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Zentrum für Präventive Dopingforschung / Institut für Biochemie
Center for Preventive Doping Research / Institute of Biochemistry

Carbon isotope ratios of endogenous steroids found in human serum – an update

T. Piper, M. Thevis

Outline

1) Introduction into serum steroids in doping controls
2) First approach on establishing an IRMS-based confirmation method
3) Results obtained after high-dose applications
4) Results obtained after low-dose applications
5) Improving the current IRMS-based approach

• Introduction

- Detection of testosterone doping has (recently) been extended encompassing human serum[1]

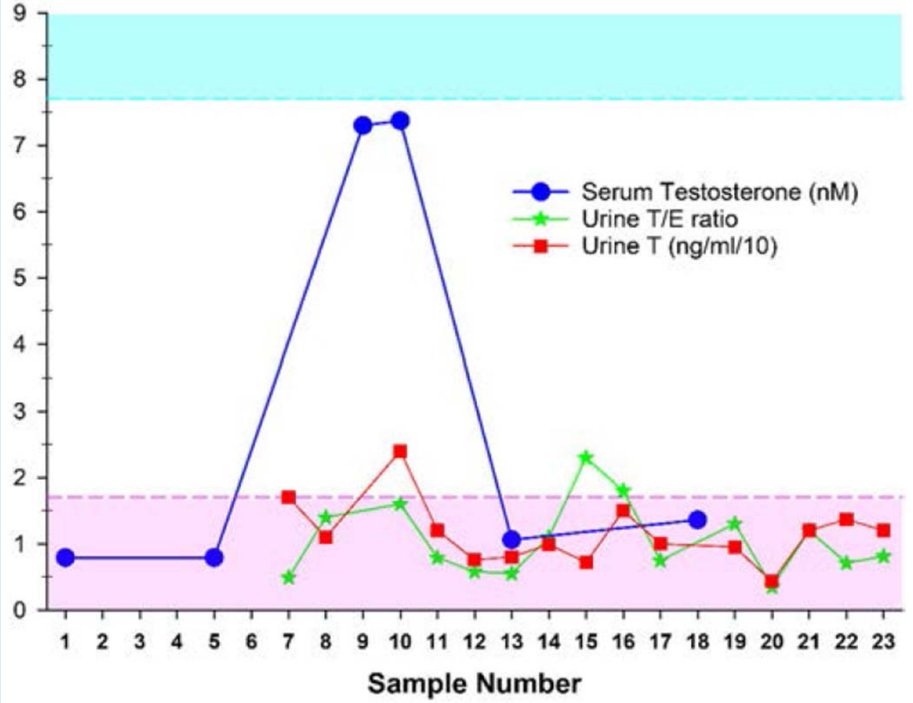


FIGURE 1 Athlete A's serial measurements of serum (blue circle) and urine (red square) testosterone and the urine T/E ratio (green star) over 25 urine or serum samples collected over 6 years. The shaded regions represent the female (pink, 0–1.7 nmol/L) and male (blue, >7.7 nmol/L) reference ranges for serum testosterone

› Drug Test Anal. 2019 Oct;11(10):1566-1571. doi: 10.1002/dta.2689. Epub 2019 Sep 3.

Detection of testosterone doping in female athletes

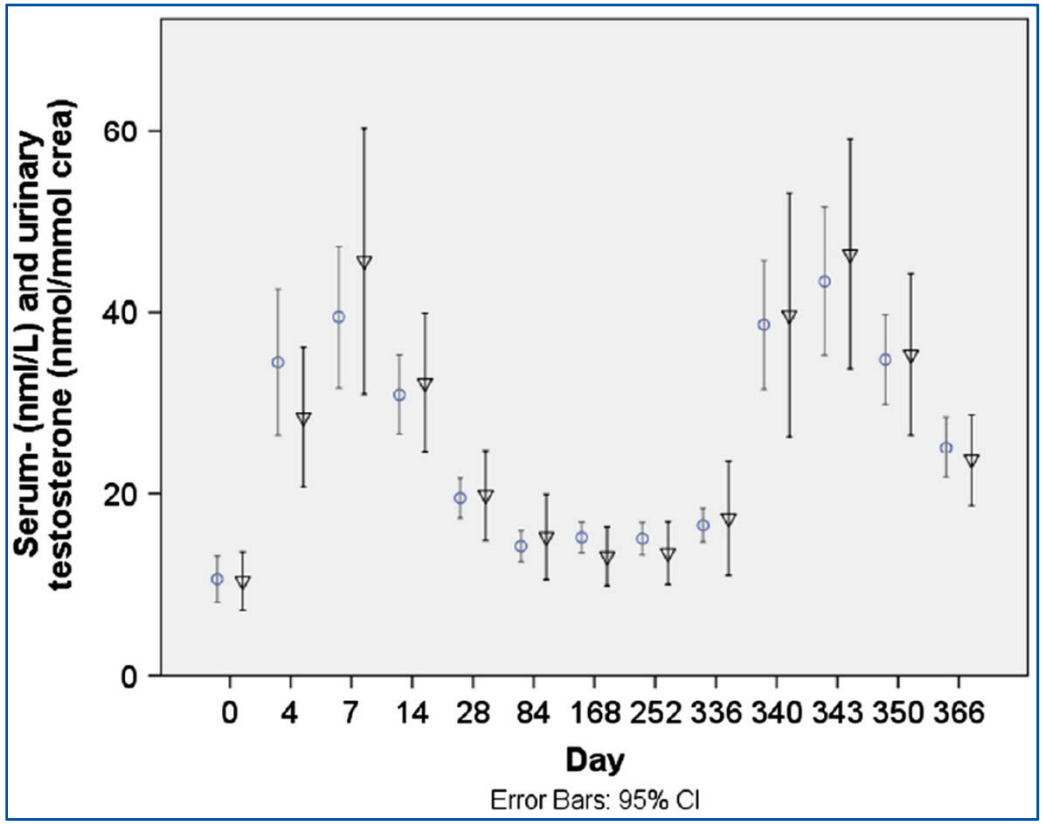
David J Handelsman [1], Stéphane Bermon [2]

Affiliations + expand

PMID: 31454165 DOI: 10.1002/dta.2689

• Introduction

- After treatment with 1000 mg i.m. T-undecanoate



Relationship between testosterone in serum, saliva and urine during treatment with intramuscular testosterone undecanoate in gender dysphoria and male hypogonadism

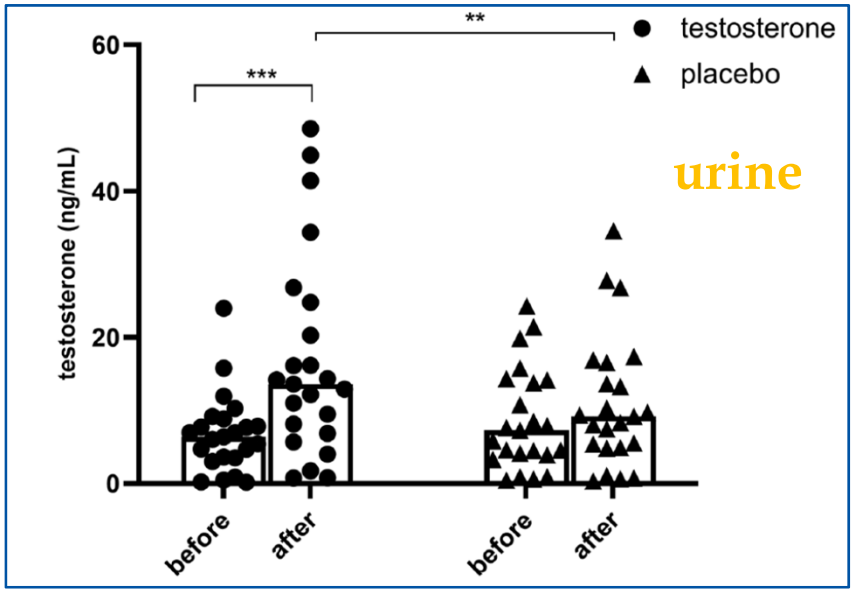
[1,2]Y. Lood, [3]E. Aardal-Eriksson, [4]C. Webe, [1,2]J. Ahlner, [4]B. Ekman and [4]J. Wahlberg

• Low-dose application to female volunteers - 1

- 10 weeks treatment with T-Gel (10 mg T) per day in healthy young females

Disposition of Urinary and Serum Steroid Metabolites in Response to Testosterone Administration in Healthy Women

Jona Elings Knutsson,[1,2*] Alexander Andersson,[3,4*] Lasse Vestli Baekken,[5] Anton Pohanka,[3,4] Lena Ekström,[3,4] Angelica Lindén Hirschberg[1,2]



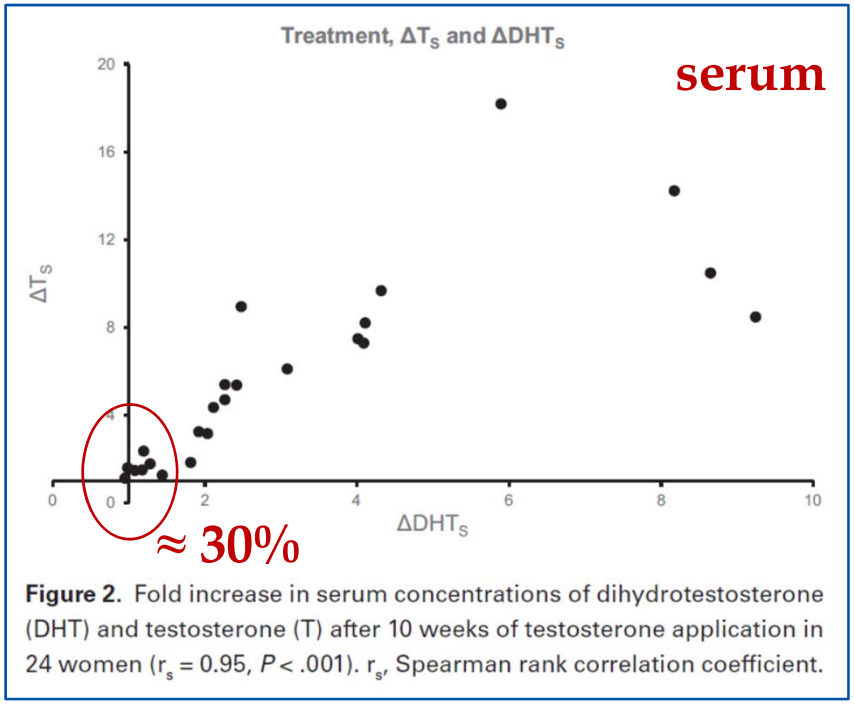


Figure 2. Fold increase in serum concentrations of dihydrotestosterone (DHT) and testosterone (T) after 10 weeks of testosterone application in 24 women ($r_s = 0.95$, $P < .001$). r_s, Spearman rank correlation coefficient.

• Low-dose application to female volunteers - 1

- 4 weeks treatment with T-Gel (10 mg T) per day in healthy young females

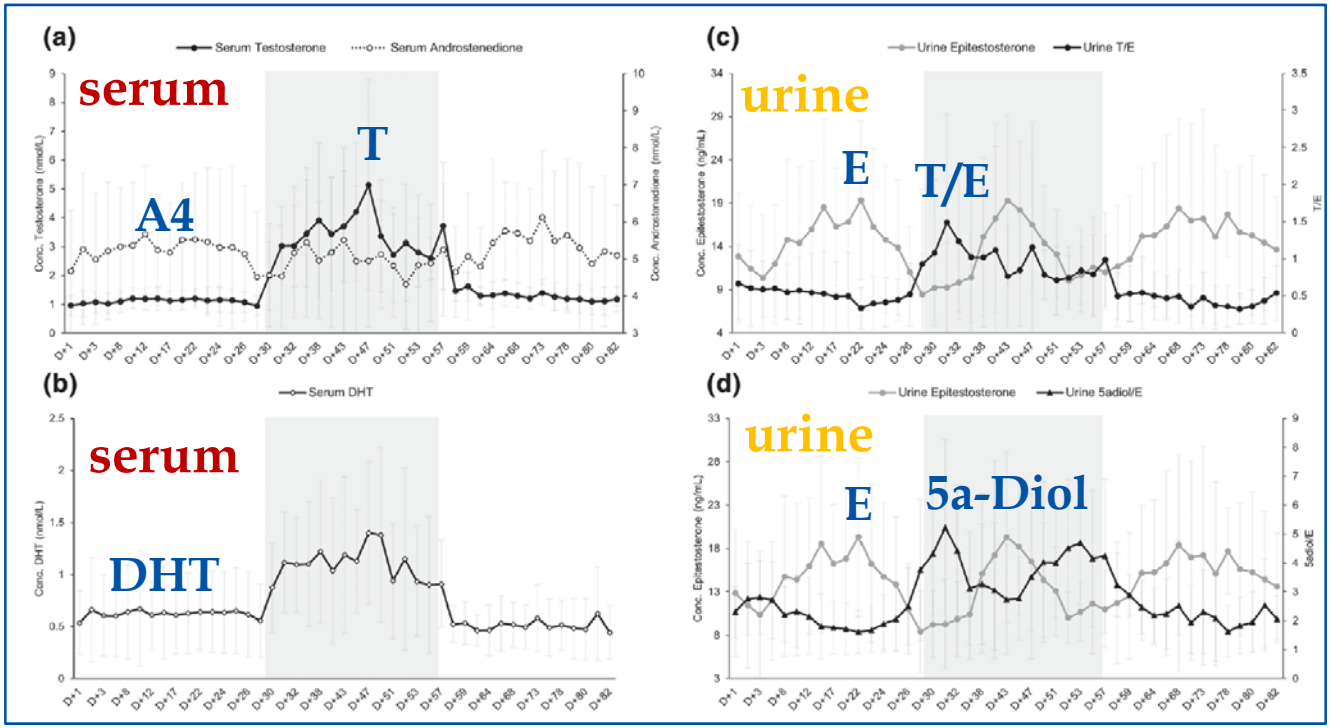
› Drug Test Anal. 2022 May;14(5):833-850. doi: 10.1002/dta.3040. Epub 2021 Apr 14.

Longitudinal evaluation of multiple biomarkers for the detection of testosterone gel administration in women with normal menstrual cycle

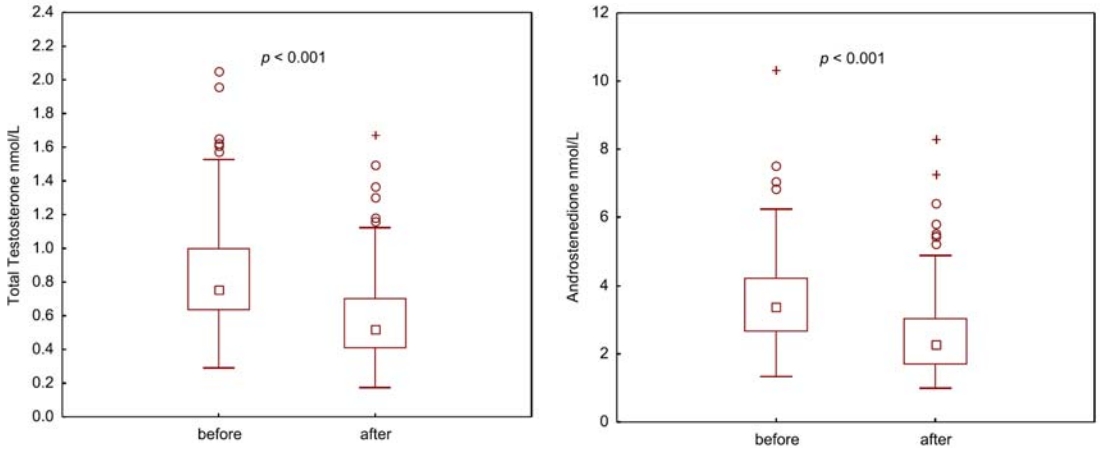
Olivier Salamin [1 2], Raul Nicoli [2], Tobias Langer [2], Julien Boccard [3 4 5], Carine Schweizer Grundisch [2], Cheng Xu [6], Serge Rudaz [3 4 5], Tiia Kuuranne [2], Nelly Pitteloud [6], Martial Saugy [1]

Affiliations + expand

PMID: 33817997 DOI: 10.1002/dta.3040



• Oral contraceptives as confounding factor



Randomized Controlled Trial > Drug Test Anal. 2023 Jan;15(1):134-138. doi: 10.1002/dta.3373. Epub 2022 Oct 2.

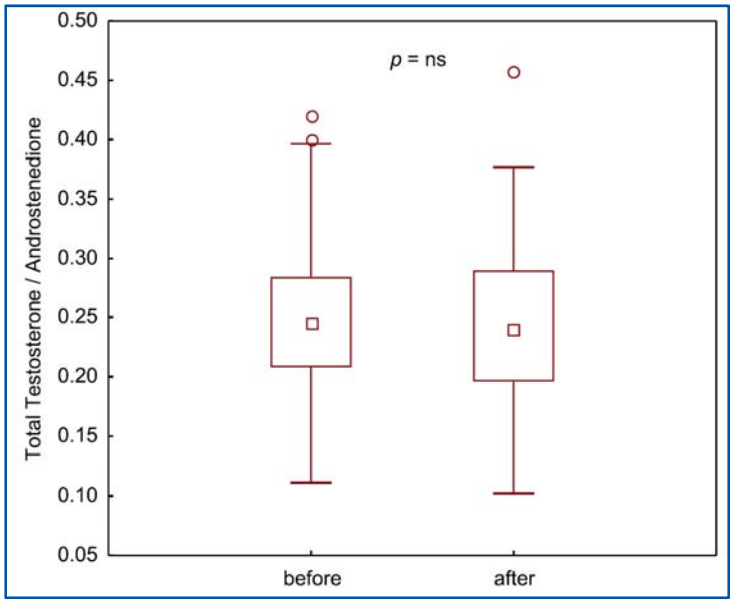
Disposition of serum steroids in response to combined oral contraceptives and menstrual cycle phases: A double-blind, randomized, placebo-controlled study

Jona Elings Knutsson [1,2], Lena Ekström [3,4], Angelica Lindén Hirschberg [1,2]

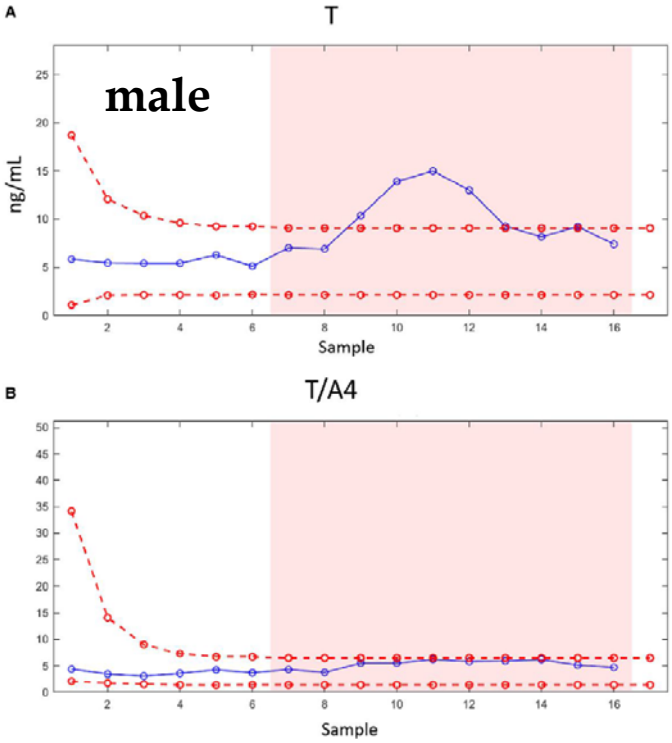
Affiliations + expand

PMID: 36165603 DOI: 10.1002/dta.3373

- Ratio of T/A4 is not affected by contraceptives



• The final WADA ABP approach



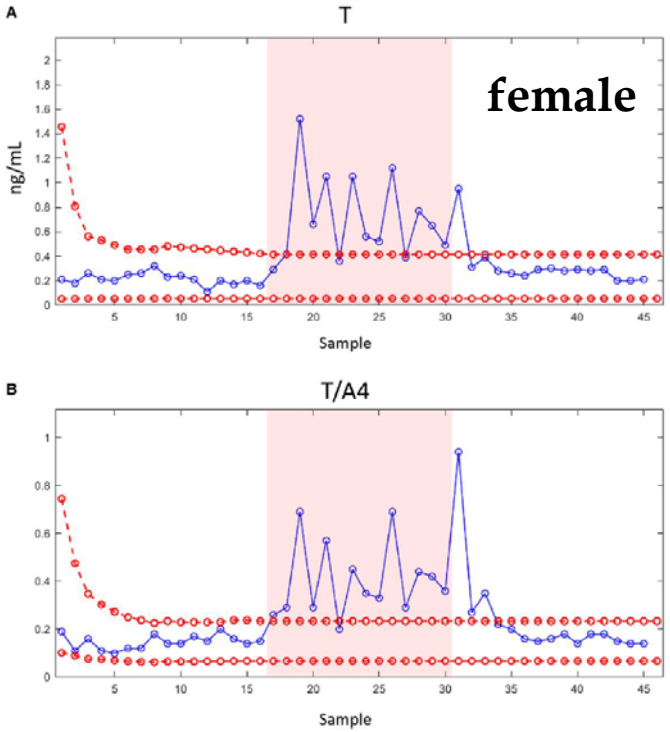
Clinical Trial > J Clin Endocrinol Metab. 2023 Jul 14;108(8):1937-1946.
doi: 10.1210/clinem/dgad085.

Longitudinal Profiling of Endogenous Steroids in Blood Using the Athlete Biological Passport Approach

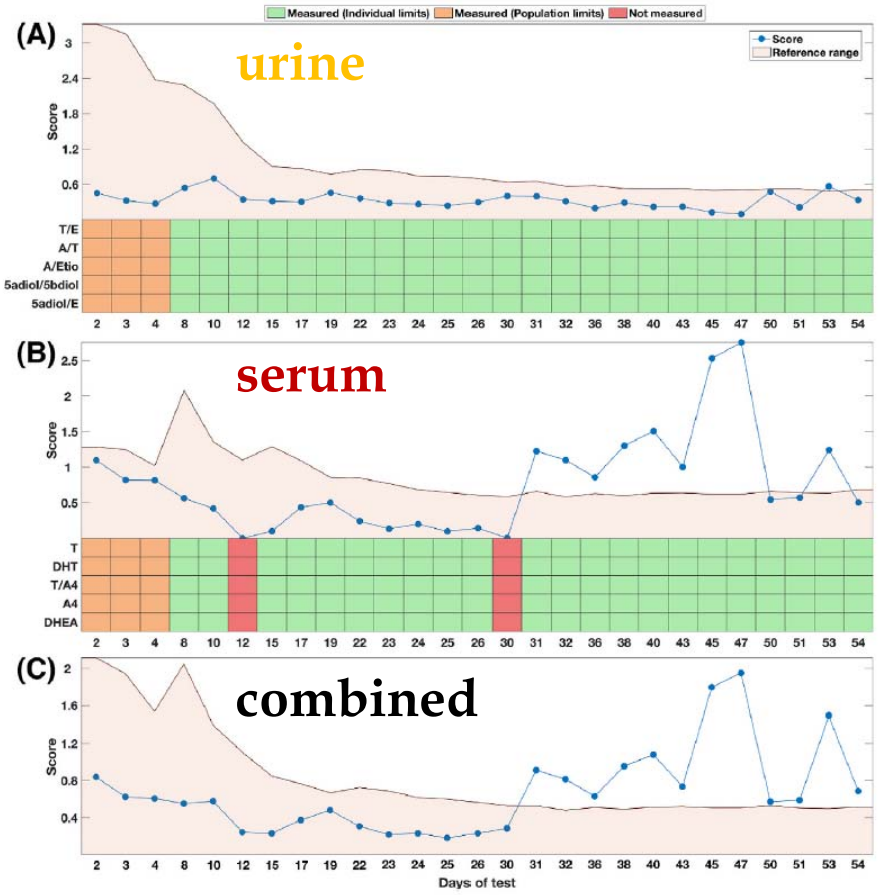
Tristan Equey [1], Olivier Salamin [2 3], Federico Ponzetto [4], Raul Nicoli [2], Tiia Kuuranne [2], Jonas Saugy [3], Martial Saugy [3], Reid Aikin [1], Norbert Baume [1]

Affiliations + expand

PMID: 36794909 DOI: 10.1210/clinem/dgad085



• And beyond…



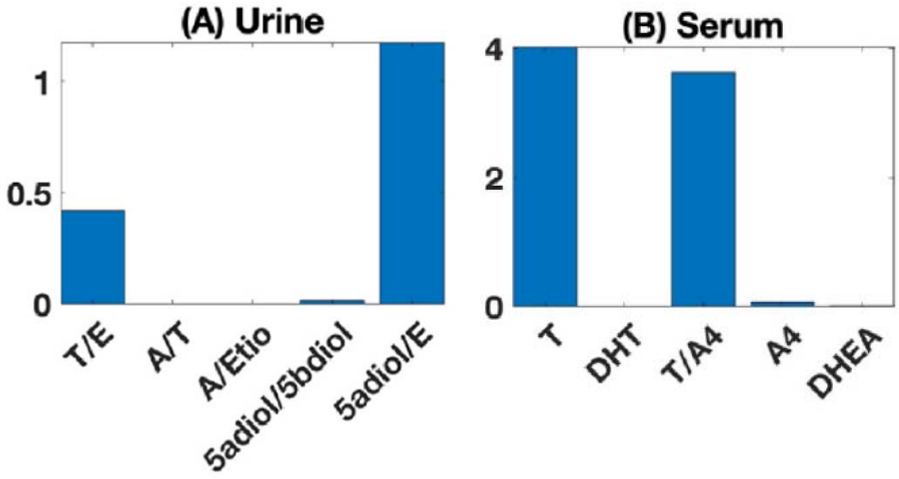
› Anal Chim Acta. 2023 Aug 1;1267:341389. doi: 10.1016/j.aca.2023.341389. Epub 2023 May 17.

A new multimodal paradigm for biomarkers longitudinal monitoring: a clinical application to women steroid profiles in urine and blood

Miguel de Figueiredo [1], Jonas Saugy [2], Martial Saugy [2], Raphaël Faiss [2], Olivier Salamin [3], Raul Nicoli [4], Tiia Kuuranne [4], Serge Rudaz [1], Francesco Botrè [2], Julien Boccard [5]

Affiliations + expand

PMID: 37257979 DOI: 10.1016/j.aca.2023.341389



Introduction

- Detection of testosterone doping has recently been extended encompassing human serum
- Ongoing studies on steroid profiles measured in serum/plasma
- ABP approaches apparently enable high sensitivity
- But currently lacks an unambiguous confirmation
- As provided by IRMS in urine samples

Aim of the initial study (2020)

- Development and validation of IRMS method for serum steroids
- Investigations on a reference population
- Derive preliminary thresholds
- Proof-of-concept
  - Male volunteers from testosterone replacement therapy
  - Male volunteer after 5-fold administration of T-Gel

• Method development

- Concentration of steroids found in serum is generally very low:
  - T, ADIONE, DHEA, DHT, ETIO-GLUC < 5 ng/mL

  - A-GLUC and ETIO-SULF < 50 ng/mL

  - **DHEA-SULF, A_SULF and EpiA-SULF** > 50 ng/mL

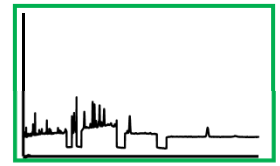
- Taking into consideration the available volume of 1 mL
- Additionally cholesterol (CHOL) is available "unlimited"

- **Sample preparation protocol**

1) One mL of serum, precipitation with ice-cold acetonitrile
2) Vortex, centrifuge and transfer organic layer to test tube, dry
3) Reconstitute with buffer and extract with TBME (**CHOL**)
4) SPE, dry down followed by acidic solvolysis
5) Extraction with TBME (formerly sulfated **DHEA, A and EpiA**)
6) Acetylate and forward to multi-dimensional gas chromatography-combustion-isotope ratio mass spectrometry **(MDGC-C-IRMS)**

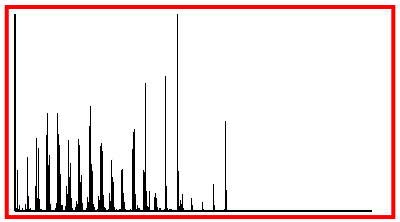
• MDGC-C-IRMS set up

→ Retention time stability
→ Transfer windows



Injector FID

→ Analyte identity confirmation
→ Assessment of peak purity



qMS

GC_2

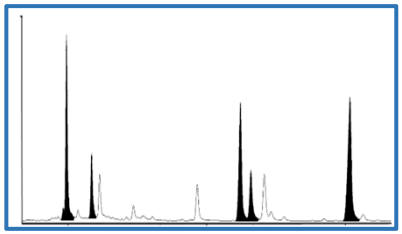
GC_1

Backflush

Deans Switch

Aux 1 Aux 2

IRMS Conflo IV GC Isolink



^{2}D GC

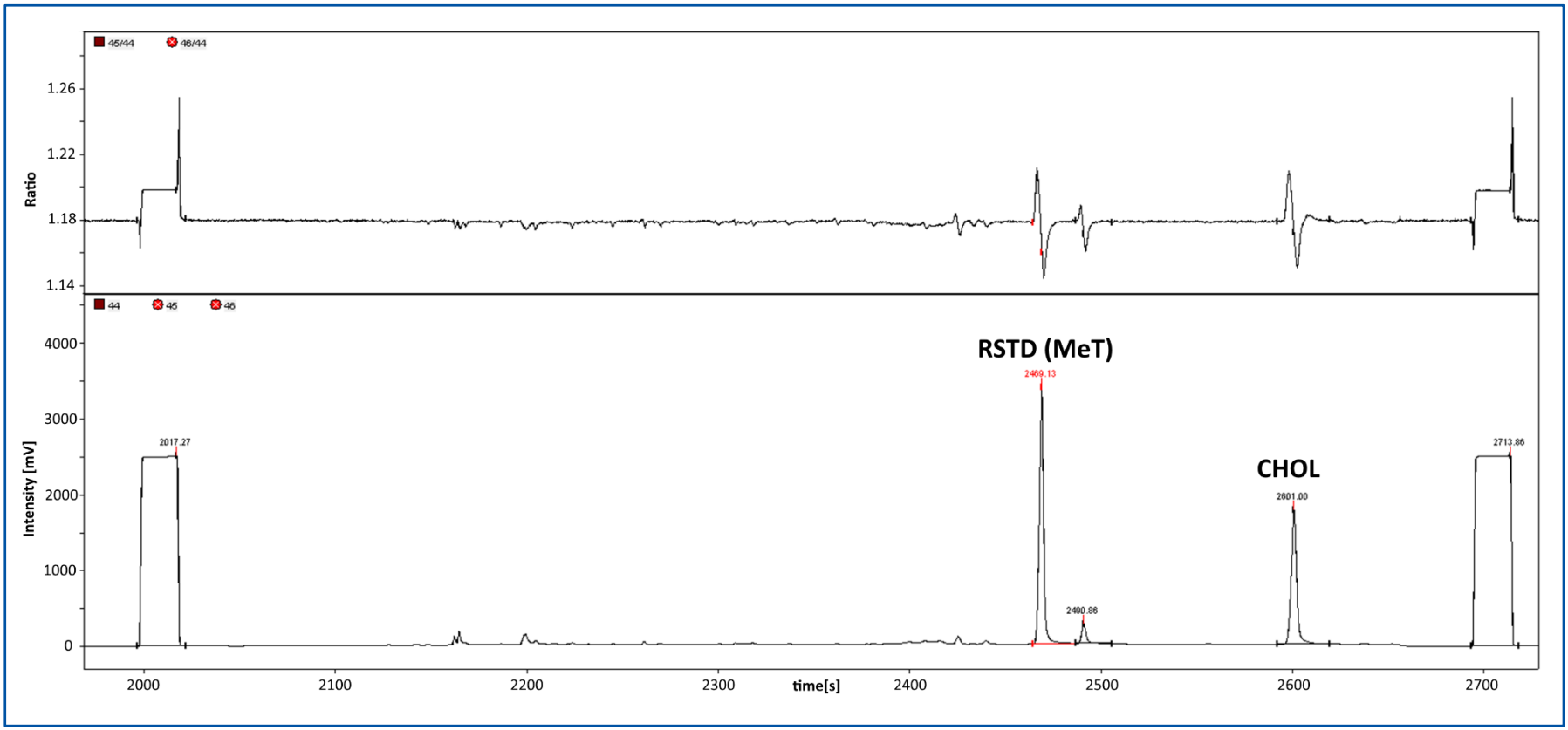
^{1}D GC

2nd column:
Medium polarity

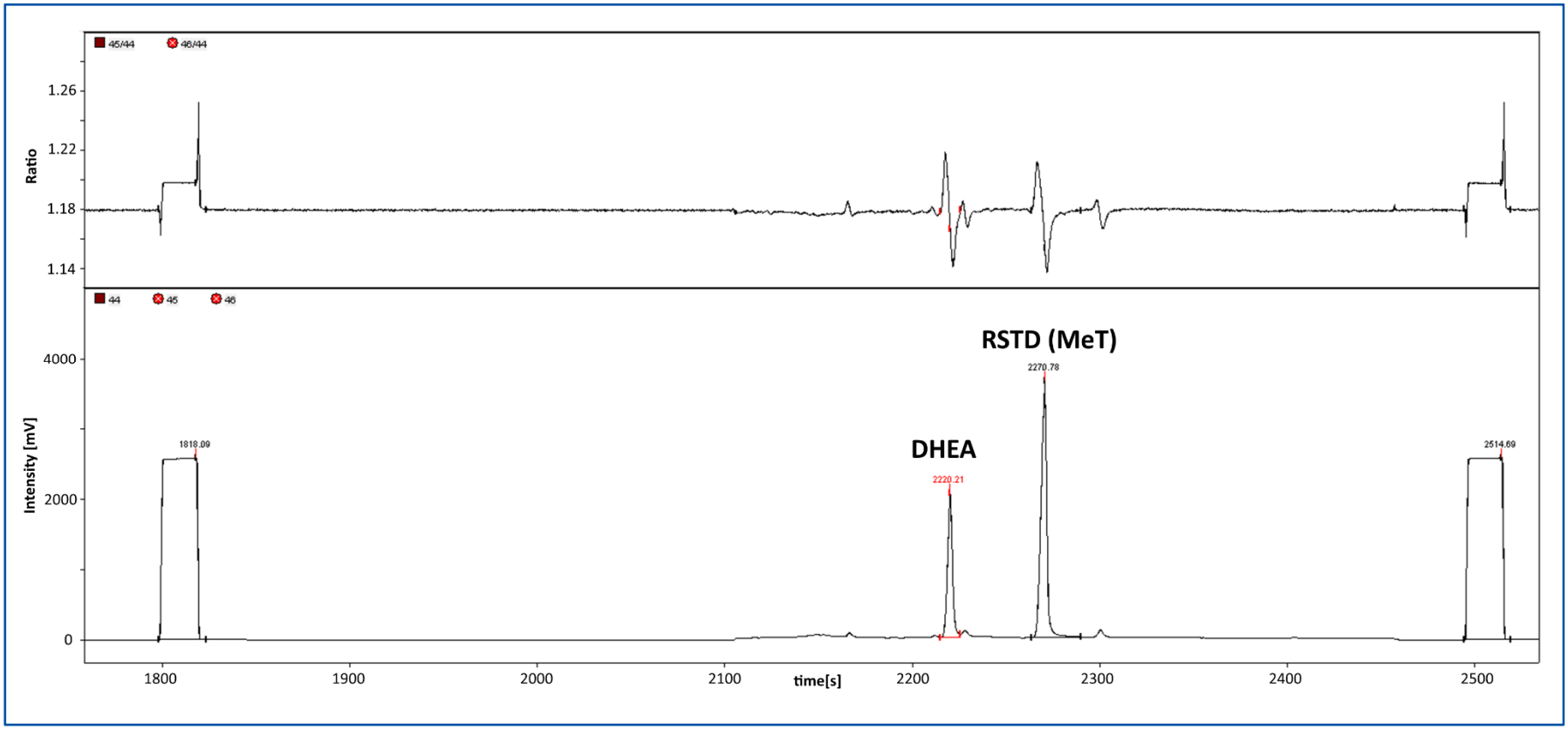
1st column:
1.00 µm film thickness
Lower polarity

Putz M *et al.* Anal Chim Acta **2018**, DOI: 10.1016/j.aca.2018.05.016

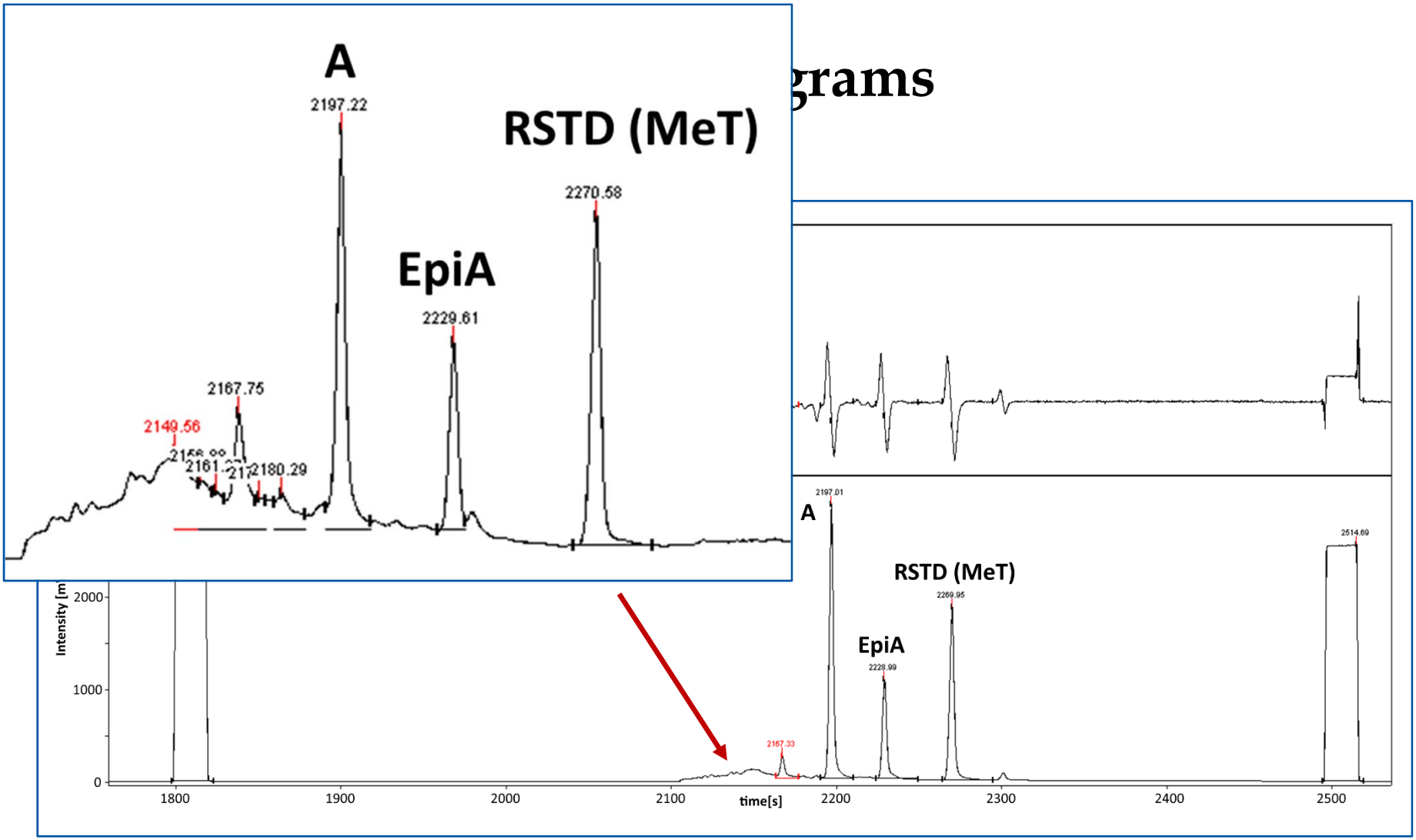
- **MDGC-C-IRMS chromatograms**



- **MDGC-C-IRMS chromatograms**

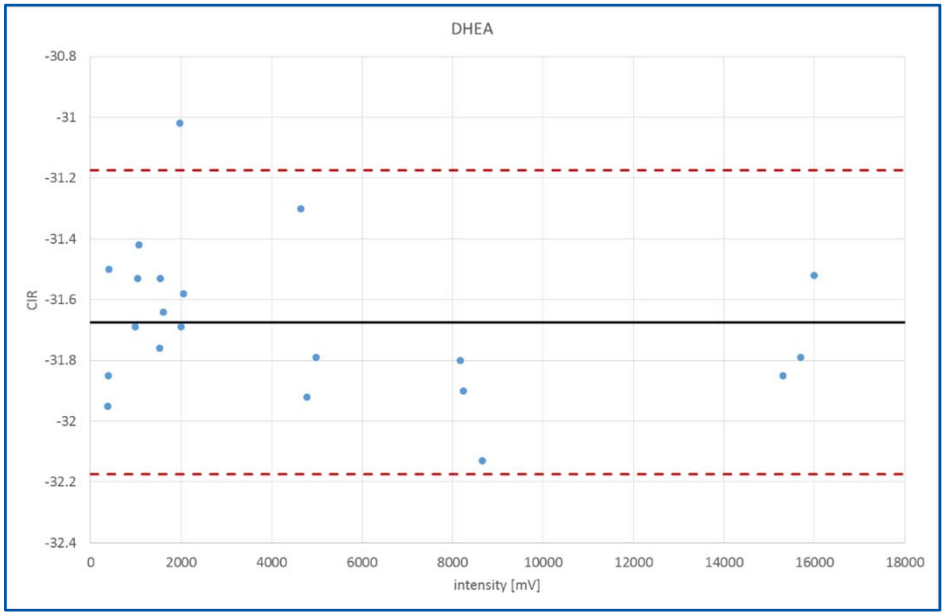


grams

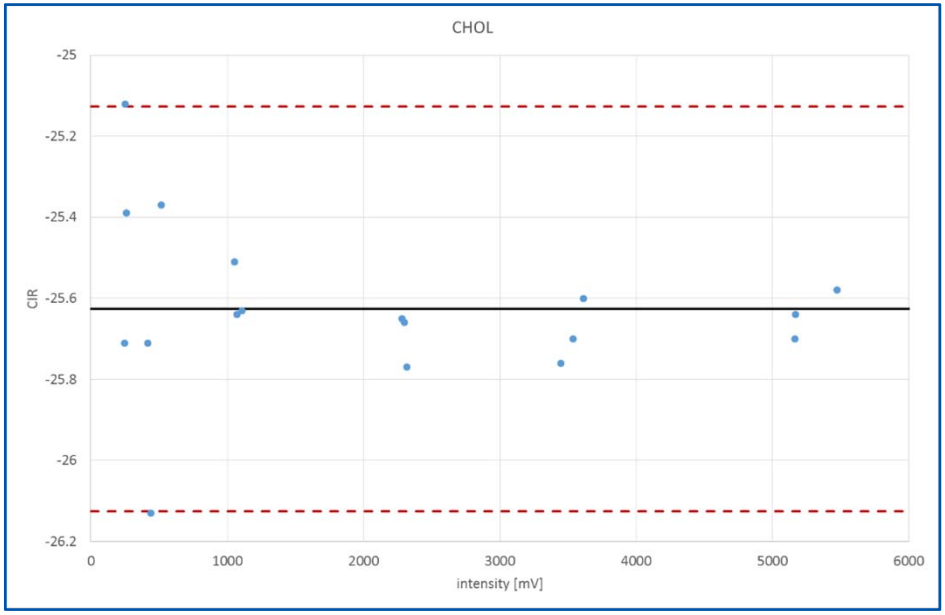

A
2197.22
RSTD (MeT)
2270.58
EpiA
2229.61
2167.75
2149.56
2180.29
A
2197.01
RSTD (MeT)
2269.95
EpiA
2228.99
2514.69
2167.33
Intensity [m
2000
1000
0
1800
1900
2000
2100
time[s]
2200
2300
2400
2500

• Validation – Instrument Linearity

- Covering a concentration range from 8 to 400 ng on column



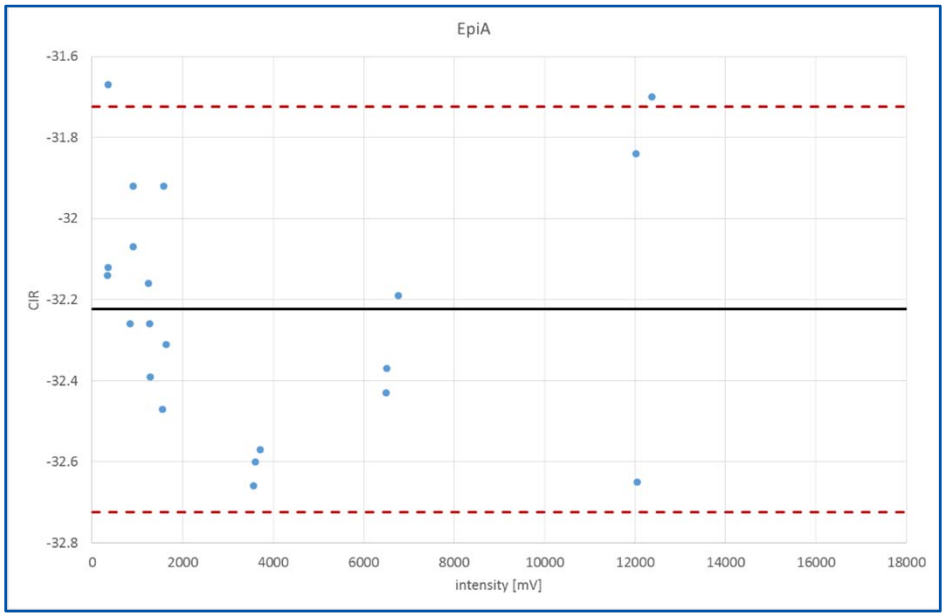
DHEA: 400 to 15700 mV



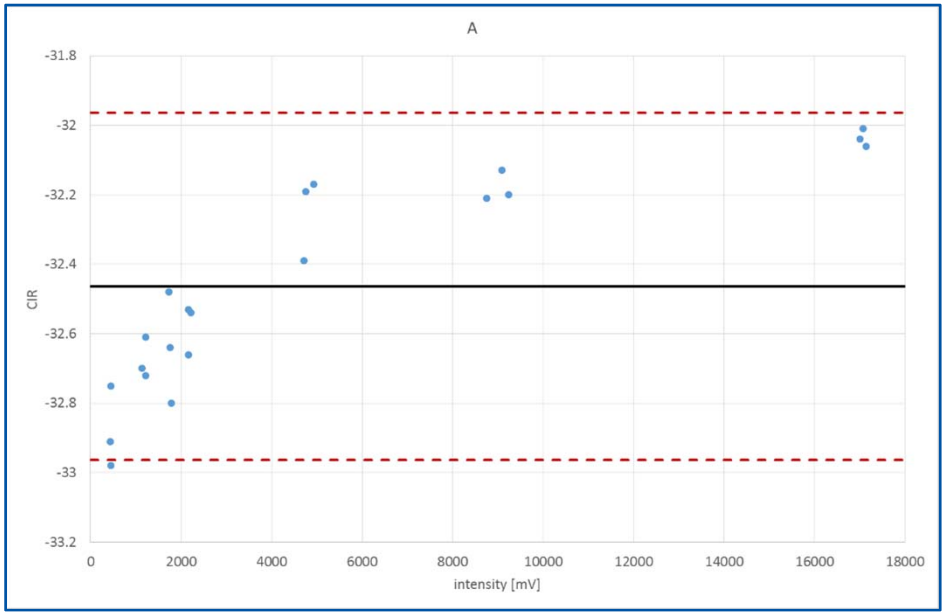
CHOL: 250 to 5500 mV

• Validation – Instrument Linearity

- Covering a concentration range from 8 to 400 ng on column

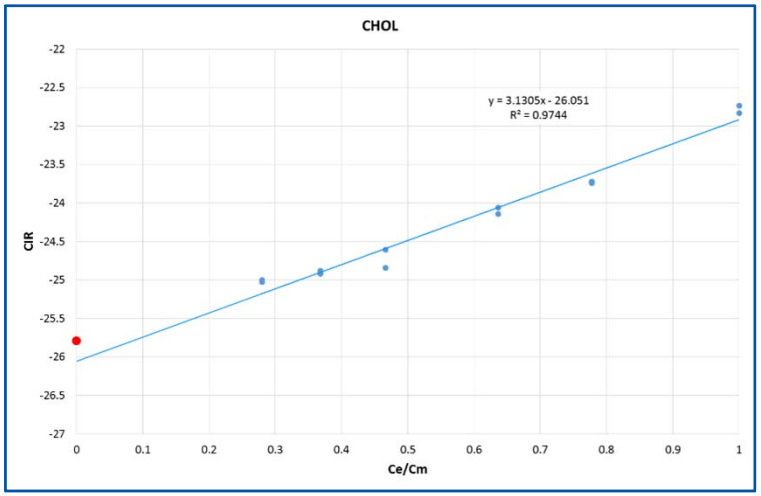


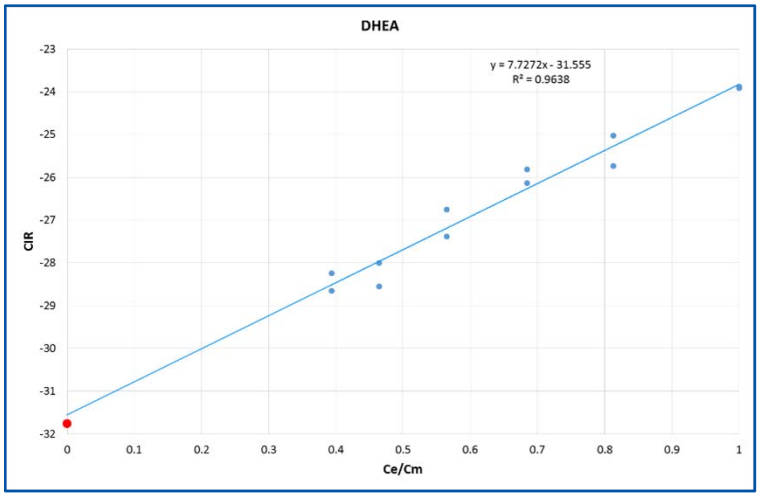
EpiA: 350 to 12000 mV

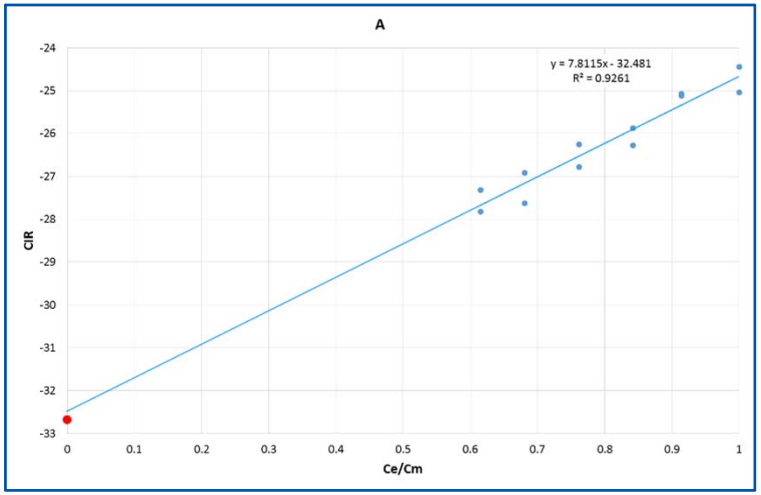


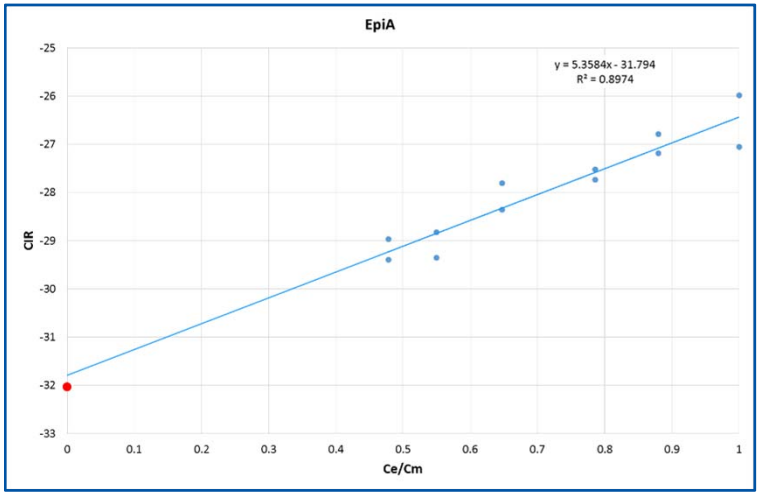
A: 440 to 17000 mV

• Validation – Linear Mixing Models









- # Validation – Linear Mixing Models

Steroid	a	SD(a)	b	SD(b)	MU
CHOL	3.13	0.16	-26.05	0.10	0.19
DHEA	7.73	0.47	-31.55	0.32	0.57
A	7.81	0.70	-32.48	0.57	0.90
EpiA	5.36	0.57	-31.79	0.43	0.71

• Validation – Repeatability

- Intra-day (n = 6 samples from one volunteer)

sample	CHOL	DHEA	A	EpiA
1	-21.5	-20.7	-21.5	-23.5
2	-21.2	-20.9	-21.5	-23.5
3	-21.3	-21.1	-21.7	-22.7
4	-21.3	-20.6	-21.4	-22.7
5	-21.4	-21.0	-22.0	-23.1
6	-21.3	-20.7	-21.8	-23.3
mean	-21.3	-20.9	-21.6	-23.2
SD	0.10	0.17	0.20	0.32

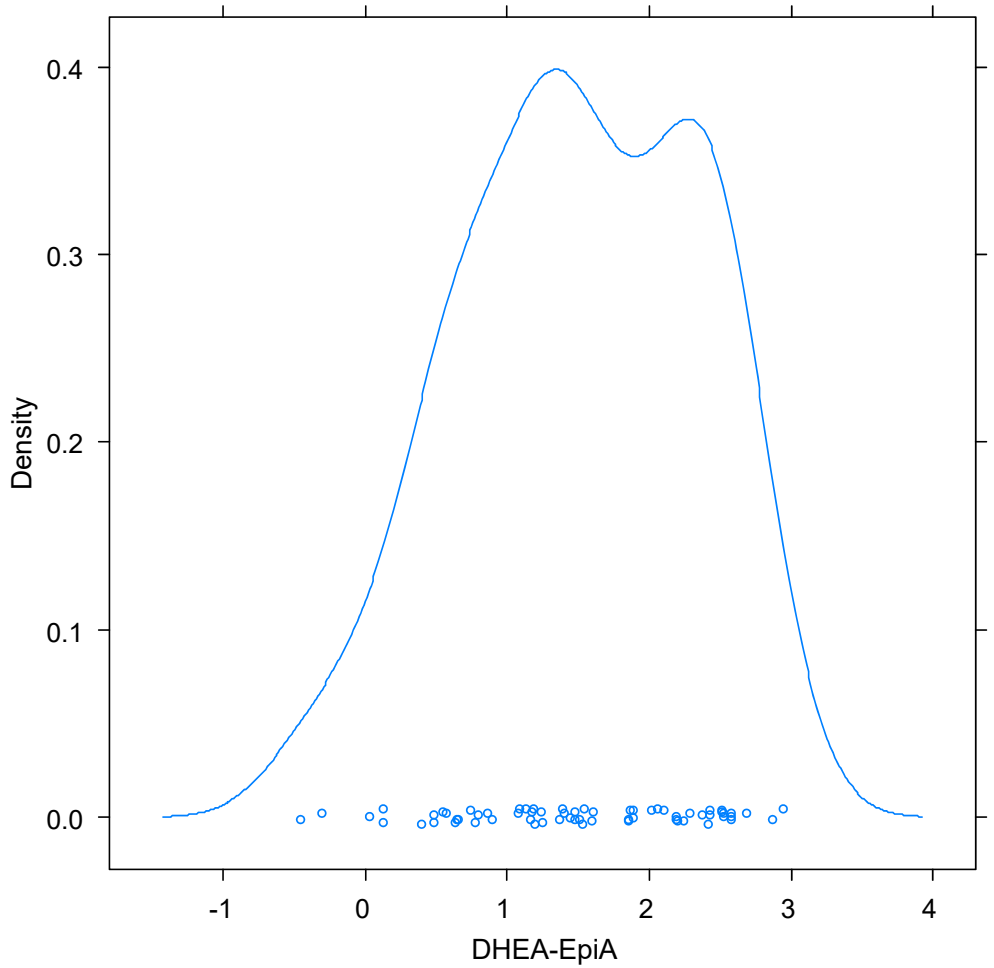
- Inter-day (n = 6 samples from a pooled serum sample)

sample	CHOL	DHEA	A	EpiA
1	-20.5	-20.9	-21.3	-22.5
2	-20.7	-20.6	-20.9	-22.3
3	-20.3	-20.4	-21.0	-21.9
4	-20.5	-20.3	-21.0	-21.5
5	-20.7	-21.0	-21.1	-21.4
6	-20.4	-21.0	-21.4	-21.5
mean	-20.5	-20.7	-21.1	-21.8
SD	0.15	0.30	0.18	0.42

Validation – Reference Population

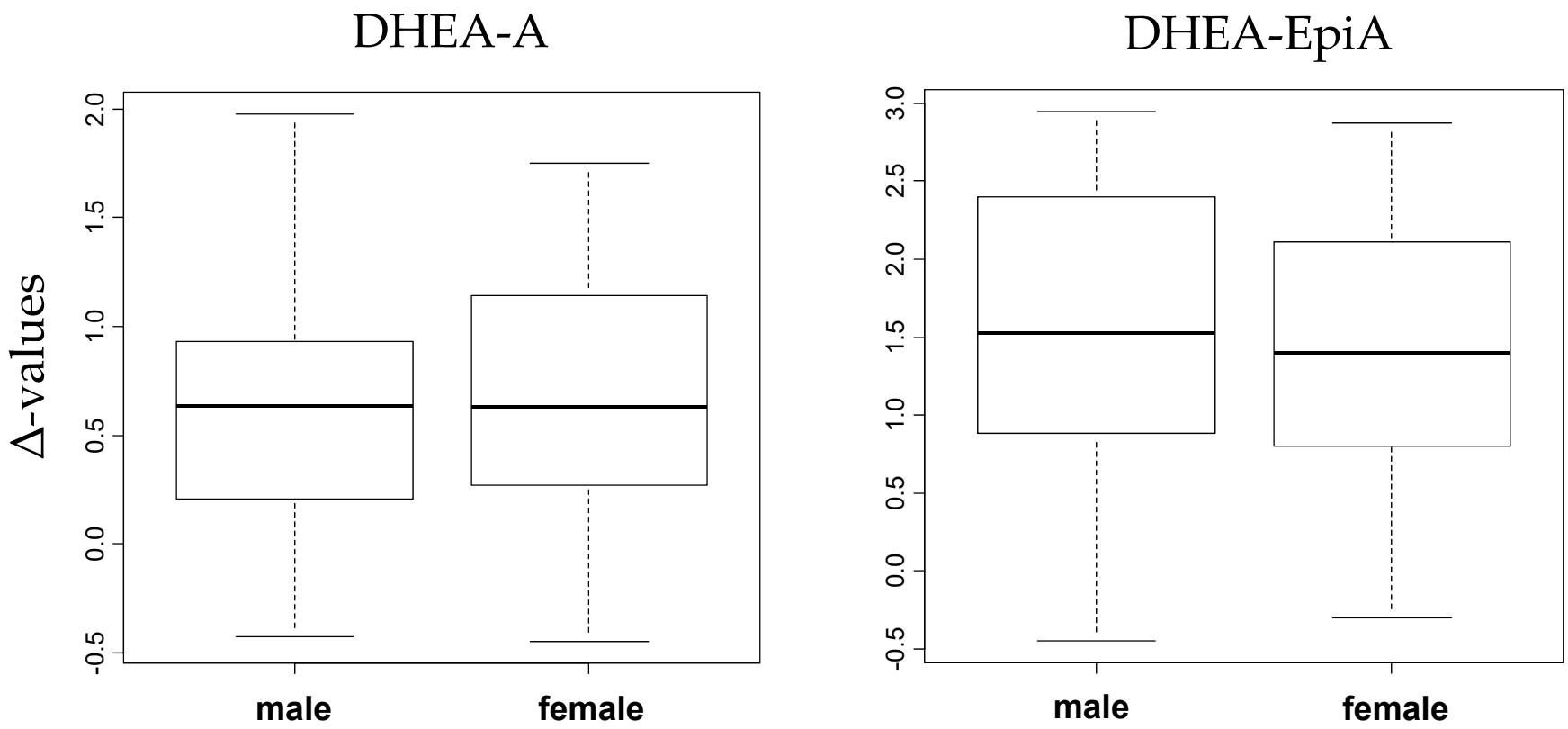
- Encompassing n = 65 athlete serum samples collected between 2016 and 2018 as doping control samples and stored frozen
- 32 female and 33 male samples
- Processed within 3 months
- All negative, but no confirmation regarding other ATF or AAF possible

• Validation – Reference Population

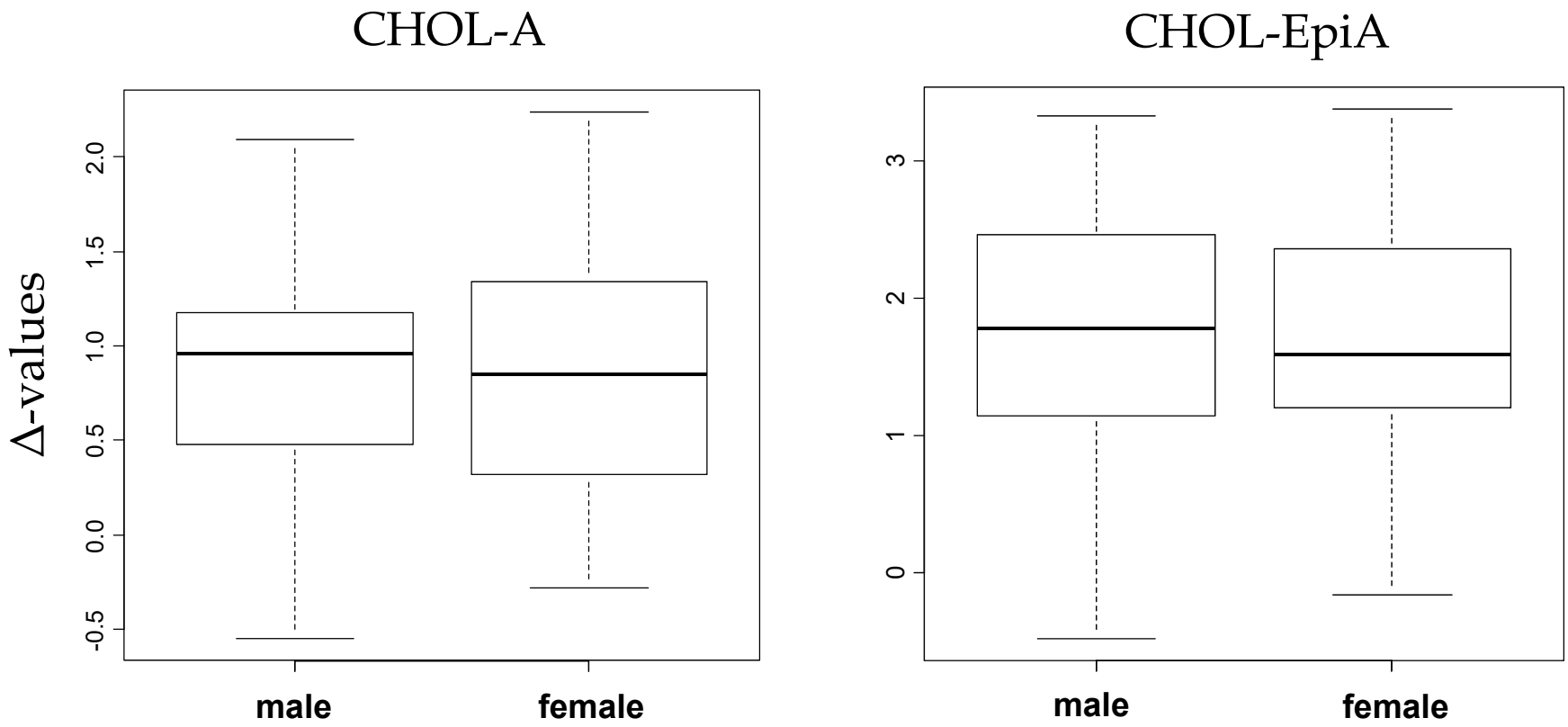


- All values were found Gaussian-distributed
- Tendency towards bimodal distributions
- But no significant difference between male and female

• Validation – Reference Population



• Validation – Reference Population



Validation – Reference Limits

	DHEA-A	DHEA-EpiA	CHOL-A	CHOL-EpiA	DHEA-CHOL
mean	**0.54**	**1.49**	**0.82**	**1.77**	**0.30**
SD	0.56	0.83	0.59	0.88	0.32
limit	**2.3**	**4.0**	**2.6**	**4.5**	**1.3**

- Comparable to thresholds found for urinary steroids
- Thresholds build with EpiA elevated due to slightly higher SDs and depleted values found here
- In-line with values reported for urinary sulfated A and EpiA

• Samples from volunteers – high-dose applications

- Six male volunteers participating in testosterone replacement therapies

Volunteer	A	EpiA	DHEA	CHOL	DHEA-A	DHEA-EpiA	CHOL-A	CHOL-EpiA
A-before	-23.9	-24.6	-22.8	-22.8	1.1	1.7	1.1	1.7
A-during	-27.5	-27.8	-22.9	-23.0	4.6	4.9	4.5	4.8
A-during	-27.0	-28.2	-23.0	-22.7	4.0	5.2	4.2	5.4

• Samples from volunteers – high-dose applications

- Six male volunteers participating in testosterone replacement therapies

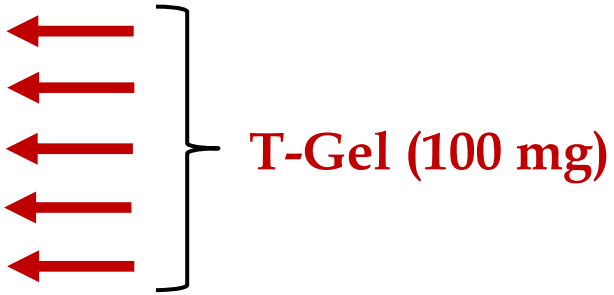
Volunteer	A	EpiA	DHEA	CHOL	DHEA-A	DHEA-EpiA	CHOL-A	CHOL-EpiA
A-before	-23.9	-24.6	-22.8	-22.8	1.1	1.7	1.1	1.7
A-during	-27.5	-27.8	-22.9	-23.0	4.6	4.9	4.5	4.8
A-during	-27.0	-28.2	-23.0	-22.7	4.0	5.2	4.2	5.4
B	-30.0	-31.1	-23.1	-23.9	6.8	8.0	6.0	7.2
C	-26.2	-27.7	-21.4	-21.1	4.8	6.2	5.1	6.6
D	-29.7	-31.2	-23.0	-23.6	6.7	8.2	6.0	7.6
E*	-24.0	-24.7	-20.6	-21.8	3.3	4.0	2.2	2.9
F	-29.5	-28.5	-24.3	-24.0	5.2	4.2	5.4	4.4
blank	-21.9	-22.2	-21.0	-20.7	0.9	1.3	1.2	1.5

*youngest volunteer – endogenous dilution?

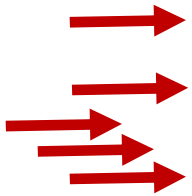
• Testosterone excretion study samples (mid-dose)

- One male volunteer applying T-Gel (100 mg) for five consecutive days

serum sample	urine sample	hours based on last application
TA	1	-111
TB	2	-87
TC	3	-63
	4	-39
	5	-15
TD	6	9
	8	18
	9	25
TE	10	33
	12	49
TF	13	57



- **Testosterone excretion study samples (mid-dose)**



time	DHEA-A	DHEA-EpiA	CHOL-A	CHOL-EpiA
-111	1.4	2.8	0.4	1.9
-87	2.5	3.1	2.0	2.6
-63	3.1	3.7	2.7	3.3
9	3.9	4.1	3.2	3.4
33	3.6	4.9	2.6	3.9
57	2.8	4.0	2.3	3.5

Intermediate Conclusion

- IRMS-based confirmation method was developed and validated
- Reference Population derived thresholds were calculated
- Samples from volunteers participating in testosterone replacement therapy showed significantly depleted values in both TCs
- Samples from a T-Gel administration were depleted beyond the established thresholds
- Sensitivity (low-dose T administrations) remains to be evaluated

- **Low-dose T administrations**

Drug Test Anal. 2023 Apr;15(4):465-469. doi: 10.1002/dta.3428. Epub 2022 Dec 30.

Usefulness of serum androgen isotope ratio mass spectrometry (IRMS) to detect testosterone supplementation in women

Alexander Andersson [1 2], Thomas Piper [3], Lena Ekström [1 2], Angelica Lindén Hirschberg [4 5], Mario Thevis [3]

Affiliations + expand

PMID: 36564145 DOI: 10.1002/dta.3428

- Reinvestigation into samples collected to estimate the performance enhancing effects of low-dose testosterone treatment in women

• Low-dose T administrations - samples

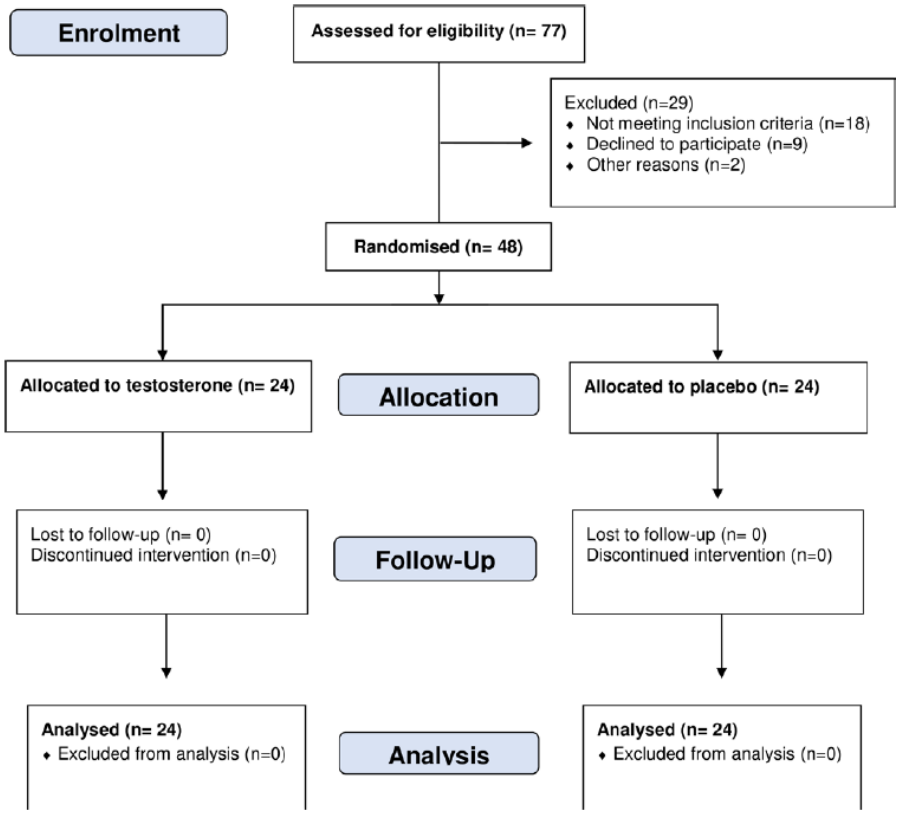
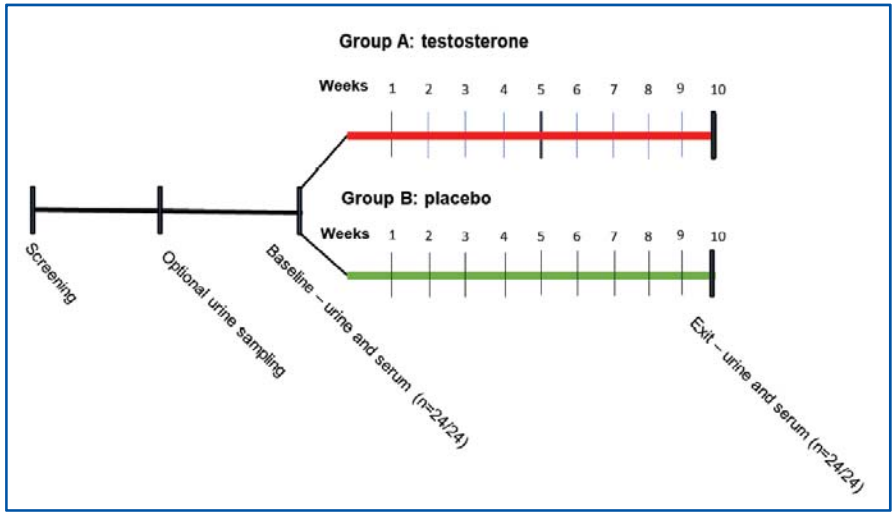


Figure 1 Flow diagram of the trial. All participants completed the study and were included in the final analysis.

Randomized Controlled Trial › Br J Sports Med. 2020 May;54(10):599-604.
doi: 10.1136/bjsports-2018-100525. Epub 2019 Oct 15.

Effects of moderately increased testosterone concentration on physical performance in young women: a double blind, randomised, placebo controlled study

Angelica Lindén Hirschberg [1 2], Jona Elings Knutsson [3 4], Torbjörn Helge [5], Manne Godhe [5], Maria Ekblom [5], Stephane Bermon [6 7], Björn Ekblom [8]



• Low-dose T administrations - samples

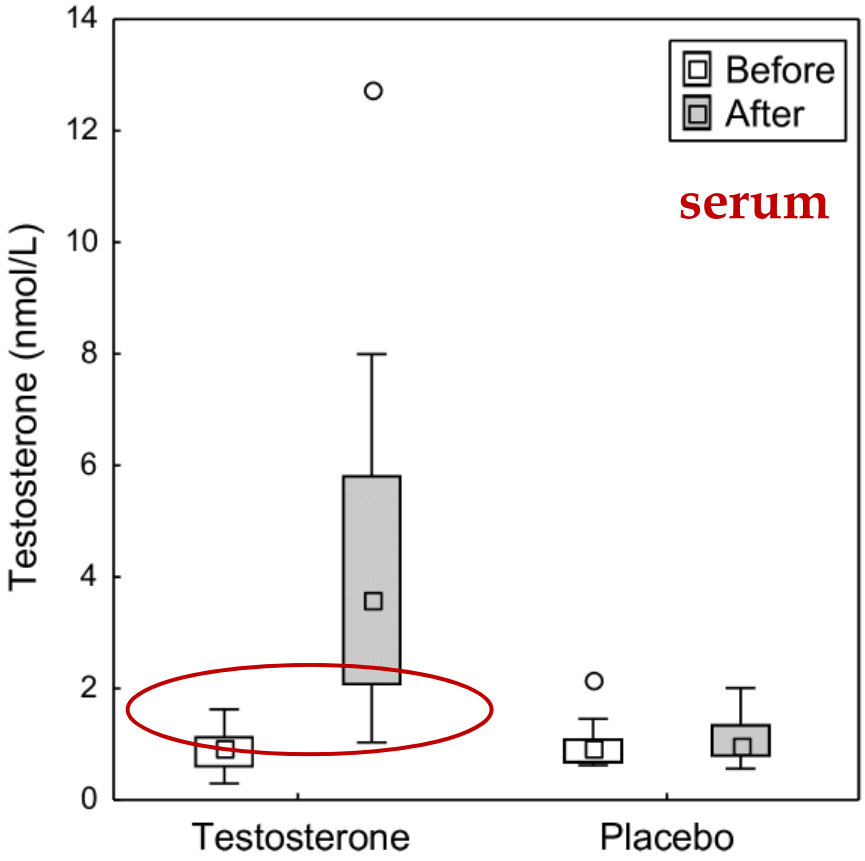


Figure 2 Serum levels of total testosterone before and after 10 weeks of treatment with testosterone or placebo. Values are median (IQR).

Randomized Controlled Trial > Br J Sports Med. 2020 May;54(10):599-604.
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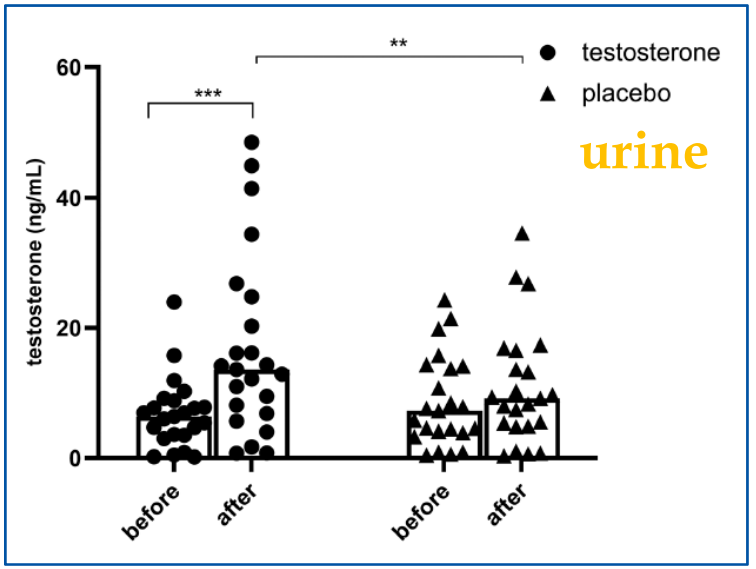
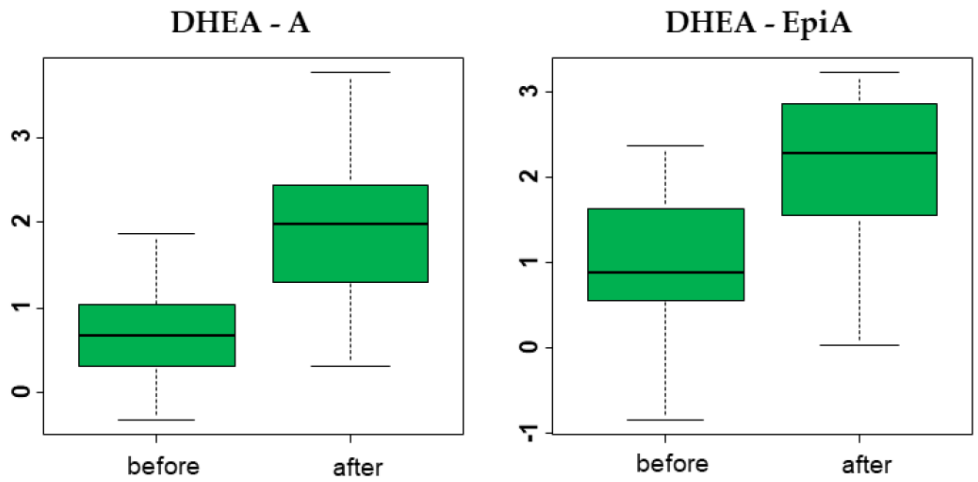
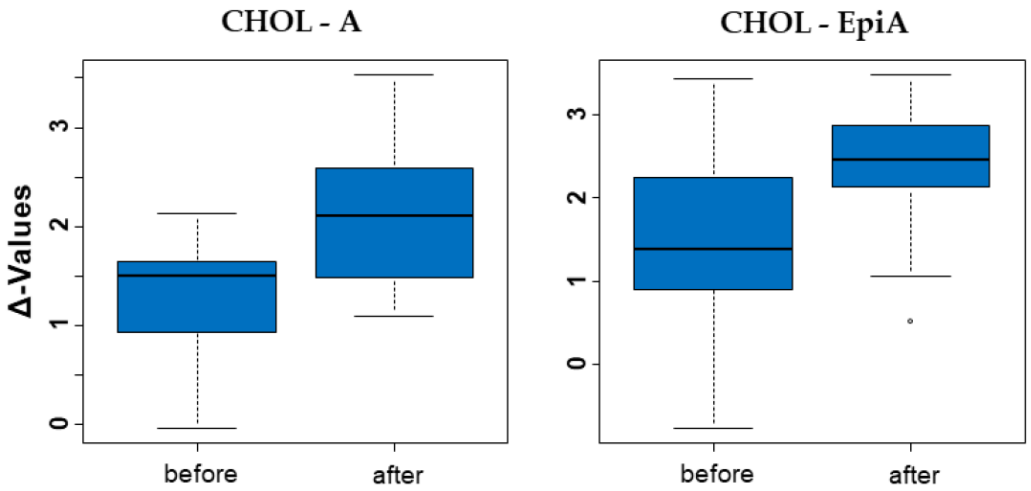


• Low-dose T administrations - IRMS

- Considering the general results, the depletion of both TCs war clearly visible!
- But not necessarily beyond established RefLims





- **Only 30 %** of all volunteers yielded AAFs compared to the populations based thresholds

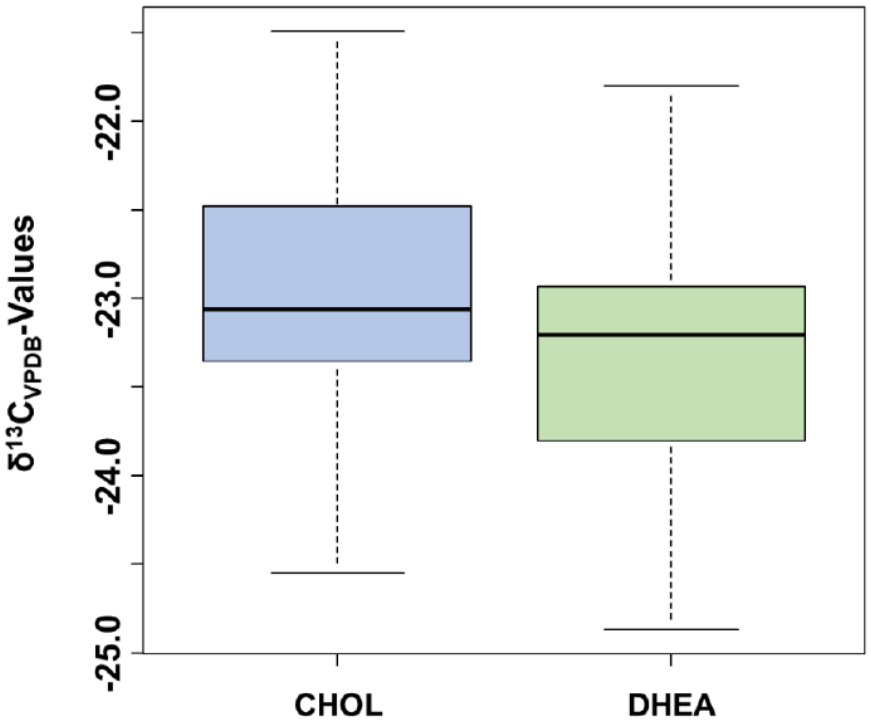
Not correlated to increased **T/E** or **T/A4**

• Possible explanation

- Enriched CIR in the North European population

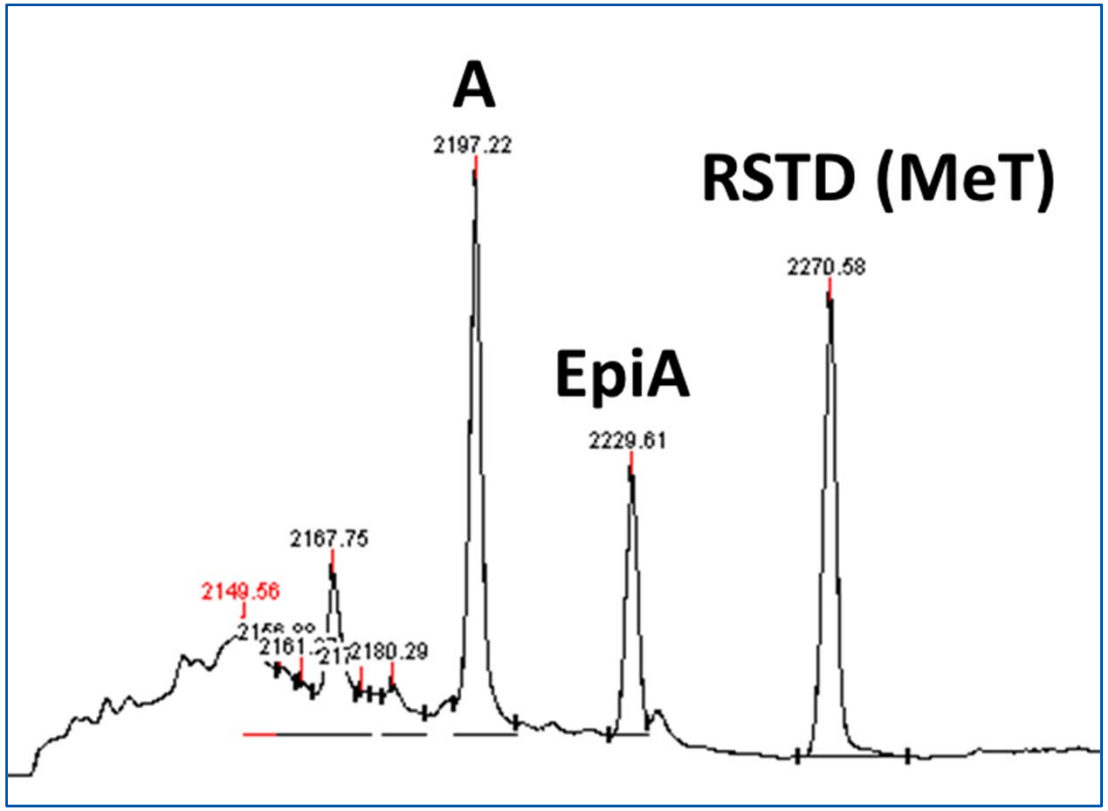
- CIR of T-Gel was not measured
- Can be expected to fall between -26 and -28 ‰

- Time of sample collection too late (8 to 14 h after treatment)
- Self-administration can result in highly diverse amounts of administered T



• Future plans

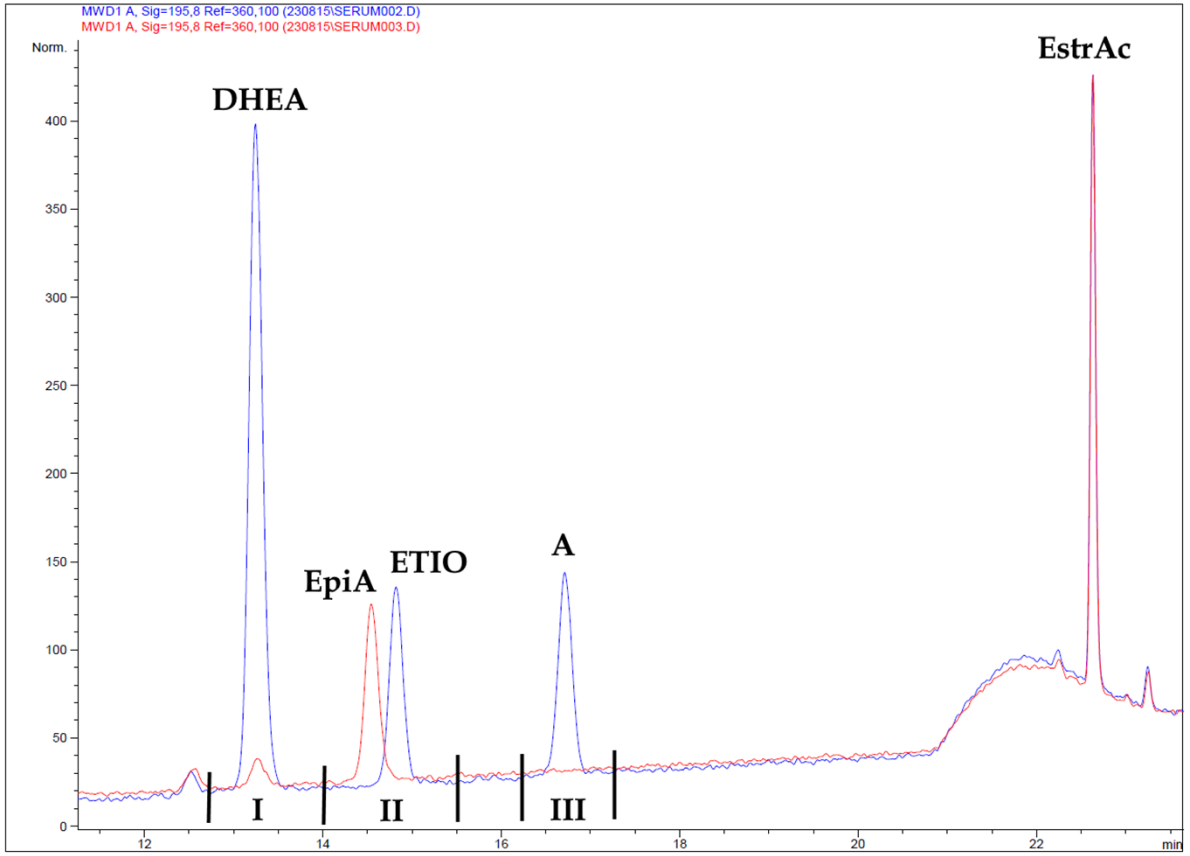
- Improving the sample clean-up employing HPLC



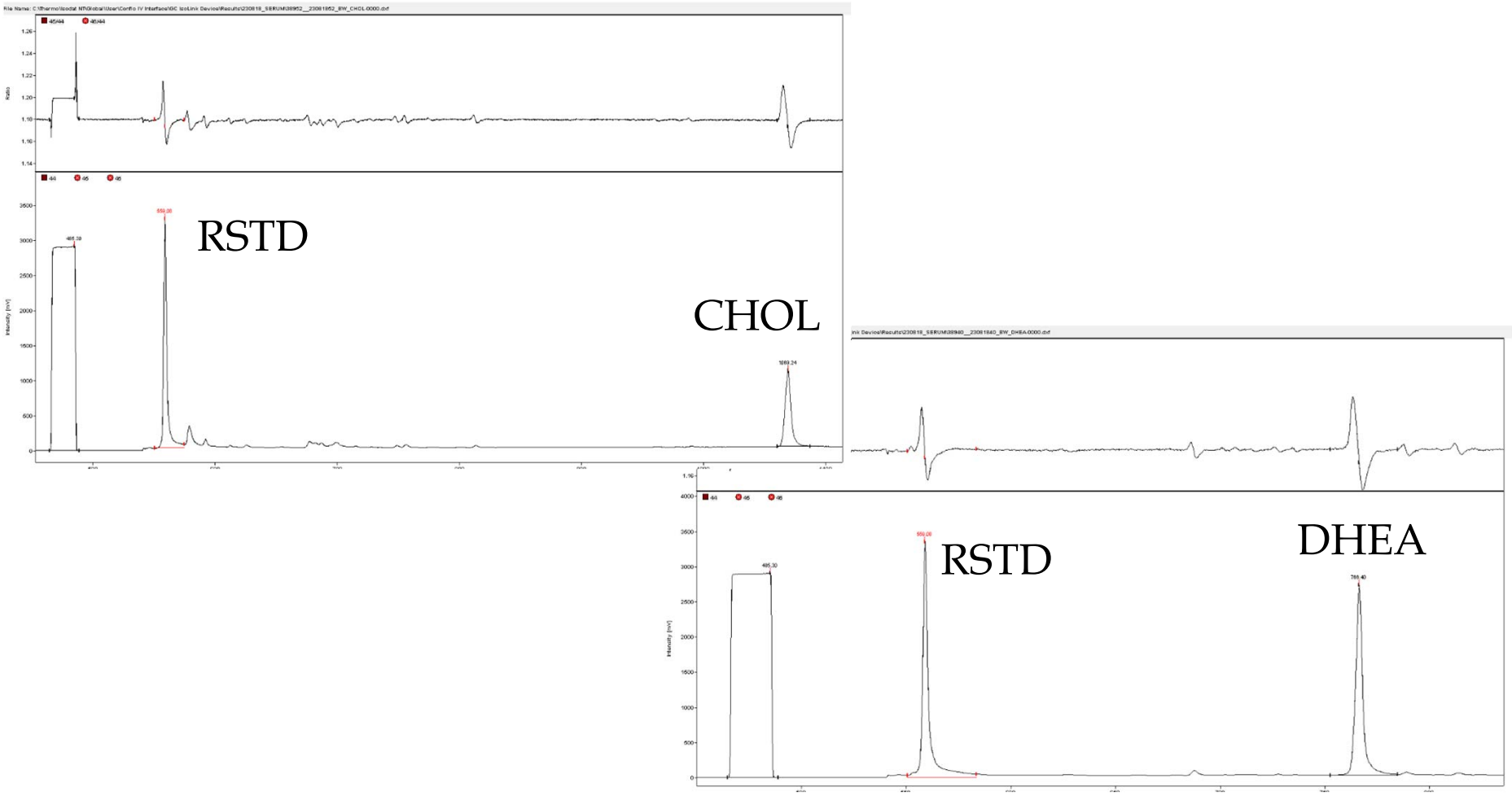
• Future plans – HPLC clean-up

- Employing routine setup yielded promising results

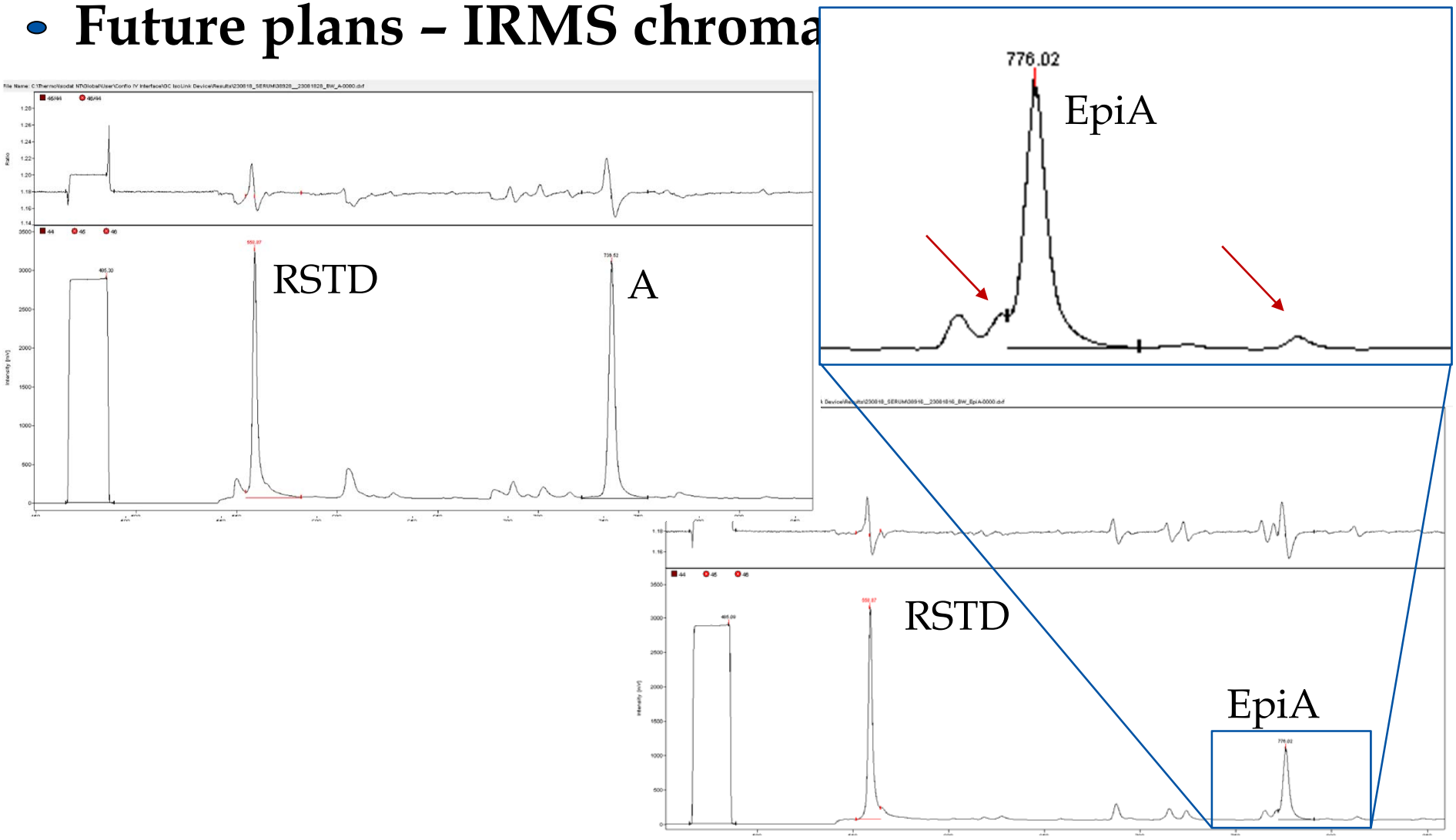
```
Control
     Column Flow  :  1.000 ml/min
     Stoptime     :    30.00 min
     Posttime     :     5.00 min

Solvents
     Solvent A 1  :  60.0 %  (H2O)
     Solvent B 1  :  40.0 %  (ACN)

Timetable

    Time     Solv.B    Flow   Pressure
|--------|-------|-------|-------|
      0.00    40.0     1.000     400
     18.00    60.0     1.000     400
     19.00    98.0     1.000     400
     30.00    98.0     1.000     400
     30.10    40.0     1.000     400
```



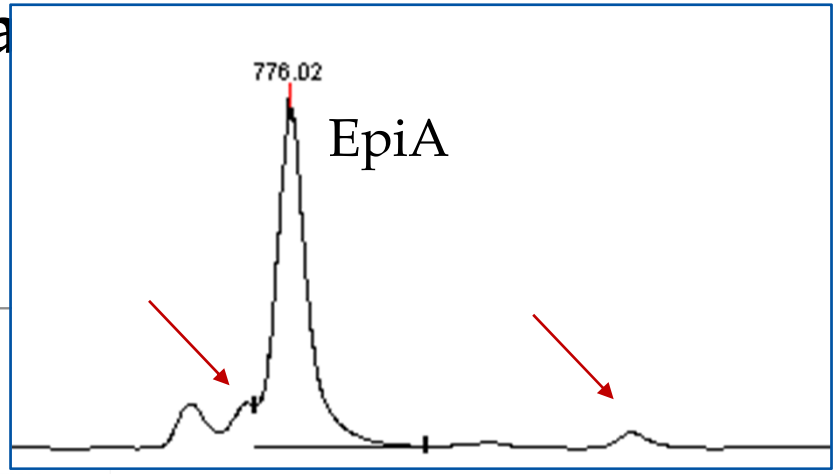
• Future plans – IRMS chromatograms

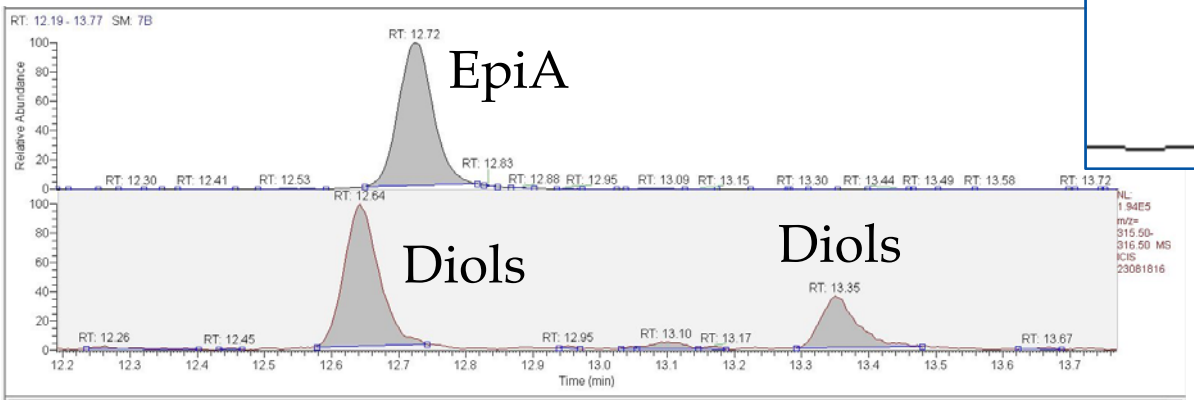


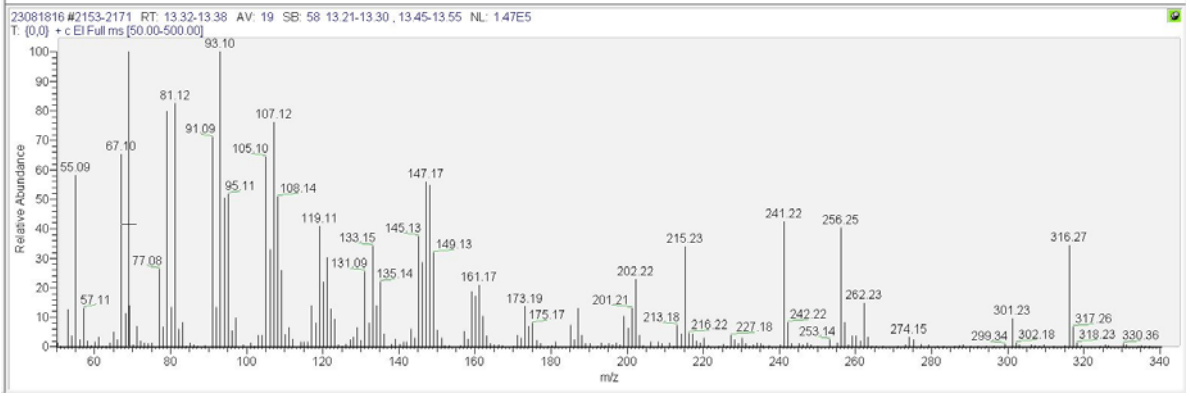
- **Future plans – IRMS chroma**



- **Future plans – IRMS chroma**







- Second HPLC-clean up after acetylation might be necessary

- **Future plans – first results**

sample	EpiA	A	DHEA	CHOL
1	-22.4	-21.7	-20.3	-21.7
2	-21.8	-20.9	-20.4	-21.7
3	-22.4	-21.4	-21.0	-21.6
4	-21.9	-20.8	-20.6	-21.8
5	-21.2	-20.3	-20.4	-21.2
6	-20.9	-20.7	-20.0	-20.7
7	-22.3	-21.5	-20.5	-21.4
8	-23.4	-22.0	-21.4	-22.1
9	-22.0	-20.4	-19.9	-21.0
BW	-20.8	-20.1	-20.0	-20.8
D	-23.1	-22.6	-20.0	-20.6
E	-23.5	-22.1	-20.1	-20.8

Negative routine doping control samples

Samples collected after T-Gel administration

Future plans

- Improving the sample clean-up employing HPLC
- First results look very promising
- Improvements for EpiA still possible
- **To Do**:
  - Full validation
  - Re-investigation into Reference Population
  - This hopefully improves RefLims…

⟹ **Confirmation method for serum steroids**

Thank you for your kind attention!