First experiences with GC-C-IRMS to detect natural hormone abuse in cattle

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Outline

- Introduction
- GC-C-IRMS for hormone analysis
- Performance characteristics
- Case study: estradiol and testosterone administration
- Conclusions

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Introduction

- RIKILT: food safety research institute within Wageningen UR
- Official control laboratory for NVWA
- EU reference lab for residues in food of animal origin: sedatives, mycotoxins, **hormonal growth promoters**
- GC-C-IRMS installed this year for 'doping control' in cattle
- Distinguish urine samples treated and non-treated animals
- First goal: set up methods for estradiol and testosterone
  - Development of efficient method
  - Testing performance of system
  - Selection of suitable ERCs

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GC-C-IRMS for hormone analysis

Endogenous pathway

Exogenous pathway

Feed (C3/C4) → Cholesterol ← Acetate

↓

Pregnenolone

↓

DHEA

↓

$(^{13}C/^{12}C)endo$

Synthesis from plant sterols (C3)

↓

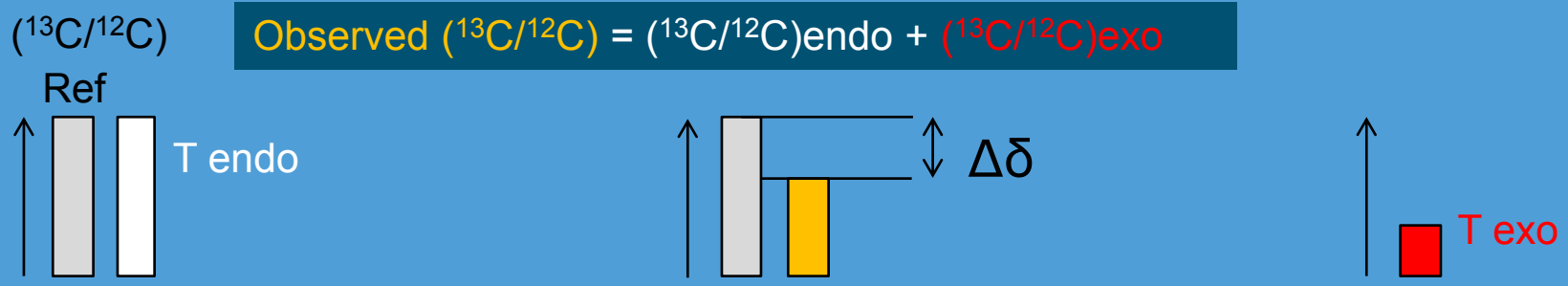
Testosterone esters

↓

$(^{13}C/^{12}C)exo$

Testosterone and metabolites

Observed $(^{13}C/^{12}C)$ = $(^{13}C/^{12}C)endo$ + $(^{13}C/^{12}C)exo$



$\Delta\delta^{13}C = \delta^{13}C_{ERC} - \delta^{13}C_{TC}$

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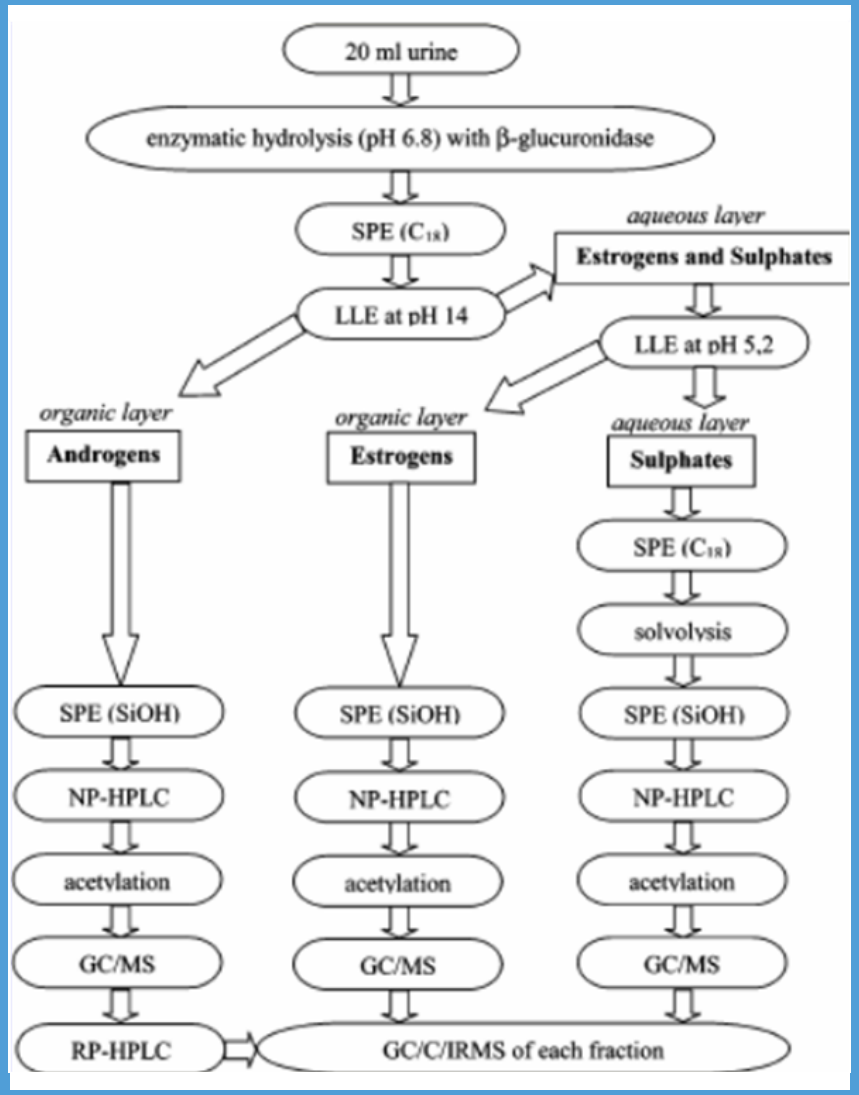
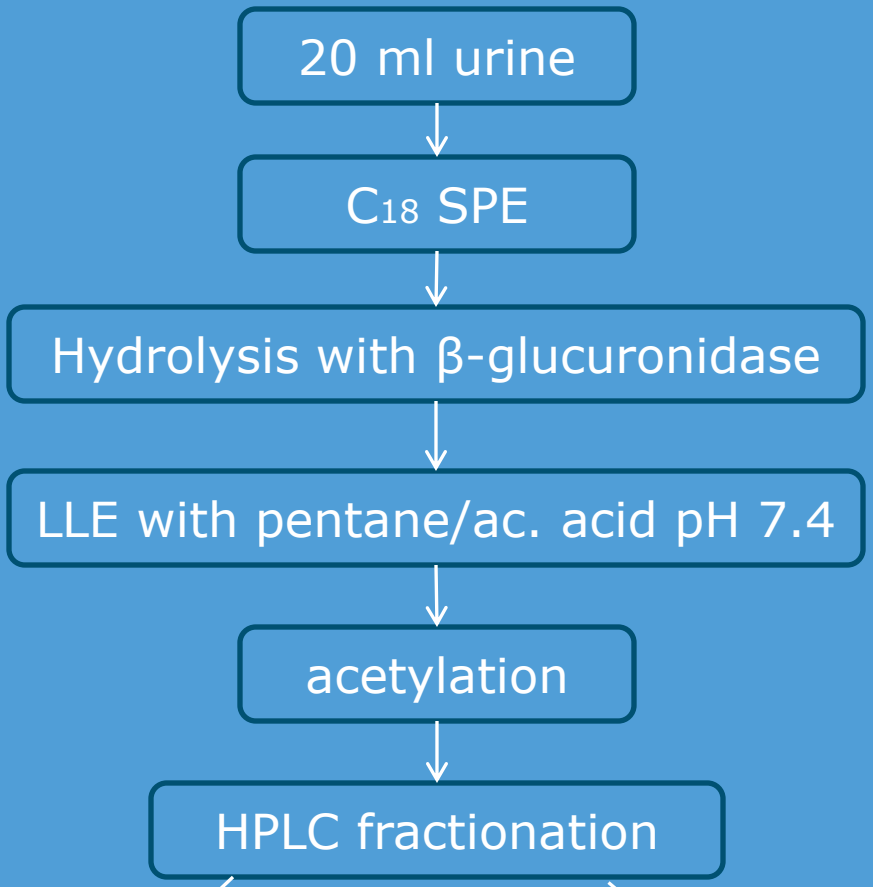
'Classical' sample clean-up



Hebestreit et al., 2006

Humane doping method (van Renthergem, 2012)



5α-androstendiol
5-androstanediol
pregnanediol

α-testosterone
β-testosterone
α-estradiol
β-estradiol
ethiocholanolone
DHEA

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Determine performance characteristics

- Reproducibility standard alkane mixture
- Calibration curve from 0-60 ng (absolute on-column) randomly injected in splitless injection mode
- Linearity determined for the different steroids
- Determination concentration dependence of delta value
- Lowest amount that can be detected within 3 st dev

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Schimmelmann alkane mixture

Alkane	δ Measured	δ Expected	Difference (‰)	Stdev (‰)
C17	-31.24	-31.16	0.08	0.05
C19	-33.02	-33.16	-0.14	0.10
C21	-29.20	-29.10	0.09	0.37
C23	-31.83	-31.76	0.07	1.02
C25	-28.42	-28.48	-0.06	0.24

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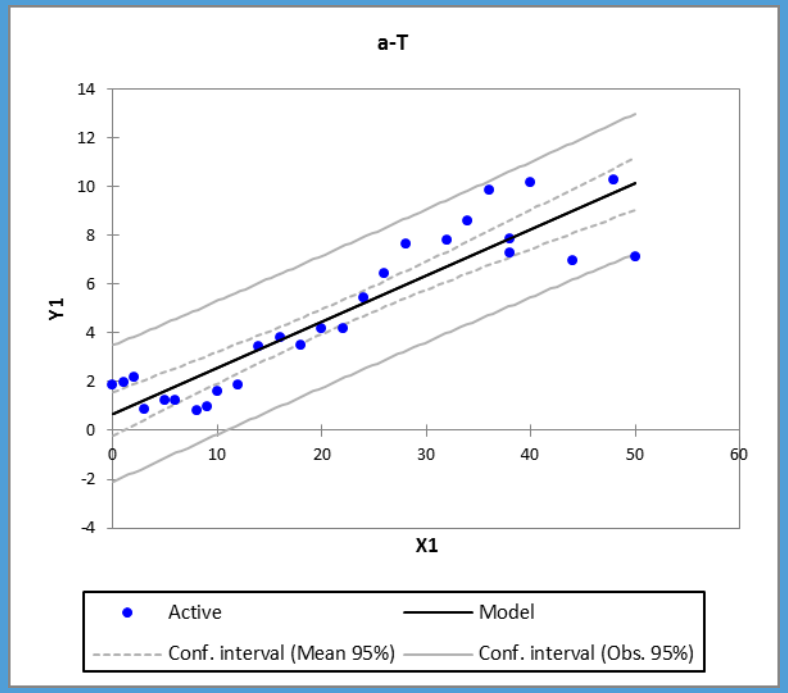
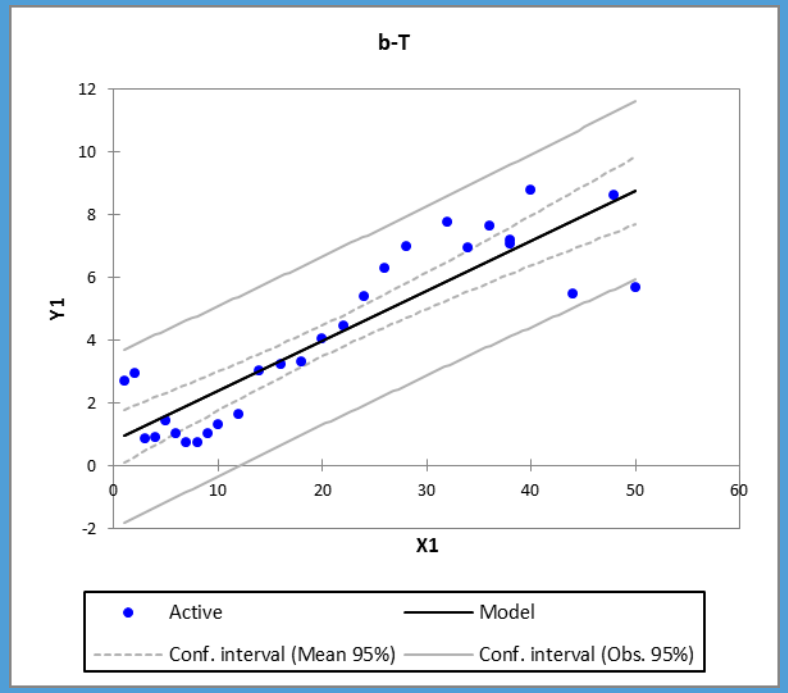
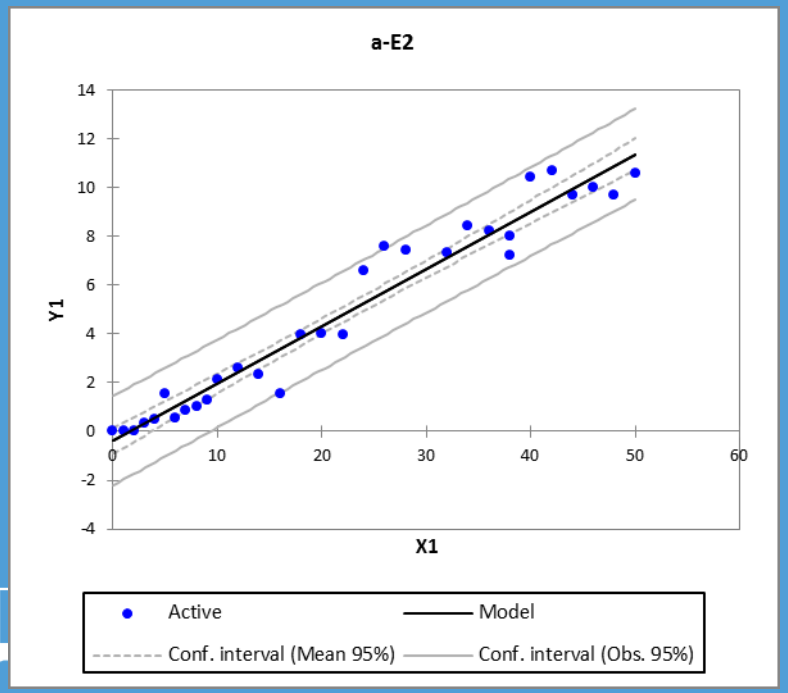
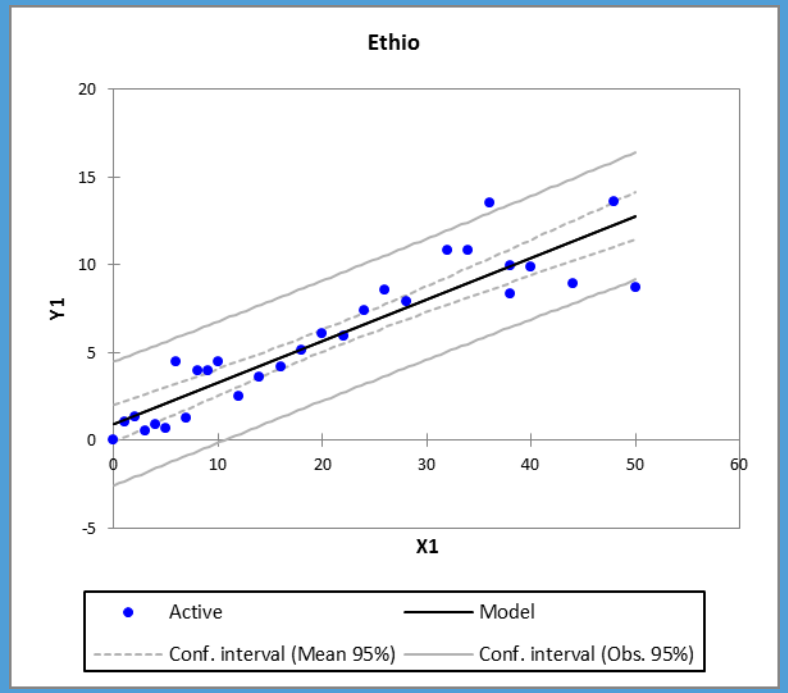

a-T
Y1
X1
Active
Model
Conf. interval (Mean 95%)
Conf. interval (Obs. 95%)


b-T
Y1
X1
Active
Model
Conf. interval (Mean 95%)
Conf. interval (Obs. 95%)


a-E2
Y1
X1
Active
Model
Conf. interval (Mean 95%)
Conf. interval (Obs. 95%)


Ethio
Y1
X1
Active
Model
Conf. interval (Mean 95%)
Conf. interval (Obs. 95%)

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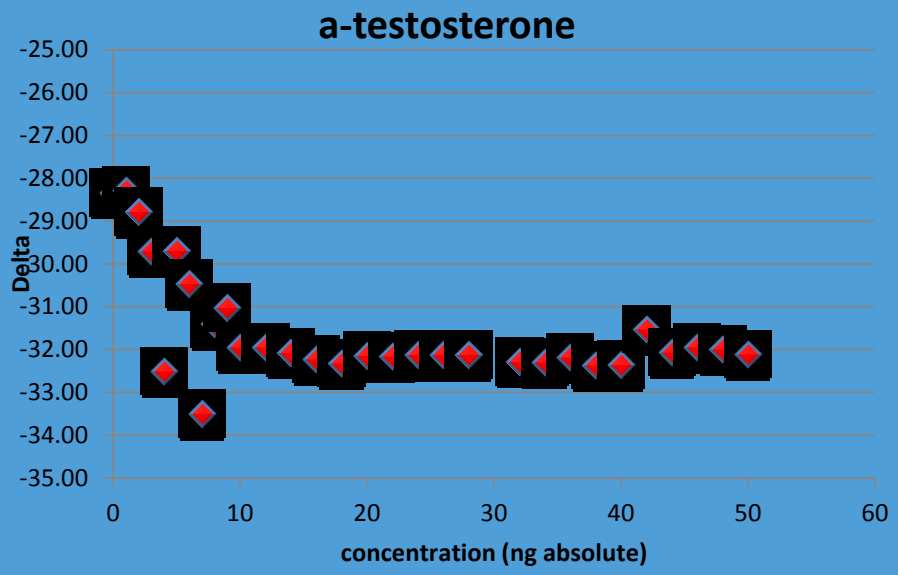
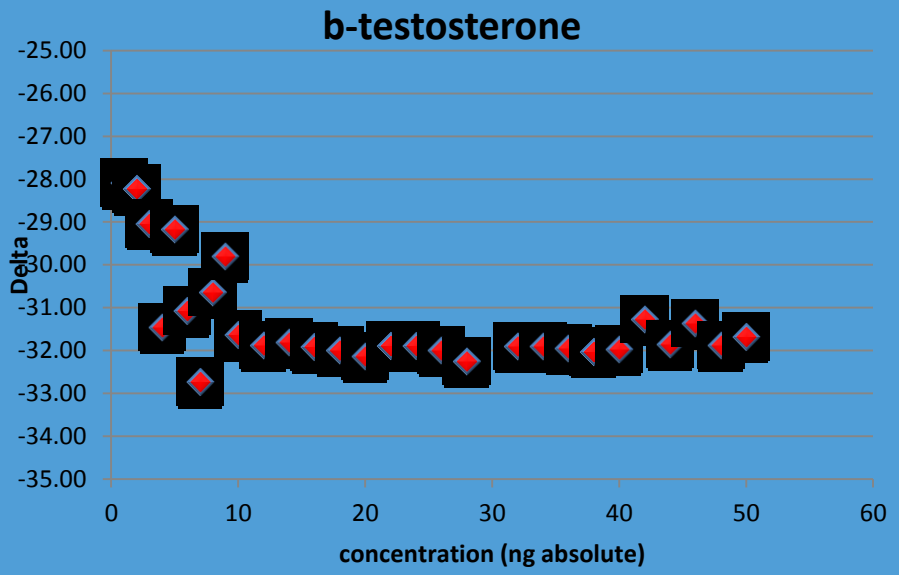
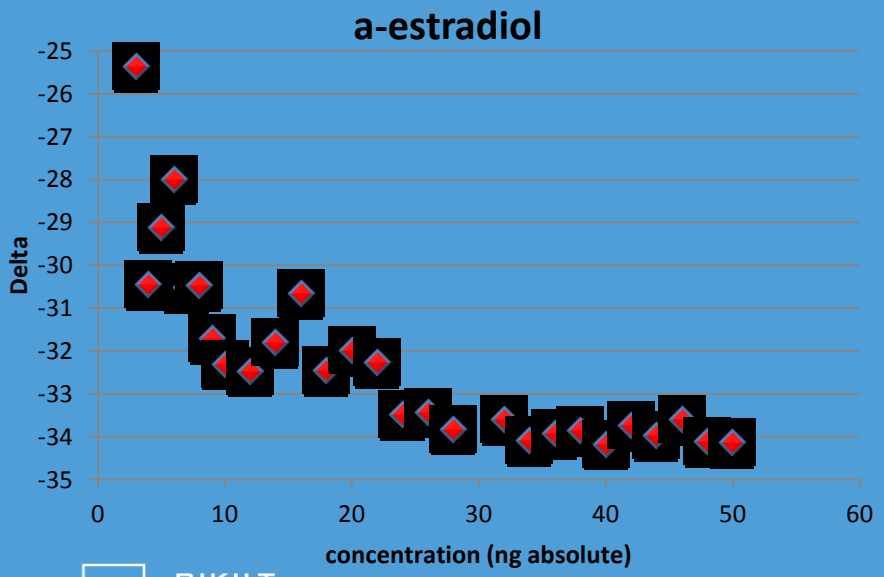
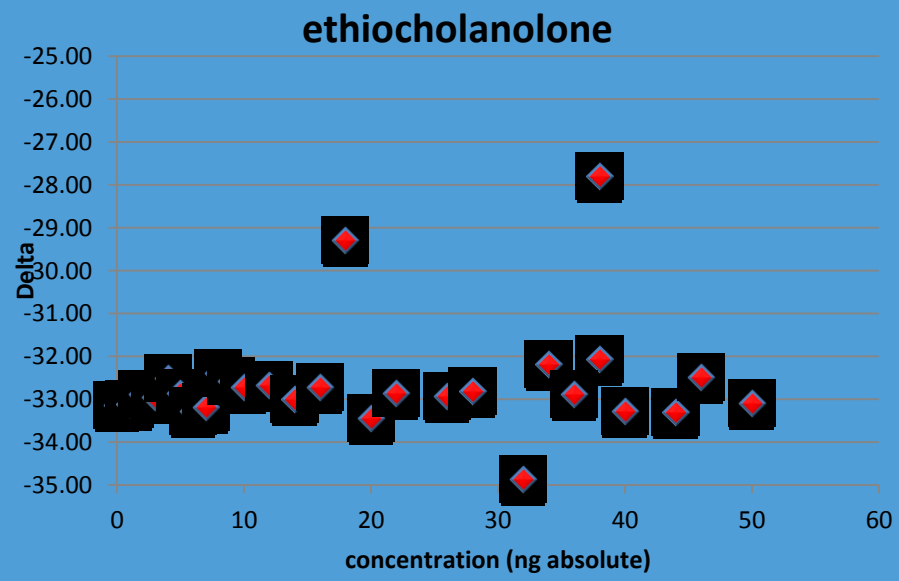
Concentration vs. delta value









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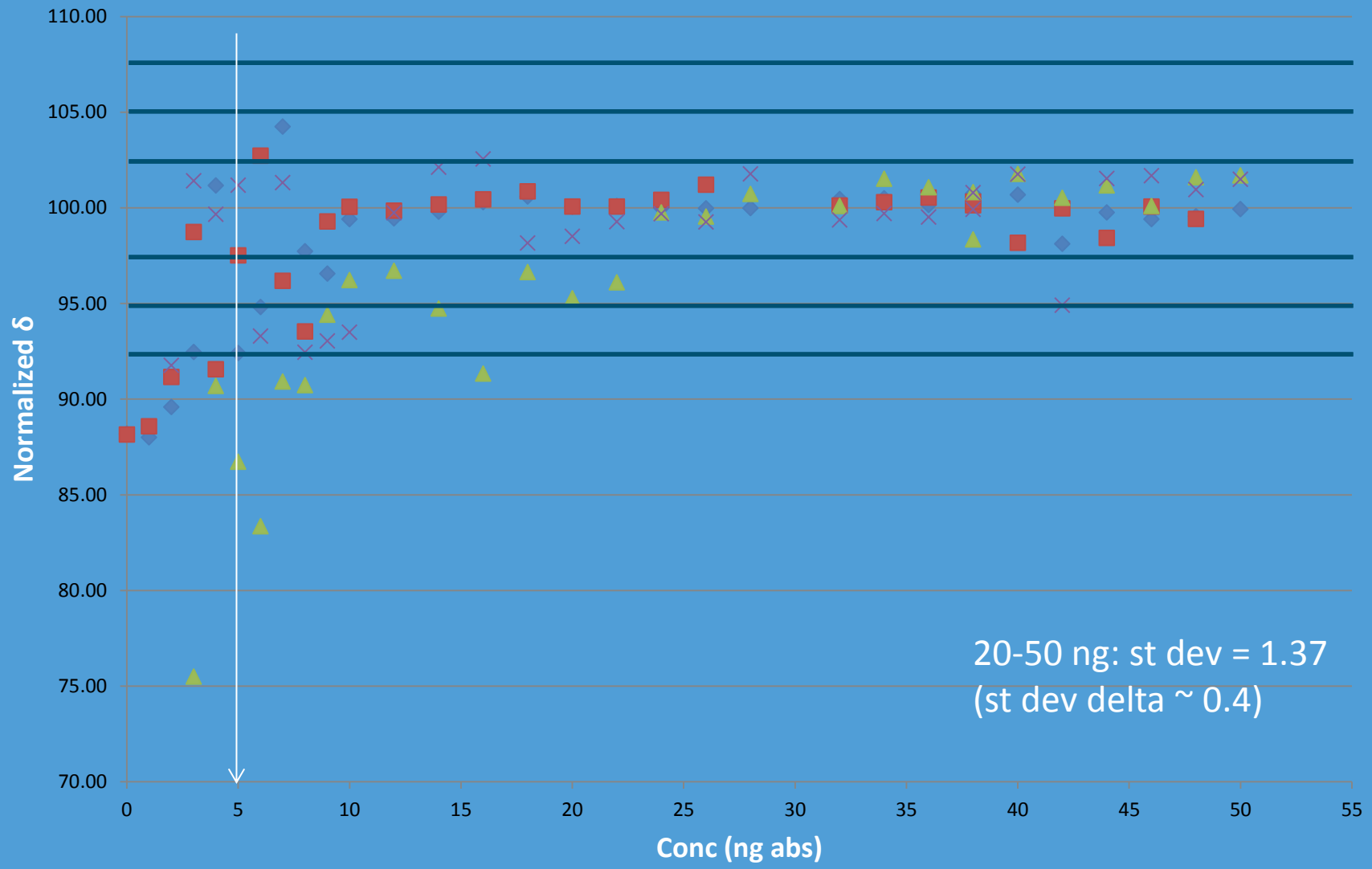
Normalized values: $\delta_{measured}$/average $\delta_{20\text{-}50\ ng}$



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A case study: E2 and T administration

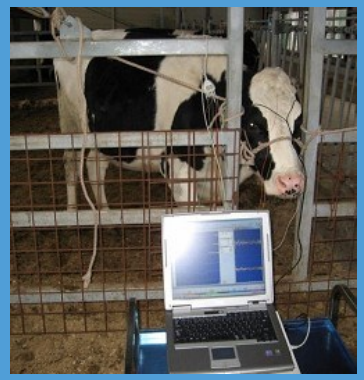
- Two sample series processed with humane doping method
- Series contained standards, samples from treated animals and a blank population
- Results of δ and δΔ shown for estradiol and testosterone

Source: Sonnon, 2008

Overview samples studied

- Cows and bulls from Dutch farms 2008/2009 – blanks

- Animal experiment cow injected with estradiol-benzoate



- Animal experiment bull injected with testosterone-cypionate

- Standards

- Spike to water

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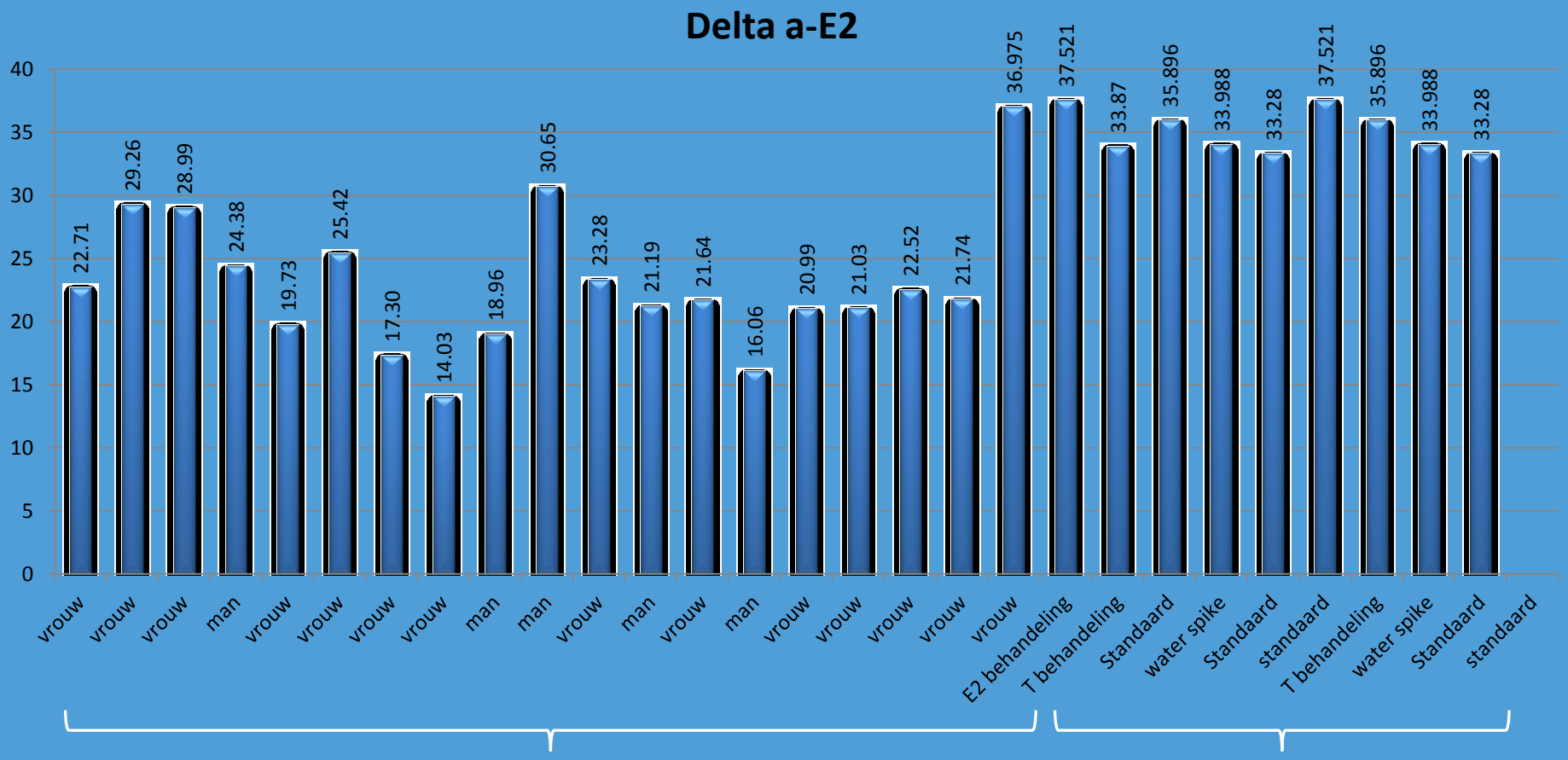
Delta values measured for a-E2



Reference population

Treated animal
+ standards

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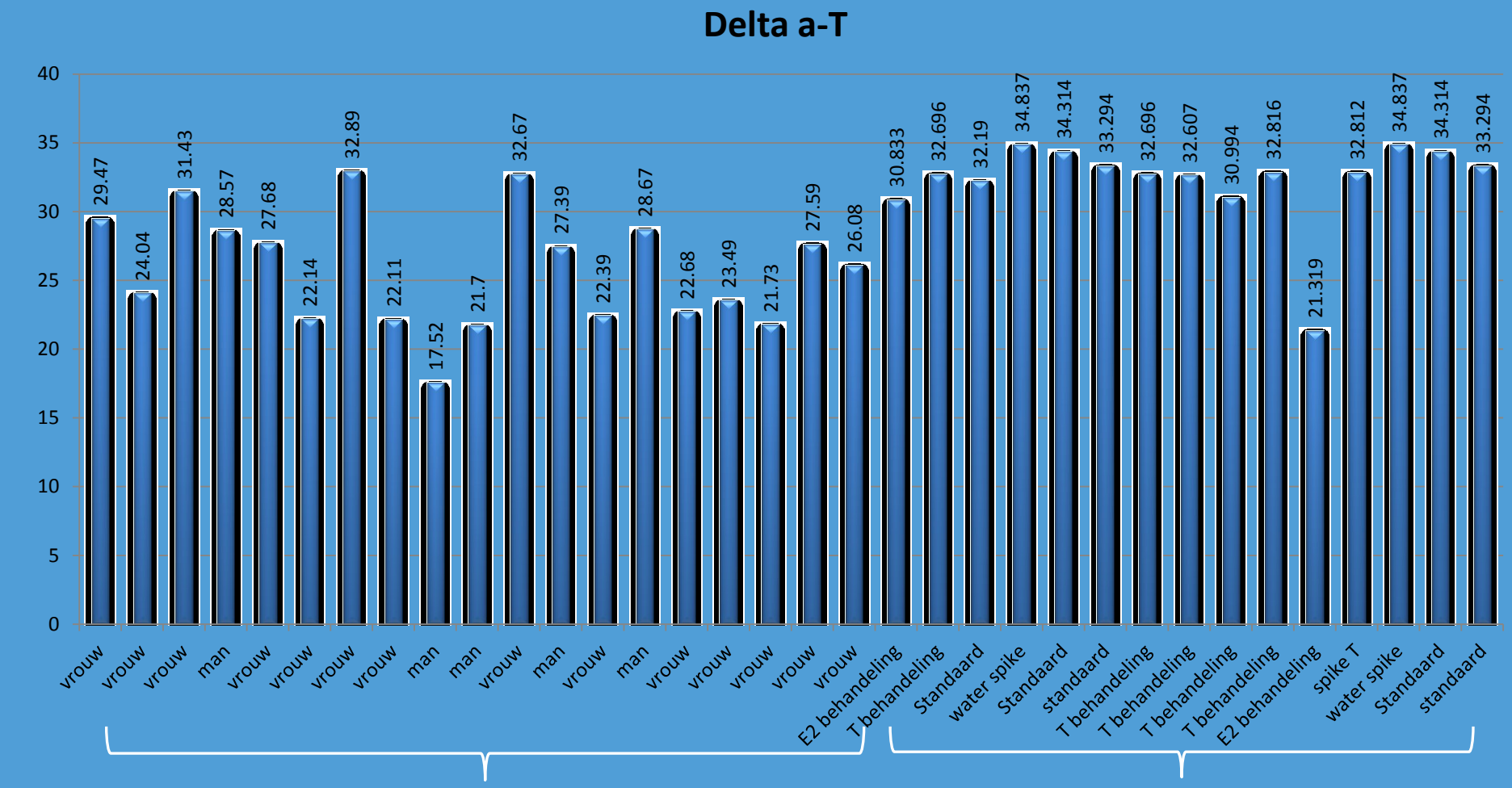
Delta values measured for a-T



Reference population

Treated animal
+ standards

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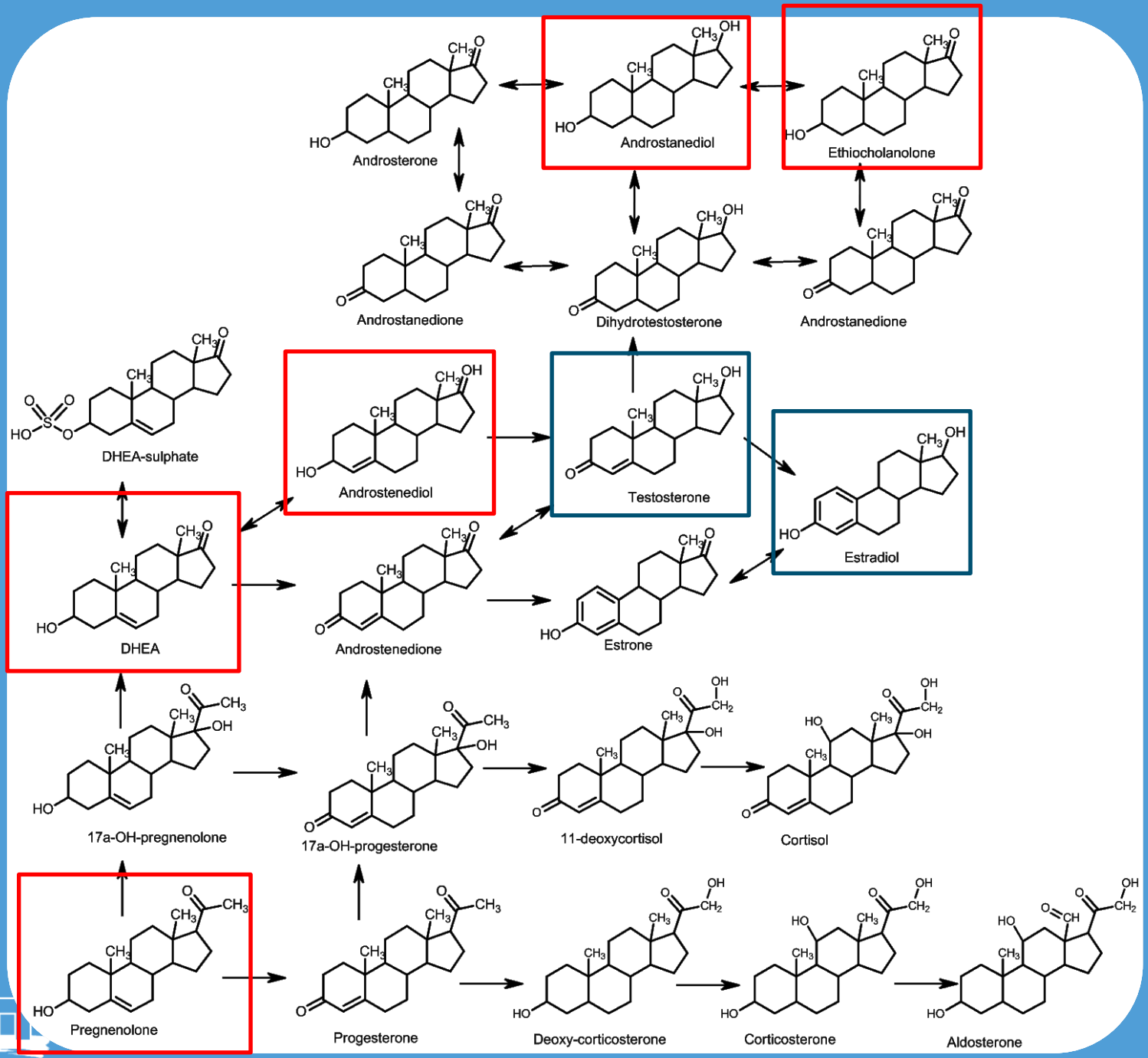
Ideal endogenous reference compound

- ERC will contribute to more pronounced differentiation
- Before target compound (TC) in the steroid synthesis
- Not influenced by TC
- Present in urine in relatively high concentration
- Easy to derivatize for GC analysis

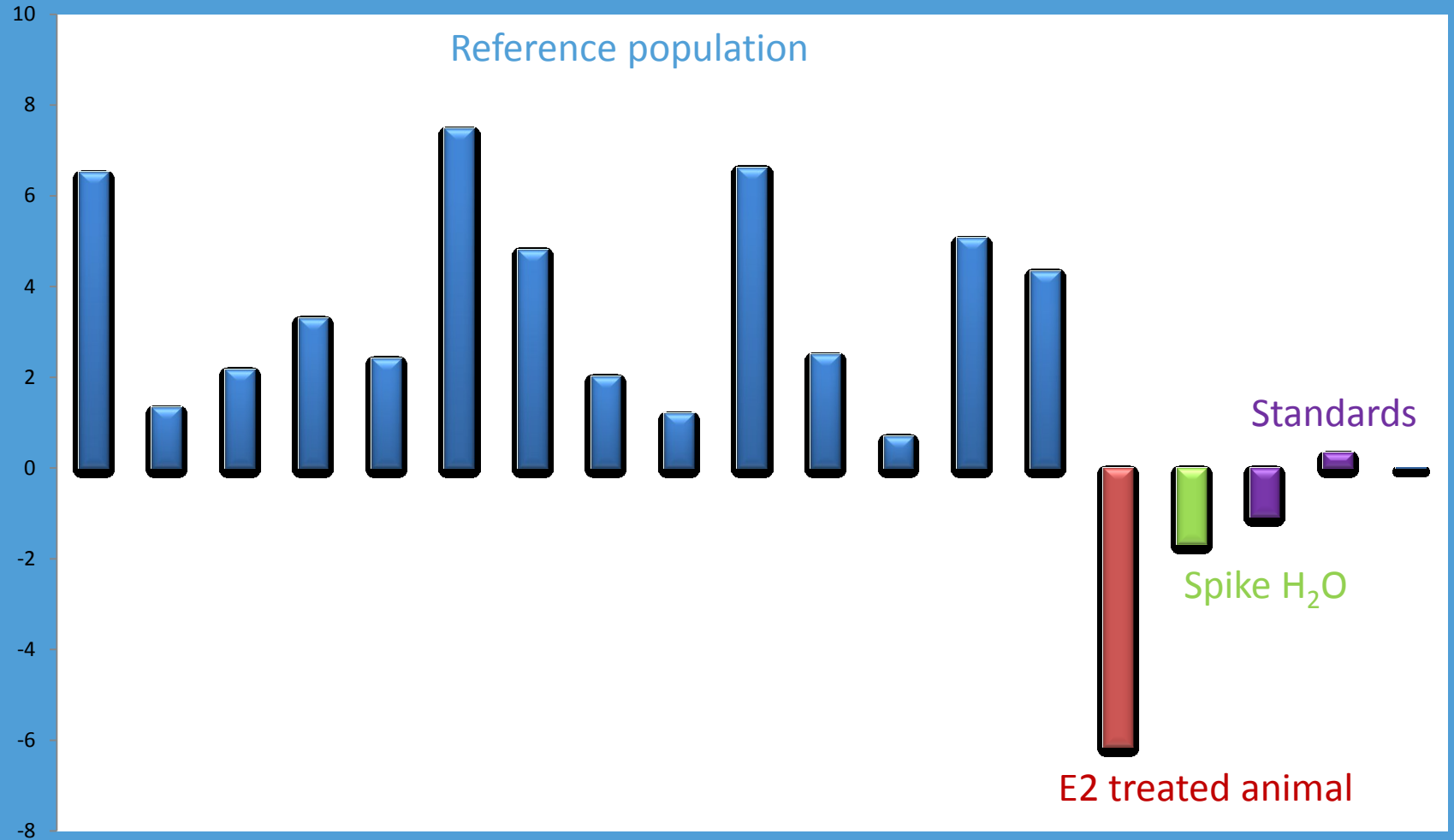

Androsterone
Androstanediol
Ethiocholanolone
Androstanedione
Dihydrotestosterone
Androstanedione
DHEA-sulphate
Androstenediol
Testosterone
Estradiol
DHEA
Androstenedione
Estrone
17a-OH-pregnenolone
17a-OH-progesterone
11-deoxycortisol
Cortisol
Pregnenolone
Progesterone
Deoxy-corticosterone
Corticosterone
Aldosterone

Testing different ERCs

- Discrimination estradiol and testosterone abuse from a "normal" population.
- Tested ERCs
  - a-Testosterone (conversion a-E2 to a-T is minimal)
  - Etiocholanolone
  - Androstanediol
- To minimize between sex differences only within sex data was used

Female population

$$\Delta\delta^{13}C(‰)=\delta^{13}C_{ERC(a\text{-}T)}-\delta^{13}C_{TC(a\text{-}E2)}$$



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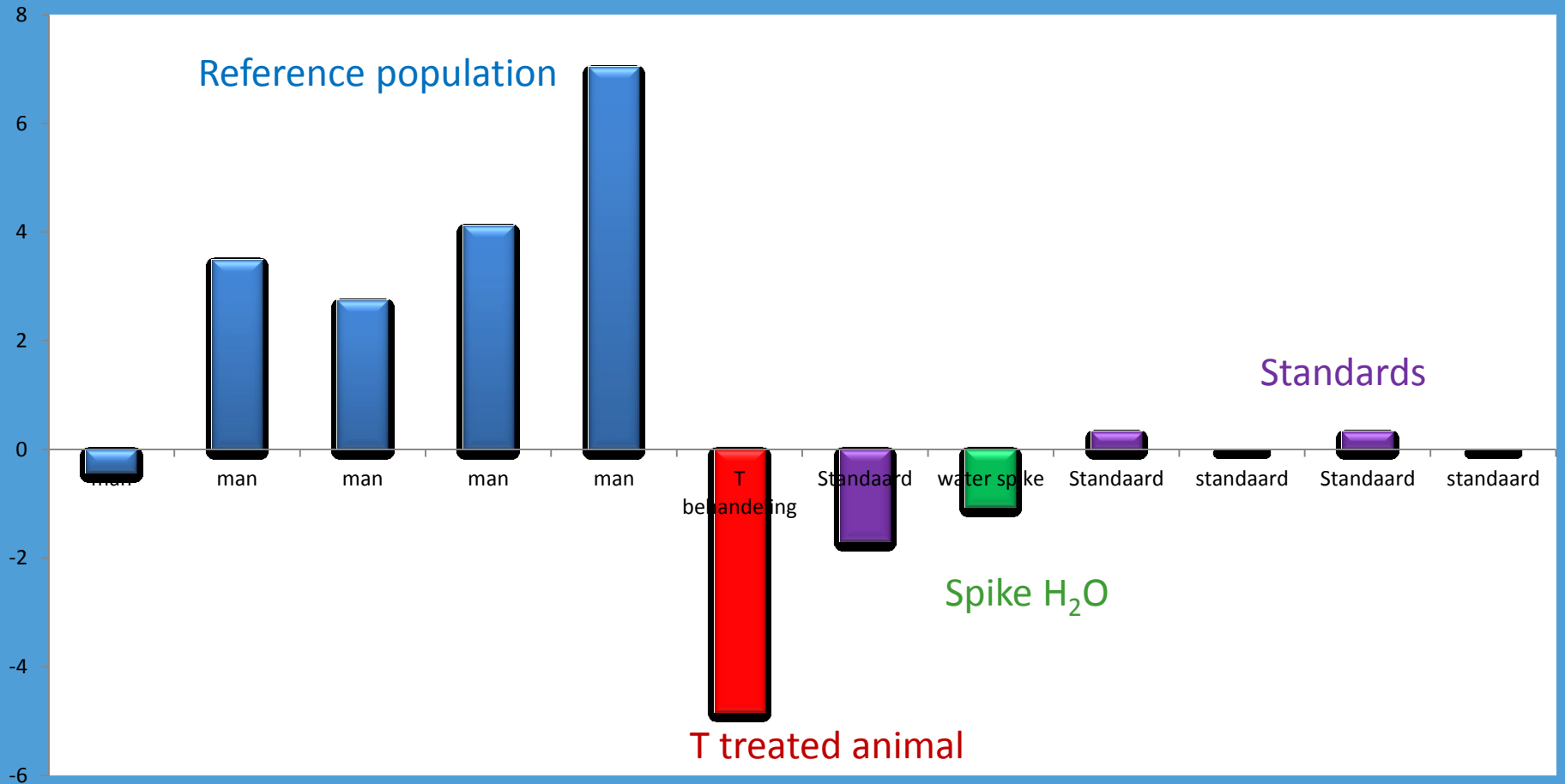
Male population

$$\Delta\delta^{13}C(‰)=\delta^{13}C_{ERC(a\text{-}T)}-\delta^{13}C_{TC(a\text{-}E2)}$$



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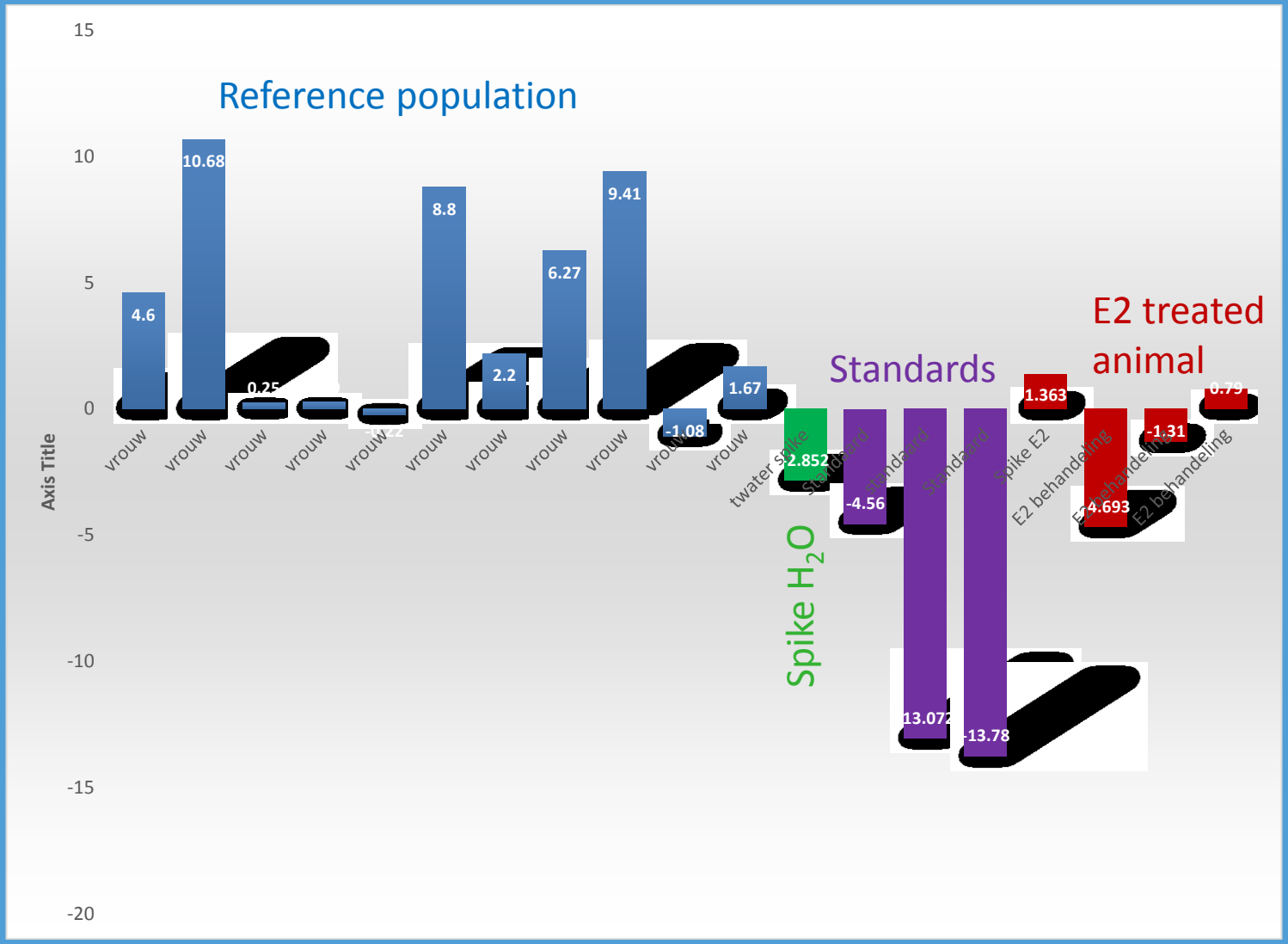
Female population

$$\Delta\delta^{13}C(‰)=\delta^{13}C_{ERC(ethio)}-\delta^{13}C_{TC(a\text{-}E2)}$$



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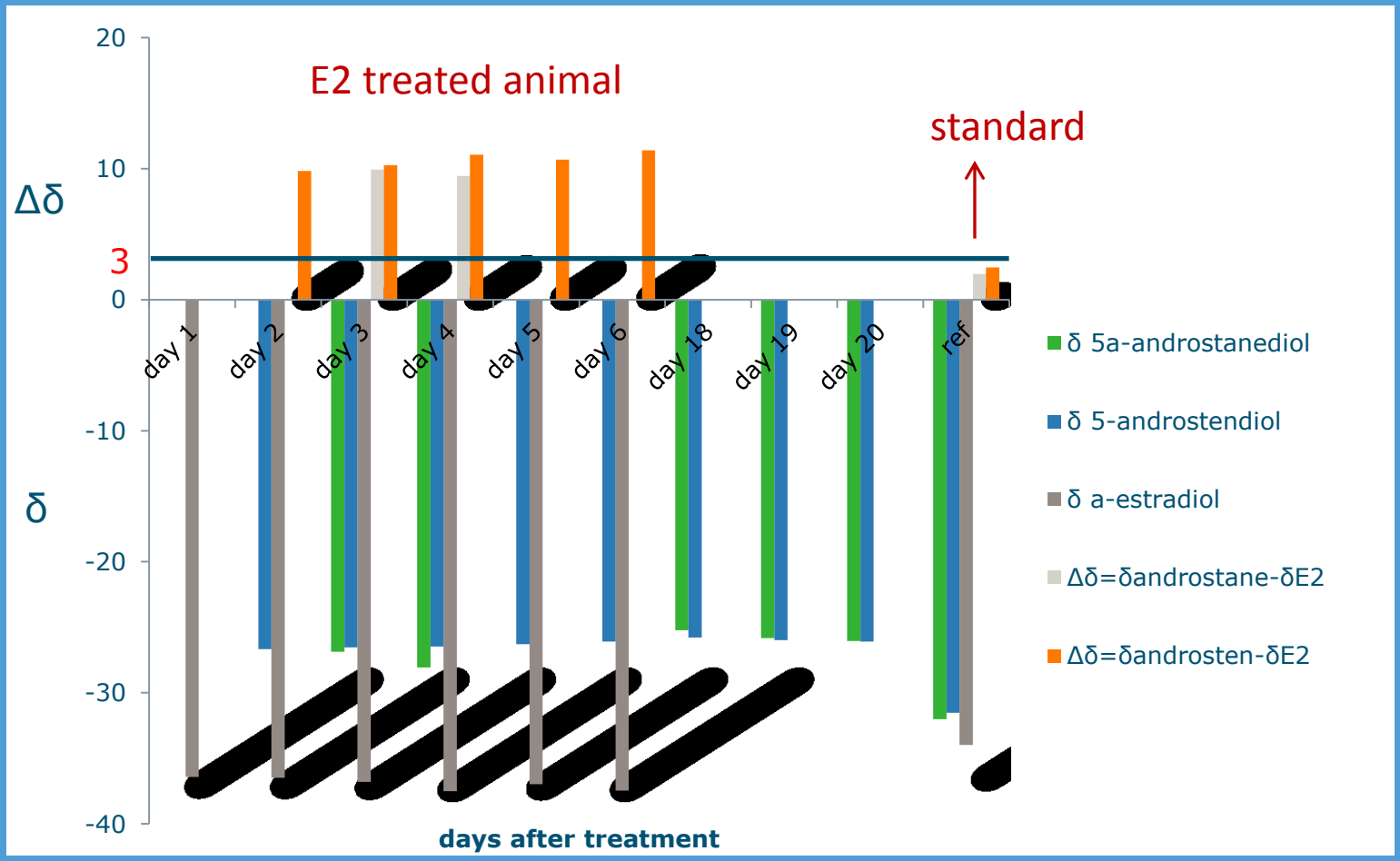
Female treated

$$\Delta\delta^{13}C(‰)=\delta^{13}C_{ERC(androsten/ane)}-\delta^{13}C_{TC(a\text{-}E2)}$$



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Conclusions

- Delta values start deviating from 3 × st dev. around 5 ng
- Using plain delta values already indication for treatment
- Using ERC gave clearly pronounced discrimination of administration of exogenous compounds
- Human doping method also works for cattle

- To do: - Find suitable ERC
  - Lower LODs
  - Switch to LVI

Thanks to my RIKILT colleagues

- Hennie van Rossum
- Paul Zoontjes
- Marco Blokland
- Saskia Sterk
- Frederike van Tricht

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Thank you for your attention

Questions?

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