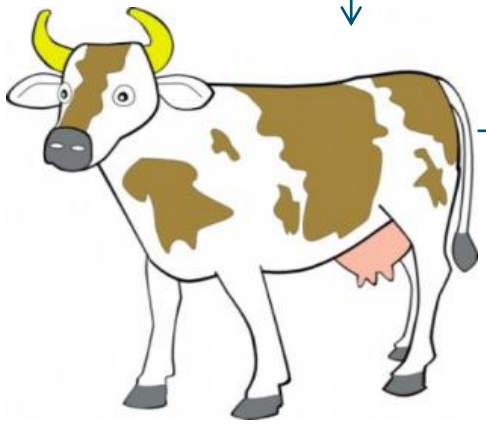

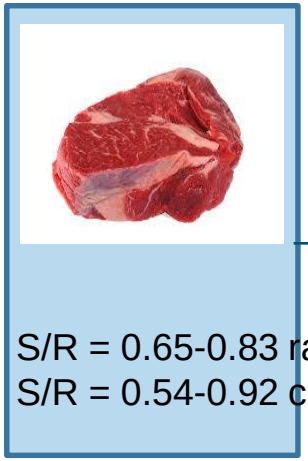

S/R = 0.65-0.83 raw meat
S/R = 0.54-0.92 cooked meat

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Proof of Principle

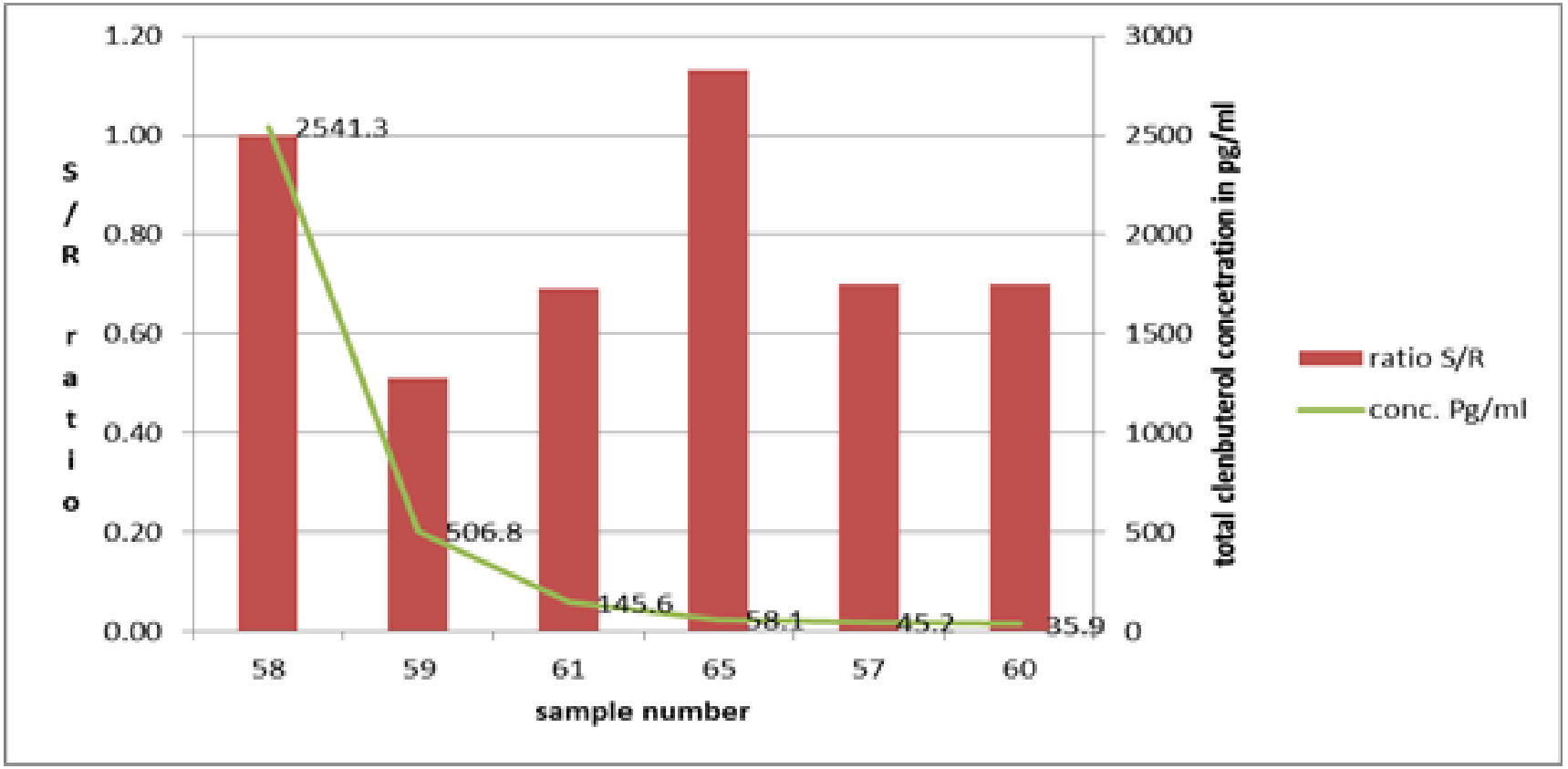
S/R Ratio in human urine

- S/R Ratio of administration trial with Clenbuterol
  - 0.97±0.02 (mean±sd).(n=108, Lausanne)

- S/R Ratio suspected food contamination samples of human urine
  - 0.51-1.13 (mean 0.79). (n=6, Cologne)

Proof of Principle

S/R Ratio in human urine



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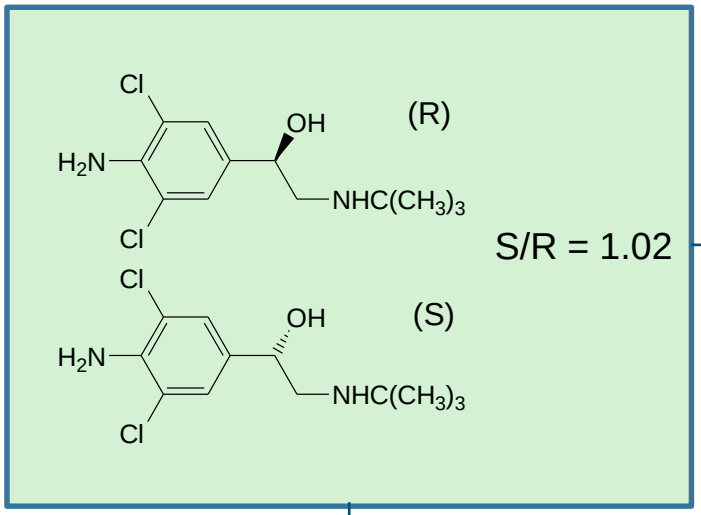
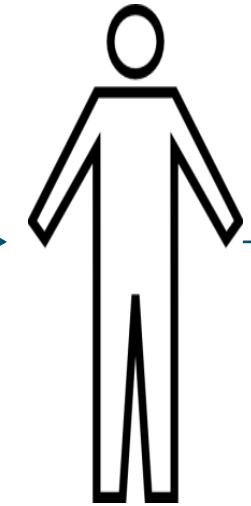
Hypothesis



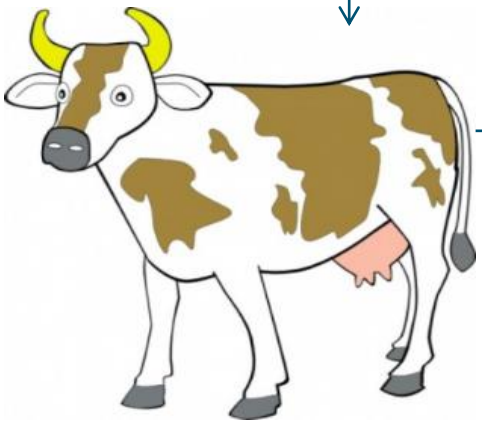
S/R = 1.02



S/R =0.97

S/R =0.51-1.13

S/R = 1.02



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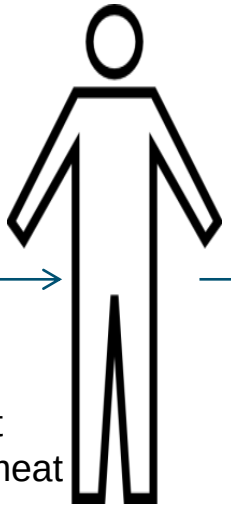

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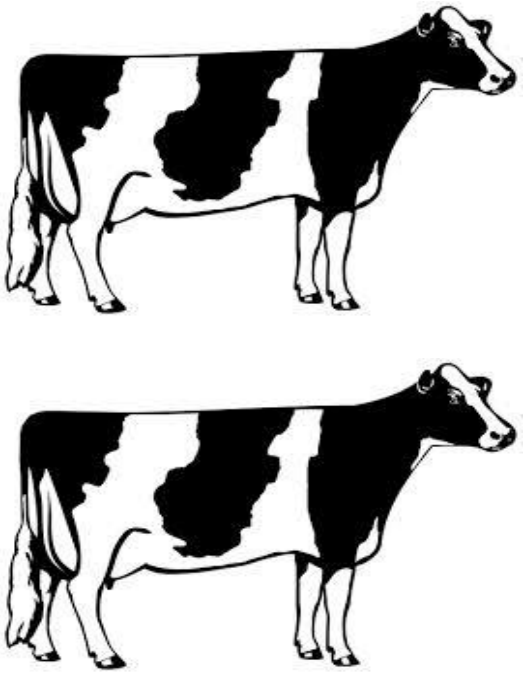
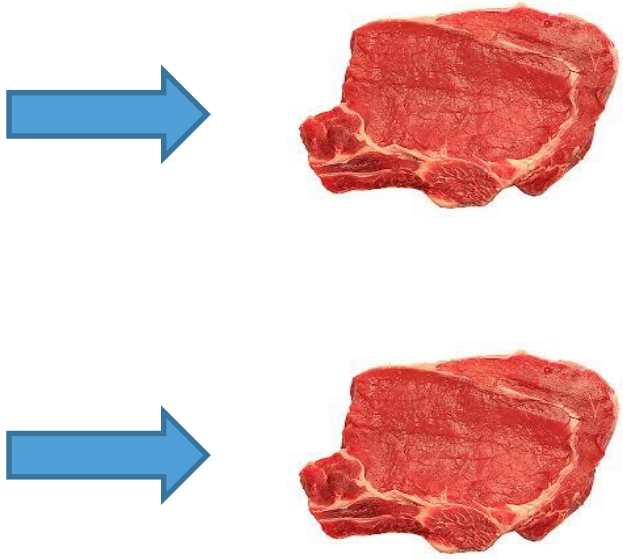
WADA project part 2

Animal trial 2, production of meat for controlled human volunteer experiment





Dose 5 mg/kg bw 2x /day

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Human trial Experimental Set-up

Source of Clenbuterol	Number of volunteers	Concentration of Clenbuterol	Administered total amount of Clenbuterol	Proportion S-Clenbuterol
Muscle	4	1,67 µg/kg	0,84 µg	0,509±0,006
Liver	4	41,79 µg/kg	8,36 µg	0,635±0,004
Spiropent® tablet	4	-	≈ 17,7 µg	0,499±0,001
R-Clenbuterol reference standard	1	-	1,0 µg	0,024
S-Clenbuterol reference standard	1	-	1,0 µg	0,997

Table 1: Overview of the Clenbuterol sources administered (mean±95% CI)

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Human trial Sampling

Urine Sampling Clenbuterol

Code: T
Height: 1,85 m.
Sex: male
Ingestion of: Clen-S
Time of ingestion: 12.30

Age: 34
Weight: 75 kg
Date of ingestion: 20-1-2015

Day/Date	Sample number	Time of urination	Time of previous urination	Urine volume
Day 0 1	1	10.25	7.00	200 ml
2	2	13.15	10.25	350 ml
3	3	22.00	17.00	350 ml
Day 1 1	4	6.30	23.30	400
2	5	10.30	9.00	250
3	6	16.00	10.30	250
4	7	20.15	16.00	200
Day 2 1	8	6.30	23.15	450
2	9	10.00	6.30	250
+ 3	10	17.30	10.00	350
+ 4	11	23.00	17.30	350
Day 3	12	7.00	23.00	250
	13	12.00		350
	14	23.30	18.30	200

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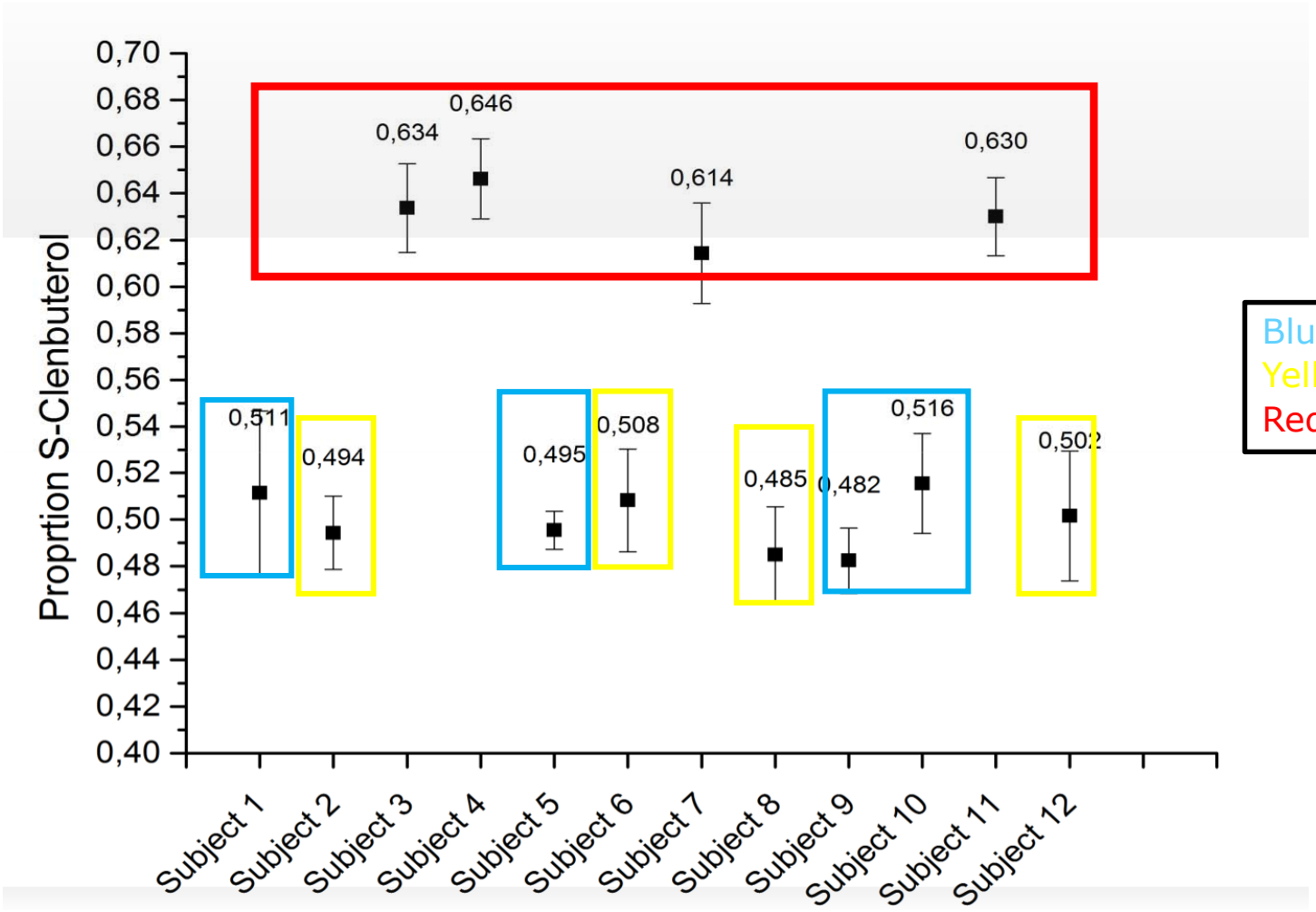
Results controlled Human volunteer experiment (1)



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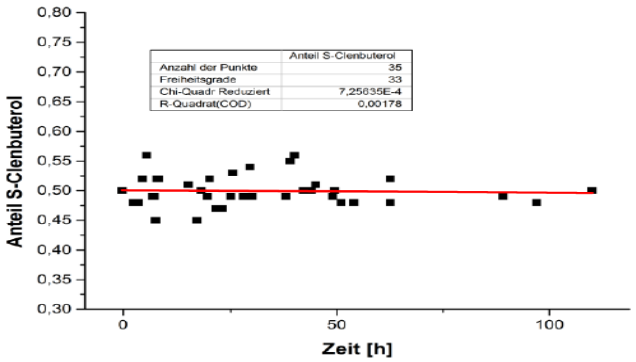
Results controlled Human volunteer experiment (2)

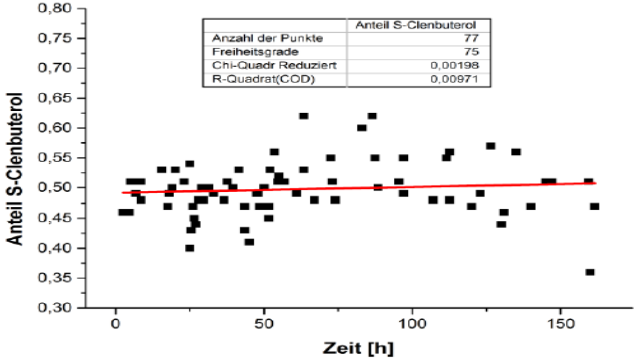
- Distinction based on the proportion of S-Clenbuterol is **possible**:
  - between **liver** (0.635±0.004) and **Spiropent®** tablet (0.499±0.001) respectively **meat** (0.509±0.006).
- Distinction between **meat and Spiropent®** tablets is **not possible** based on the enantiomeric composition, due to the reason that the ingested proportions of S-Clenbuterol are too close to the Spiropent® tablets.

Results controlled Human volunteer experiment (3)

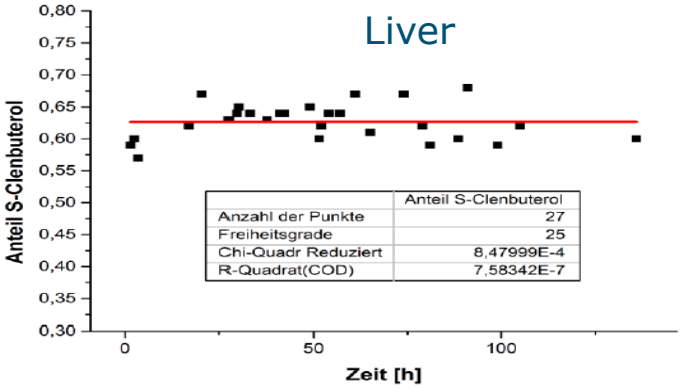
Meat



Tablet



Liver



S-clenbuterol proportion is stable during excretion

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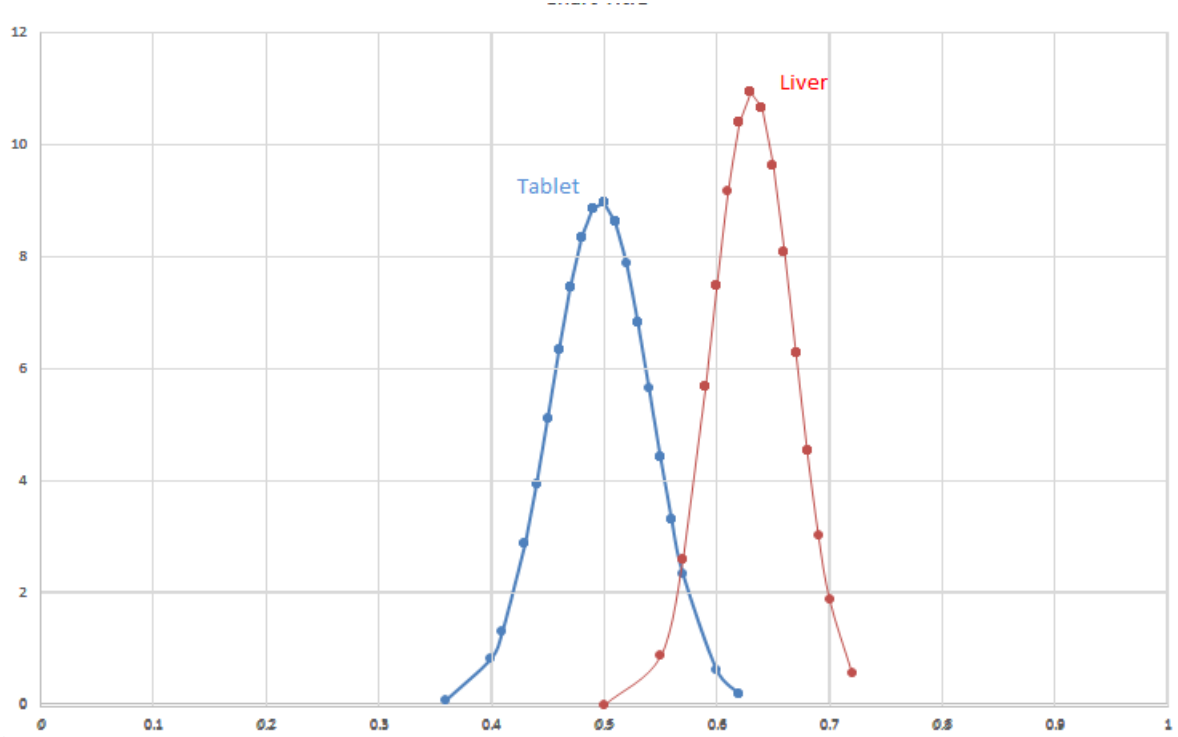
Decision model



The proportion of S-clenbuterol in which 95% of results of the tablet samples were detected.
Based on the analytical variation in this population a cut-off of 0.59 (upper value) and 0.41 (lower value) is proposed.
Out of this range it is unlikely that the source of clenbuterol was a (prohibited) intake of a preparation containing (racemic) clenbuterol (error used was 5%).

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Conclusion

- Sensitive and selective analytical methods were developed using UHPLC-MS/MS and SFC-MS/MS for meat, bovine and human urine and preparations.
- The tools were used on real samples provided by anti-doping laboratories.
- Pharmaceutical preparations showed a stable S/R ratio around 1.02 for different prepartions
- Meat from treated animals contained a different S/R ratio for clenbuterol. S/R ratio 0.54-1.15 (Mexico+WADA projects).
- Urine samples presumed to be contaminated via meat S/R clenbuterol ratio were lower (0.42-1.38) than the S/R clenbuterol ratio from humans having consumed clenbuterol (0.75-1.63) in an administration trial (WADA part 1).

Conclusion 2

- Ratios in meat after different treatment of bovine animals not constant
- Ratio in liver different then in meat
- Ratio between species also different (Literature, Chinese study)
- Statistical model work in progress, based on concentrations and ratio

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Alexander Schmidt

Maria Parr

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And you for your attention.....

Questions?

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