Derivatize or not, our experiences from both, - and some cases from the real IRMS life

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IRMS in the Norwegian Doping Control Laboratory

- Instruments
  - 2 GC/C/IRMS System (Thermo)
  - Delta V Plus
  - One coupled to ISQ single quadrupole mass spectrometer
  - Two Agilent 1260 HPLC systems
- Approximately 100 samples pr year
- Staff:
  - Certifying scientist/responsible scientist
  - Technician (also other tasks)
  - Certifying scientist 2 (Ingunn)

Routine Method

- In use from 2019
  - SPE (C-18)
  - Removal of free steroids (exctraction with TBME)
  - Enzymatic hydrolysis
  - Extraction with TBME
  - One HPLC clean-up

  - No derivatization
  - GC Column: Nonpolar (5%-Phenyl)-methylpolysiloxane

Previous Routine Method

- In use from 2010 to 2019
  - SPE (C-18)
  - Removal of free steroids (extraction with TBME)
  - Enzymatic hydrolysis
  - SPE (C-18)
  - 1. HPLC clean-up
  - Acetylation
  - 2. HPLC clean-up

  - Two HPLC clean-ups
  - Derivatization of testosterone, 5a-diol, 5b-diol
  - GC Column: Mid polar (50%-phenyl)-methylpolysiloxane

Pros and cons for the old method

- Robust HPLC clean-up
- No need for adjusting the collection times
- Very clean extracts

- Time consuming
- Changes of acetate correction factor
- Batch variations of derivatization reagents
- Less robust GC column

Decided to go for a new sample preparation without derivatization

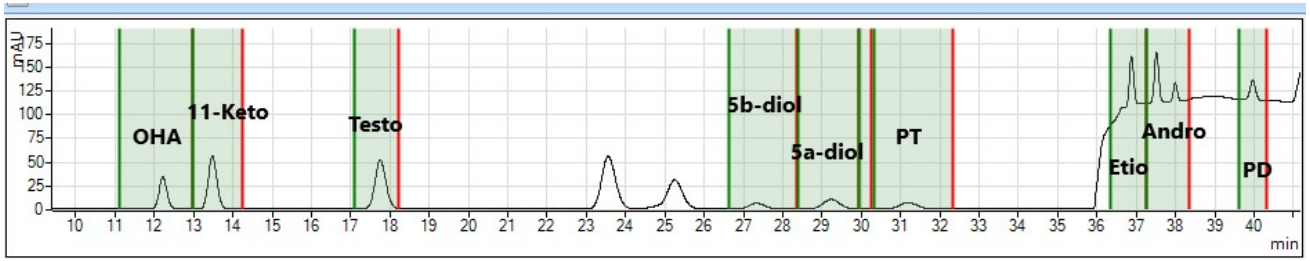
Sample preparation – urine samples

- Urine volume 1-20 mL
- SPE (500mg Bond-Elute C18)
- Hydrolysis with β-Glucuronidase
- Extraction with tert-butyl methyl ether

- **HPLC sample preparation**
- Column: ACE 5 C18 (250 mm× 4.6mm, 5µm)
- Injection volume: 50 µL
- Temperature: 38 °C
- Flow: 1 ml/min.
- Mobile phase: ACN og H_2O
- Monitored wavelengths: 192 nm and 254 nm
- Endogenous reference compounds (ERC):
  - Pregnanediol (PD), 11-OH-Androsterone (OHA), 11-Ketoetiocholanolone (11-Keto), Pregnanetriol (PT)

Gradient:

Tid (min.)	% ACN
0,00	38
32,50	38
32,51	55
33,51	55
38,00	65
38,01	100
48,00	100
50,00	38
62,00	38



Chromatographic Conditions - IRMS

- Chromatographic Conditions
  - Column: Agilent J&W HP5 MS UI (30 m, i.d. 0.25 mm, film thickness 0.25 µm)
  - Injection mode: Split-less
  - Injection volume: 1-3 µL

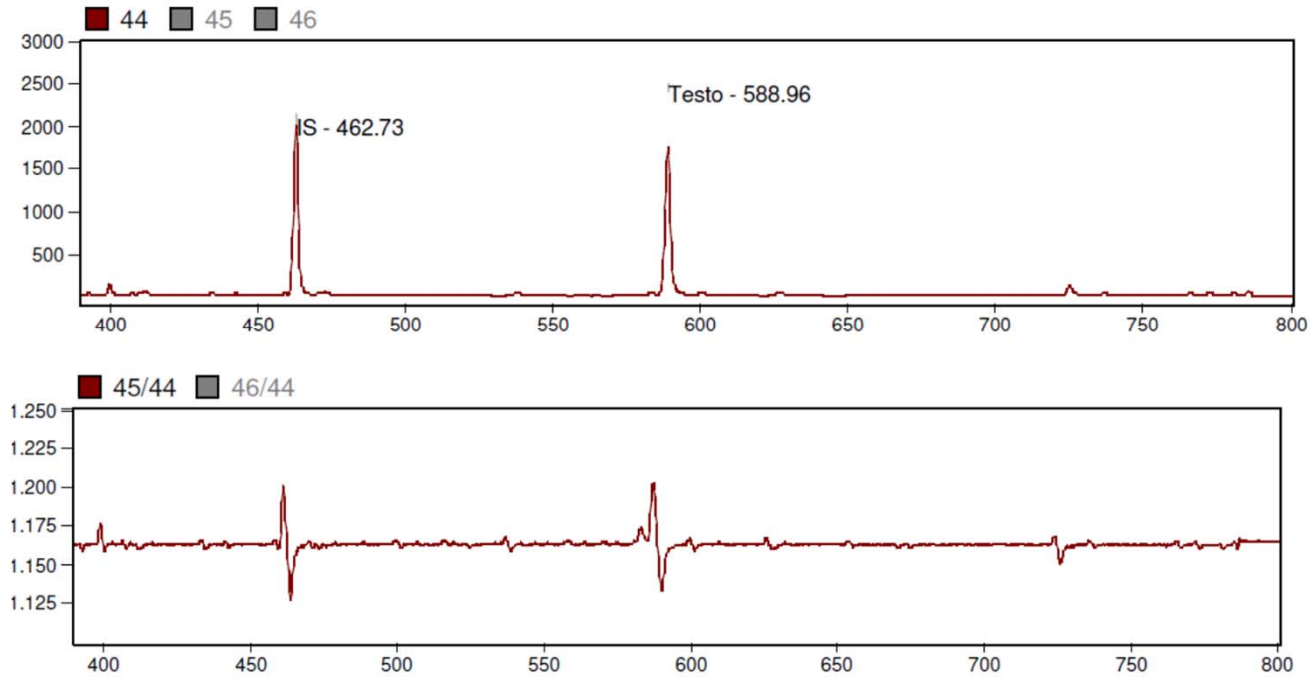
  - The sample preparation and the analytical method was based on:
    - de la Torre X, Colamonici C, Curcio D, Molaioni F, Botrè F. Anal Chim Acta. 2012 Dec 5;756:23-9.

TCs ad ERCs

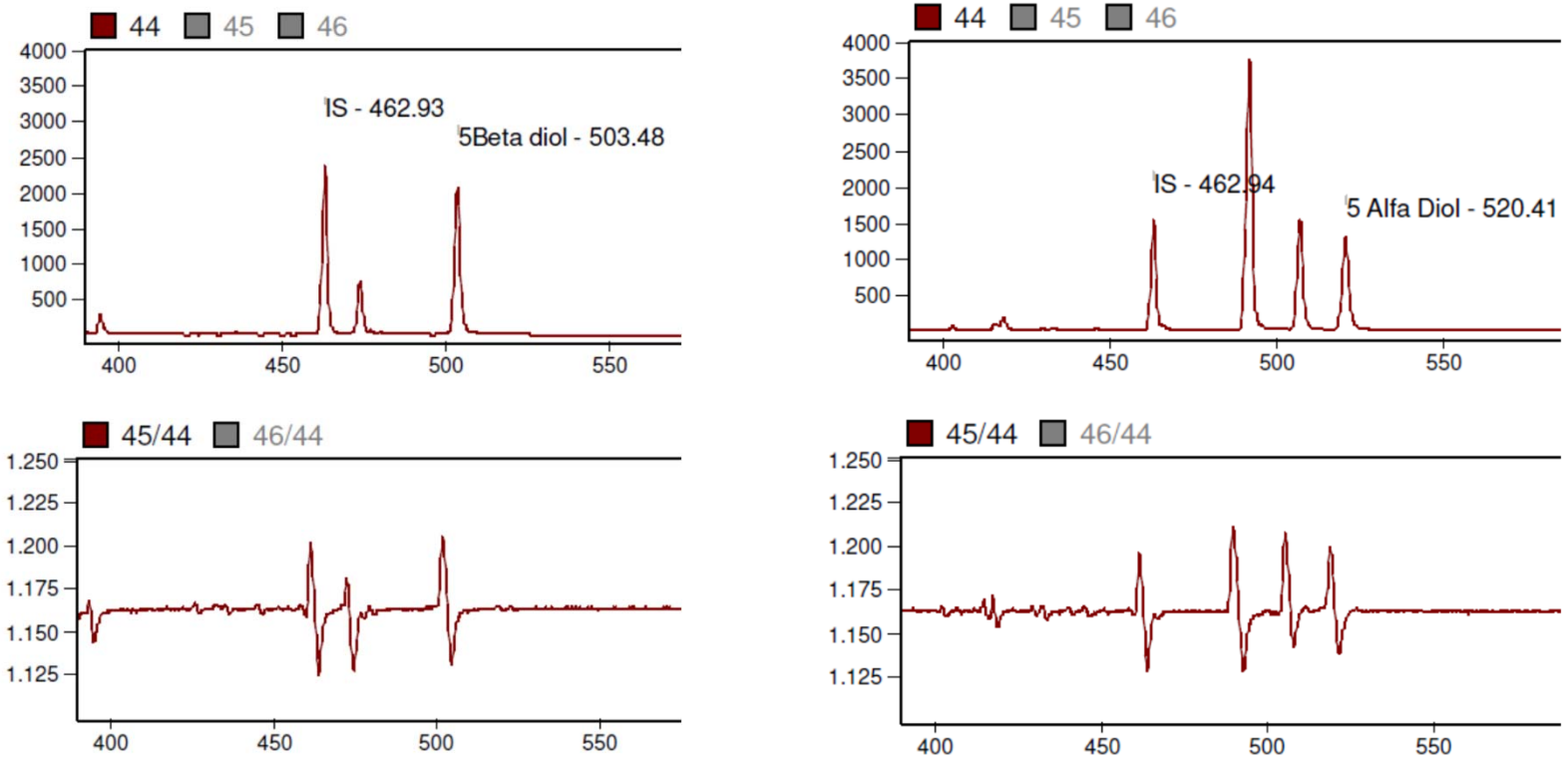
Run time:

Fraction	Compound	
F1	11β-OH-androsterone	ERC 2
F2	11-oxo-etiocholanolone	ERC 4
F3	Testosterone	
F4	DHEA	
F5	Epitestosterone	
F6	5β-Adiol	
F7	5α-Adiol	
F8	Pregnanetriol	ERC 3
F9	Etiocholanolone	
F10	Androsterone	
F11	Pregnanediol	ERC 1

IRMS chromatograms of Fraction F3



IRMS chromatograms of Fraction F6 and F7



Pros and cons for the new method

- Faster!
- Only one HPLC cleanup
- No worries for acetylation factors
- Longer lifetime for the GC column

- Fraction collection needs more attention
- Training and hands-on experience is important

Challenges in daily routine

- Naturally low delta-values in Scandinavia
  - $\delta^{13}C$ value for PD lower than -25 ‰
- Some interferences in two of the fractions
  - Settings for fraction collection is very important
  - The UV chromatogram is evaluated for all samples and retention time for methyltestosterone, testosterone, androsterone and etiocholanolone are followed

Depleted ERCs
Sample: T/E 195

	δ $^{13}C/^{12}C$	Δδ ERC1	ERC2 Δδ	ERC4 Δδ
Androsterone	-28,5 ‰	2,8 ‰	5,1 ‰	5,1 ‰
Etiocholanolone	-28,2 ‰	2,5 ‰	4,8 ‰	4,8 ‰
5b-androstandiol	-27,4 ‰	1,7 ‰	4,0 ‰	4,0 ‰
Testosterone	-29,5 ‰	3,8 ‰	6,1 ‰	6,1 ‰
Pregnanediol (ERC1)	-25,7 ‰			
11-OHA (ERC2)	-23,4 ‰			
11-keto (ERC4)	-23,4 ‰			

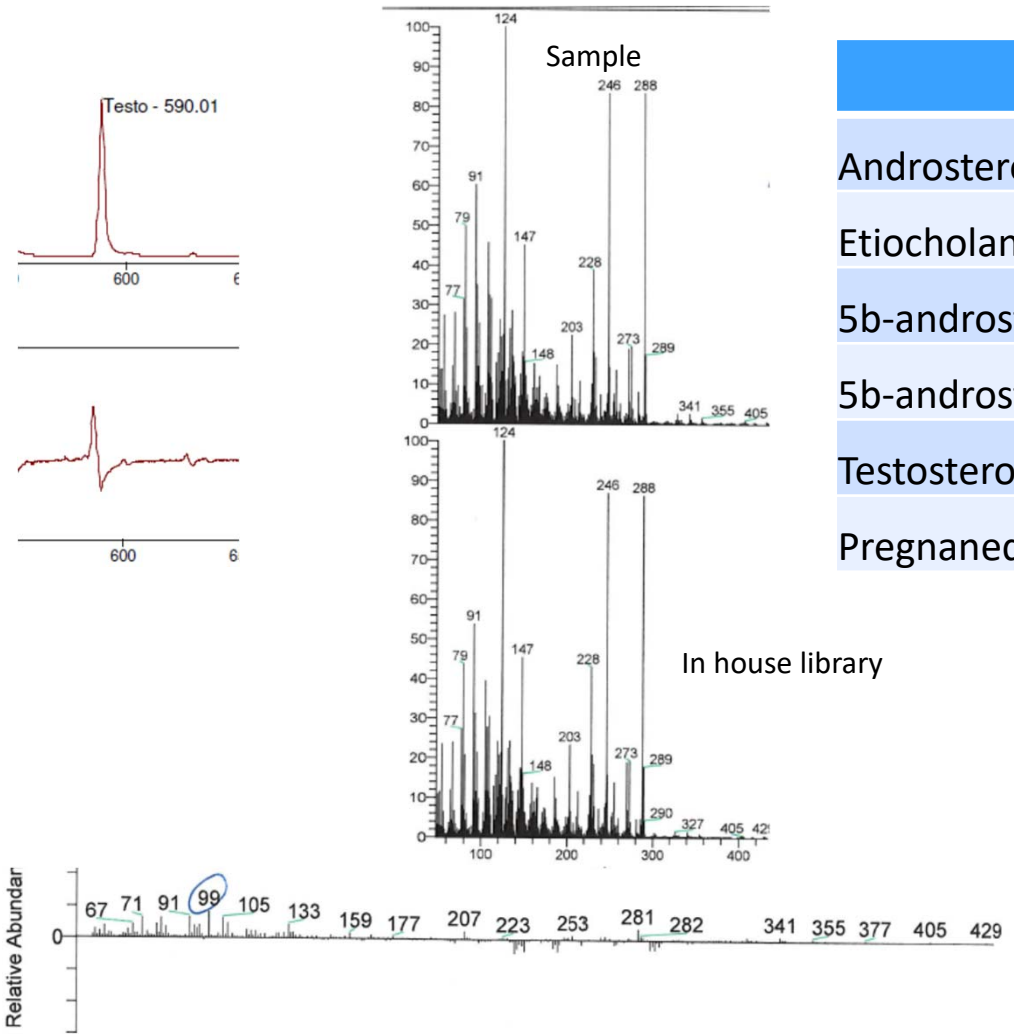
ERC 3 (PT): < LOD

Interfering compounds

- The most «dangerous»: When not obvious from peak shape.
  - Evaluation of spectrum from GC-MS single quad is helpful
  - Sometimes a low interference still may affect delta values!

  - Report without delta values for the affected compound!

Depleted T or interfering compound present?



	δ $^{13}C/^{12}C$		Δδ ERC1	
Androsterone	-21,9	‰	-0,5	‰
Etiocholanolone	-23,2	‰	0,8	‰
5b-androstandiol	-23,7	‰	1,2	‰
5b-androstandiol	-23,1	‰	0,7	‰
Testosterone	-27,6	‰	5,1	‰
Pregnanediol (ERC1)	-22,5	‰		

Not obvious from spectrum

Acetylation of the testosterone fraction

- Acetylation of testosterone fractions of sample, QCN and QCP
- Results:

Testosterone sample	-24,29	‰	
Testosterone QCN	-25,69	‰	accepted
Testosterone QCP	-29,63	‰	accepted

Followed acetylation procedure from previous routine method

Sample was reported negative, without delta value for T

Suspicous?

Compound	Concentration (SG corrected)	
Androsterone	8834	ng/mL
Etiocholanolone	3903	ng/mL
5a-androstandiol	184	ng/mL
5b-androstandiol	178	ng/mL
Epitestosterone	12	ng/mL
Testosterone	41	ng/mL

Ratios	May 23	Jan-23
T/E	3.48	2.8
5a-diol/E	15	3.1
5a-diol/5b-diol	1.0	0.63

All concentrations higher, except epitestosterone

IRMS was recommended based on higher concentrations and elevated 5a-diol/E in the sample from May 23

IRMS results

Compound	$\delta^{13}C/^{12}C$	$\Delta\delta$ ERC1	$\Delta\delta$ ERC2
Androsterone	-28,0 ‰	3,1 ‰	4,3 ‰
Etiocholanolone	-29,9 ‰	5,1 ‰	6,2 ‰
5a-androstandiol	-29,3 ‰	4,5 ‰	5,6 ‰
5b-androstandiol	-29,8 ‰	4,9 ‰	6,1 ‰
Testosterone	-26,5 ‰	1,7 ‰	2,8 ‰
Pregnanediol (ERC1)	-24,8 ‰		
11-OHA (ERC2)	-23,7 ‰		

Compound	$\delta^{13}C/^{12}C$	$\Delta\delta$ ERC1	$\Delta\delta$ ERC2
DHEA	-27,8 ‰	3.4 ‰	4.1 ‰
Pregnanediol (ERC1)	-24,4 ‰		
11-OHA (ERC2)	-23,8 ‰		

Case 1: IRMS in Helsinki Lab

Sample 1: **T/E 8.5**
Collected 13-Jun-2021
Result: ATF

	δ13C	Δδ
Androsterone	-25,92 ‰	1,5 ‰
Etiocholanolone		
5αAdiol		
5βAdiol		
Pregnanediol		

Sample 2: **T/E -1 (E<LOD)**, low steroids
Collected 5-Oct-2021
Result: NEG

	δ13C	Δδ
Androsterone	-26,41 ‰	1,8 ‰

What can we do?

- Other samples collected?
- Serum had been collected on 5-Oct and 29-Oct
- Analysis for testosterone esters showed presence of testosterone propionate

Sample 3: **T/E 59**
Collected 29-Oct-2021
Result: NEG

Testosterone	-24,13 ‰	0,7 ‰
Androsterone	-24,47 ‰	0,4 ‰
Etiocholanolone	-24,81 ‰	0,1 ‰
5αAdiol	-25,53 ‰	0,6 ‰
5βAdiol	-24,68 ‰	0,2 ‰
11β-hydroxyandrosterone	-24,54 ‰	

Testosterone		
Androsterone	-26,45 ‰	1,4 ‰
Etiocholanolone	-26,71 ‰	1,7 ‰
5αAdiol	‰	‰
5βAdiol	-27,19 ‰	2,2 ‰
Pregnanediol	-24,98 ‰	

Thanks to

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And you for your attention