

Appendix A. Supplementary data

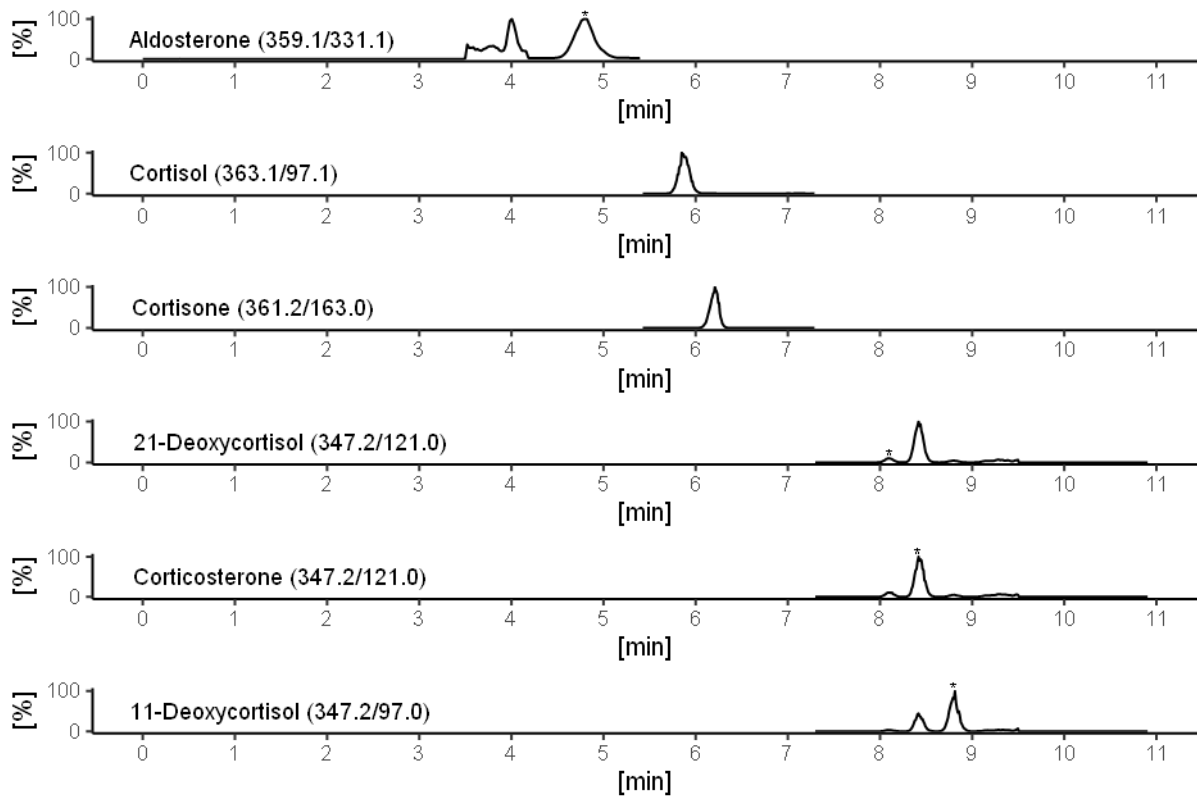


Figure A 1: Chromatogram of quantifier MRMs in panel 1 of the commercial kit. Relative intensity is given in %. Respective MRM transition (m/z) is shown behind each steroid. In chromatograms with more than one isobaric signal, target steroid is marked with an asterisk.

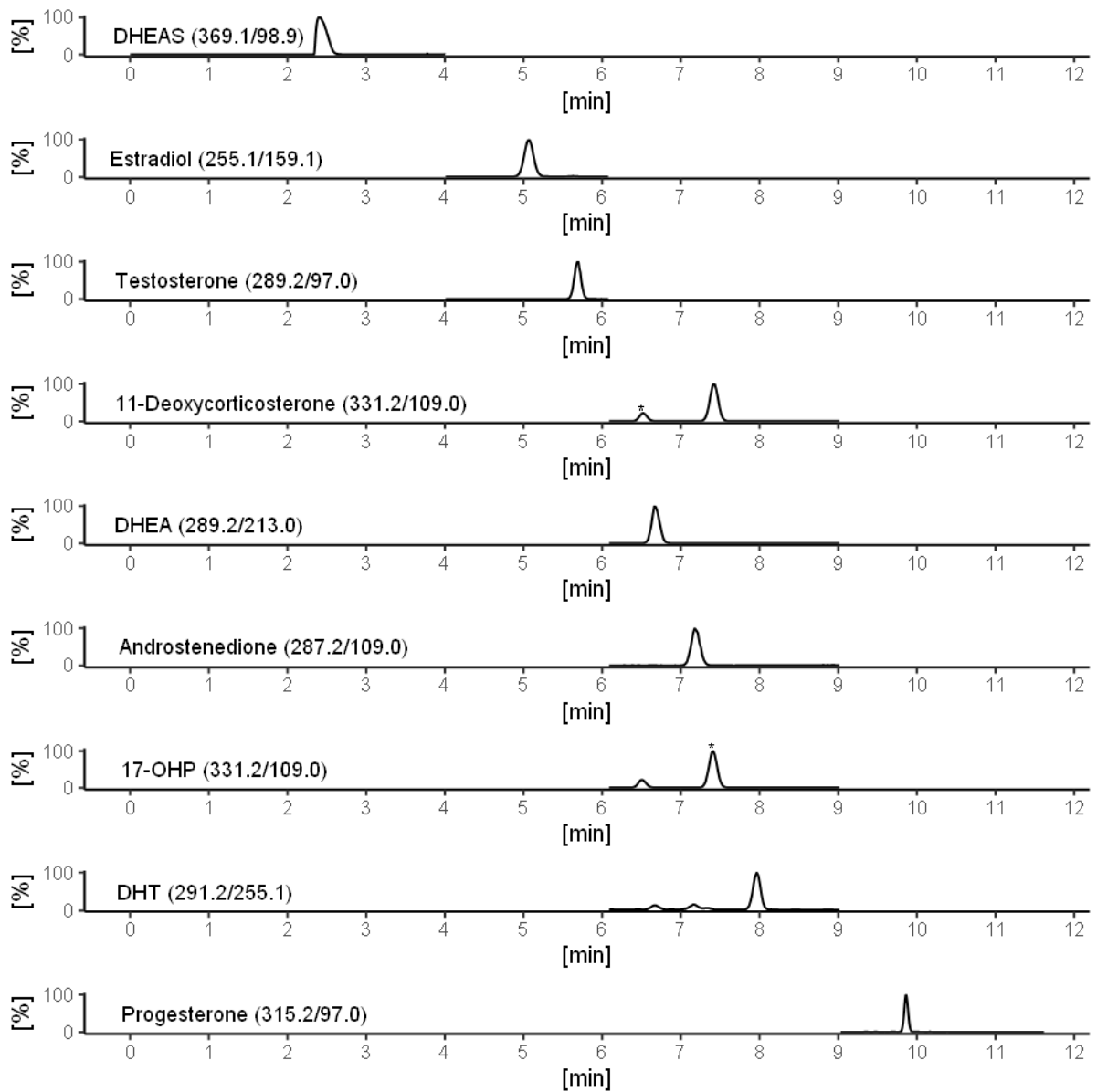


Figure A 2: Chromatogram of quantifier MRMs in panel 2 of the commercial kit. Relative intensity is given in %. Respective MRM transition (m/z) is shown behind each steroid. In chromatograms with more than one isobaric signal, target steroids are marked with an asterisk.

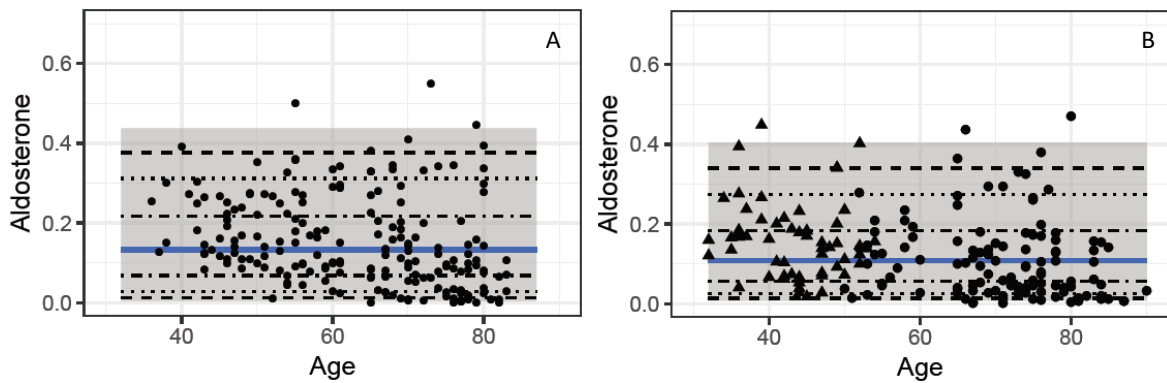


Figure A 3: Distribution of aldosterone concentrations (nmol/L) against age in years in samples with detectable aldosterone (A - males, B - females). For females, triangles represent premenopausal status, dots represent postmenopausal status. Reference interval could not be calculated because more than 30% of values were below LoQ. To roughly estimate 97.5 percentile and to show distribution of aldosterone concentrations for clinical interest, we excluded values below LoQ from the calculation. The mean concentrations estimated from concentrations in samples with measurable aldosterone after transformation are represented by the blue line. The 95% confidence interval is marked grey, whereas the dashed lines illustrate the 50%, 80% and 90% confidence intervals. The 97.5 percentile was calculated to be 0.44 nmol/L for males and 0.40 nmol/L for females.

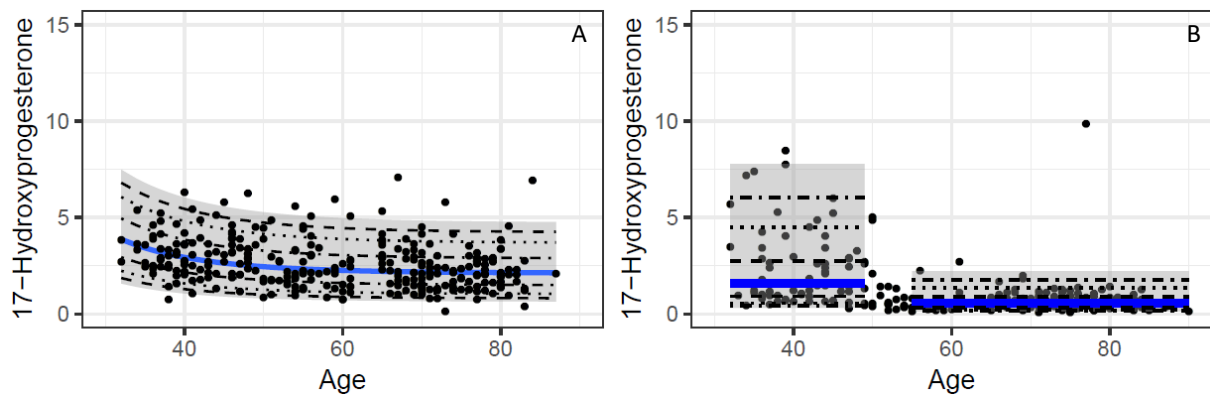
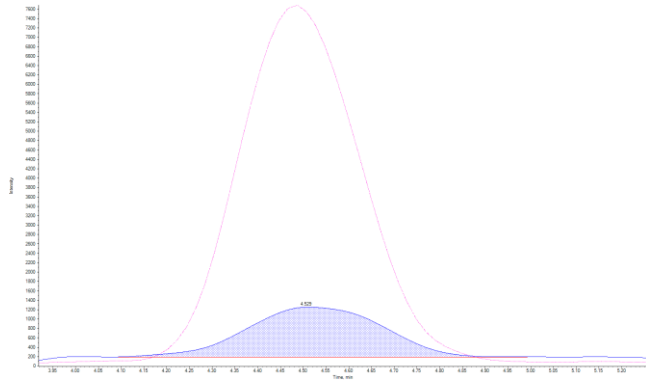


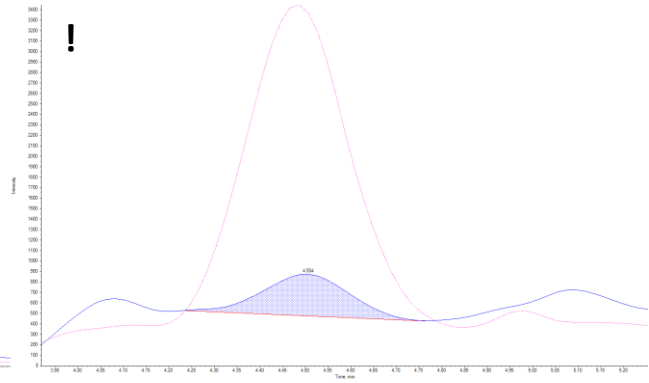
Figure A 4: Age dependent distribution of 17-OHP concentrations (nmol/L) for males (A) and females (B). Mean concentrations estimated after transformation are represented by the blue line. The 95% interval is marked grey, whereas the dashed lines illustrate the 50%, 80% and 90% confidence intervals. Reference intervals were calculated according to menopausal state for this steroid. For better visualization, the reference intervals (grey shaded areas) are not including the age group from 50-55 years, with overlapping pre- and postmenopausal states.

Panel 1

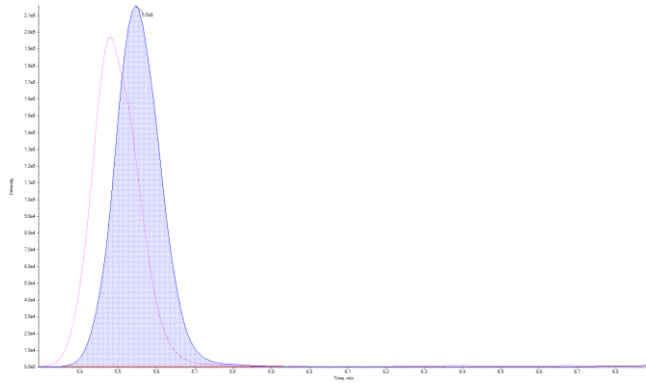
Aldosterone (359.1/331.1) calibration level 3



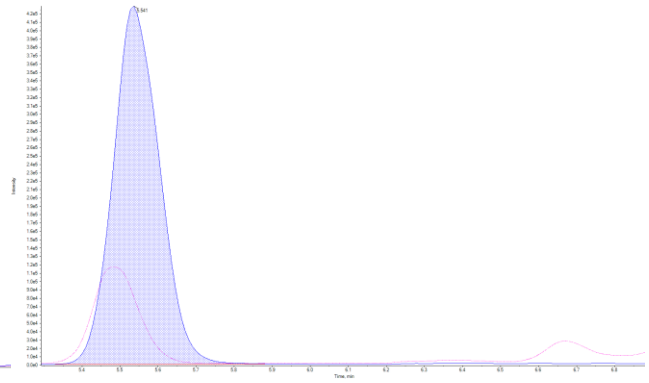
Aldosterone (359.1/331.1) serum sample



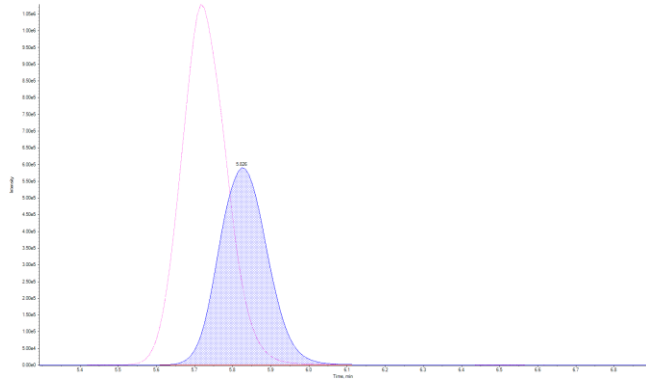
Cortisol (363.1/97.1) calibration level 3



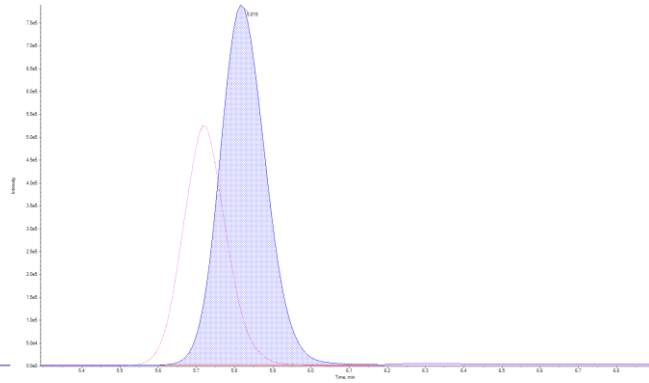
Cortisol (363.1/97.1) serum sample



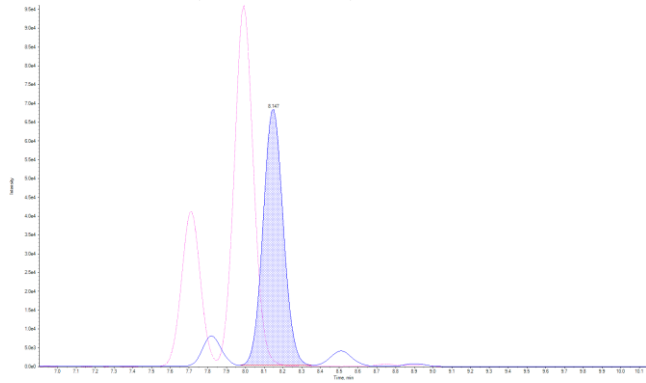
Cortisone (361.2/163.0) calibration level 3



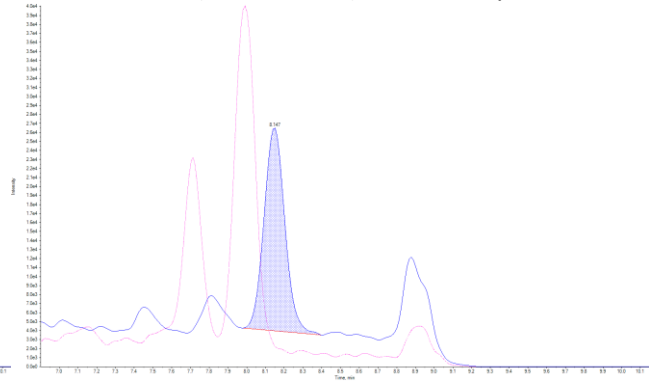
Cortisone (361.2/163.0) serum sample



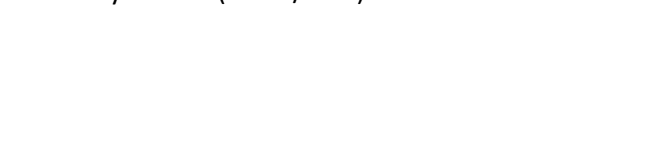
Corticosterone (347.2/121.0) calibration level 3



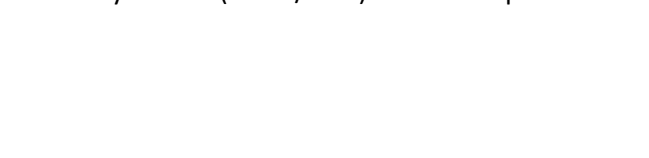
Corticosterone (347.2/121.0) serum sample

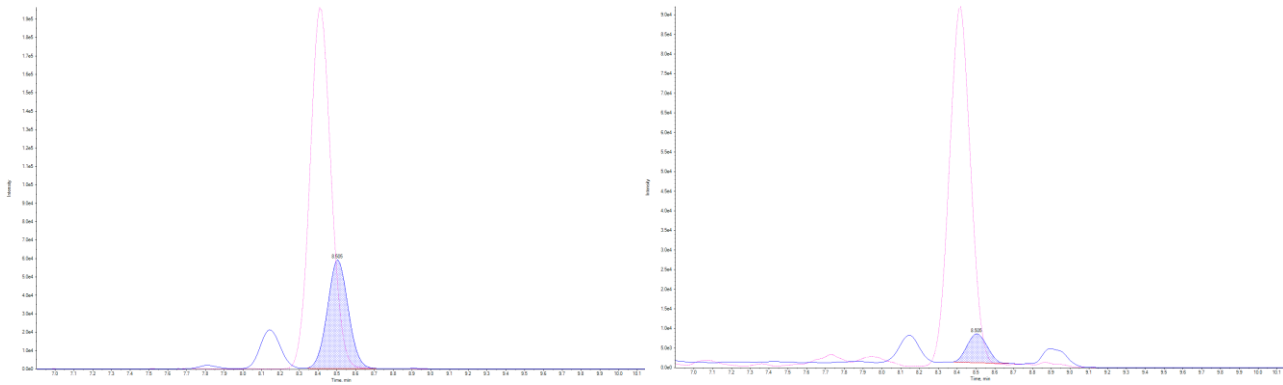


11-Deoxycortisol (347.2/97.0) calibration level 3



11-Deoxycortisol (347.2/97.0) serum sample





21-Deoxycortisol (347.2/121.0) calibration level 3

21-Deoxycortisol (347.2/121.0) serum sample

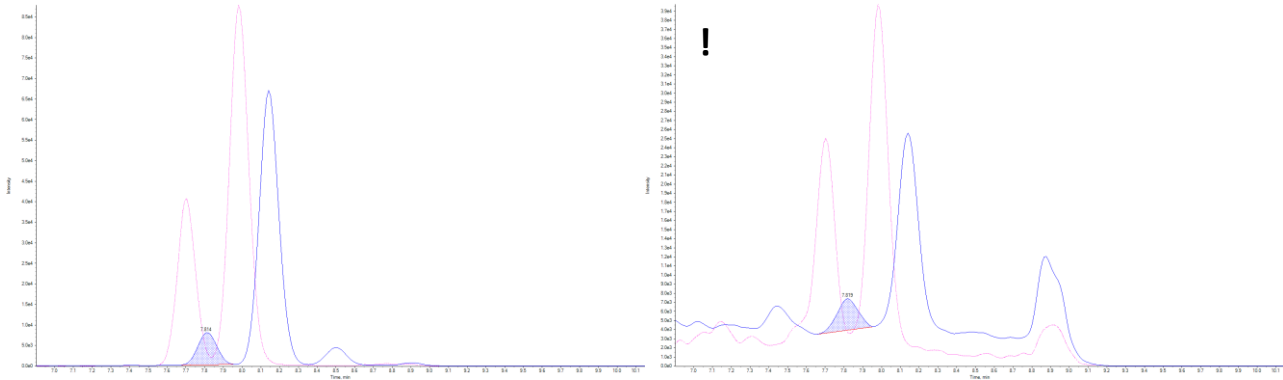
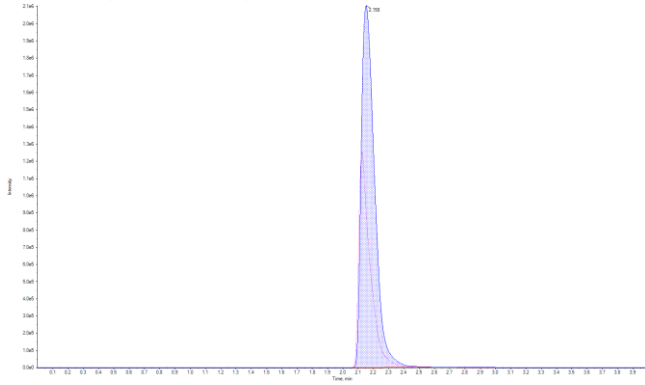


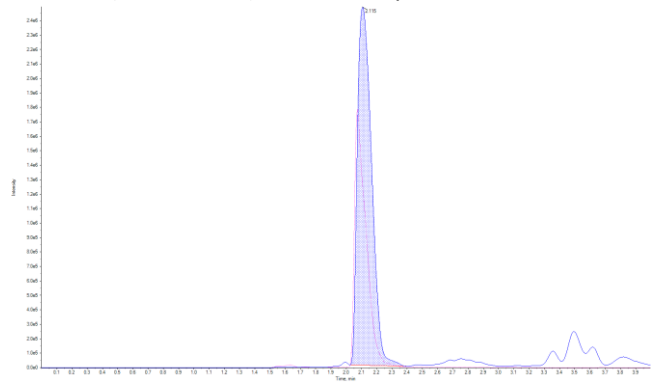
Figure A 5: MRM chromatogram of steroids (panel 1) in calibration concentration level 3 (left) and serum sample (right). MRM transition from Q1 to Q3 (m/z) is given in brackets. MRM signal of the qualifier transition is marked blue, corresponding internal standard signals are shown in pink. Steroids that did not result in reference intervals for all or one of the sex/age groups due to low concentrations or chromatographic difficulties are marked with exclamation mark. Chromatograms of isobars 21-deoxycortisol, corticosterone and 11-deoxycortisol (m/z 347.2) are showing more than one signal. In chromatogram of corticosterone, 21-deoxycortisol is eluting left next to the target signal and vice versa in chromatogram of 21-deoxycortisol. In chromatogram of 11-deoxycortisol, corticosterone is eluting left to the target signal. The three isobars elute in the order: 21-deoxycortisol, corticosterone, 11-deoxycortisol.

Panel 2

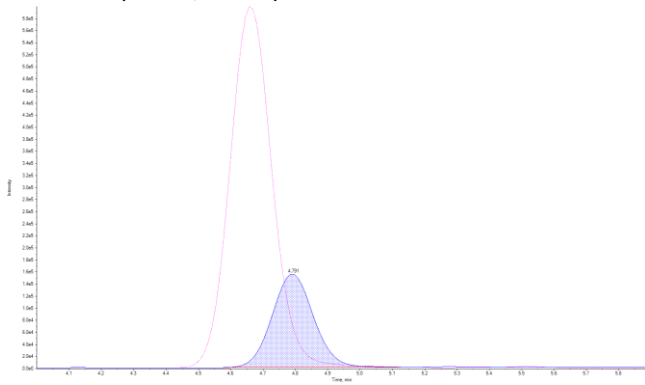
DHEA-S (369.1/98.9) calibration level 3



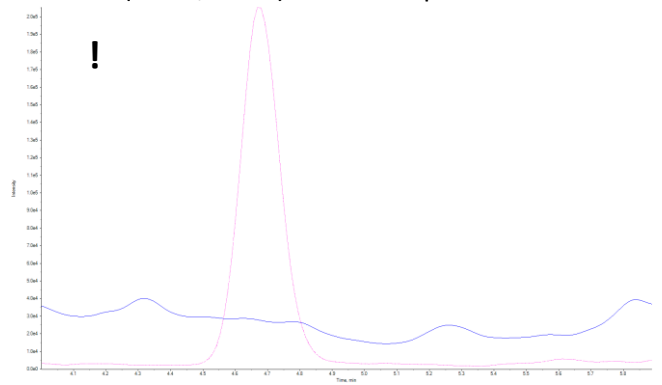
DHEA-S (369.1/98.9) serum sample



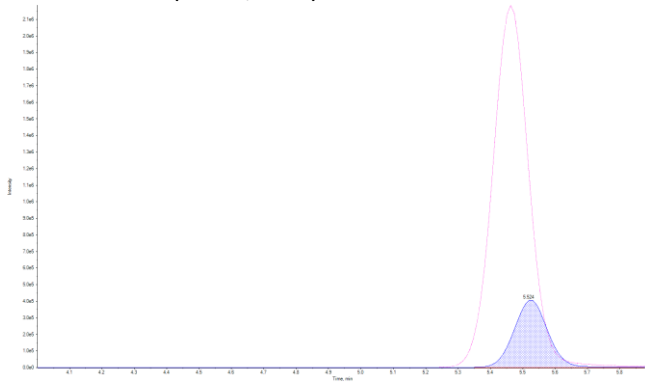
Estradiol (255.1/159.1) calibration level 3



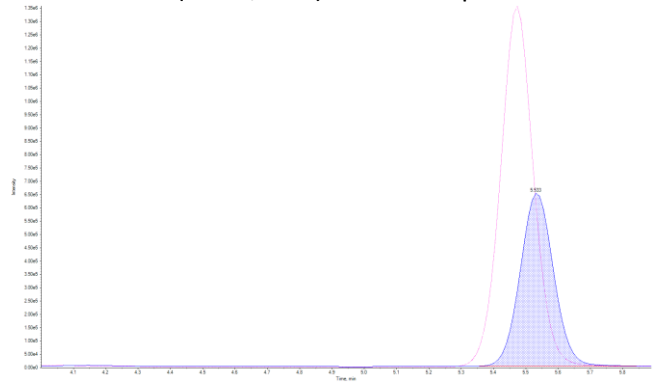
Estradiol (255.1/159.1) serum sample



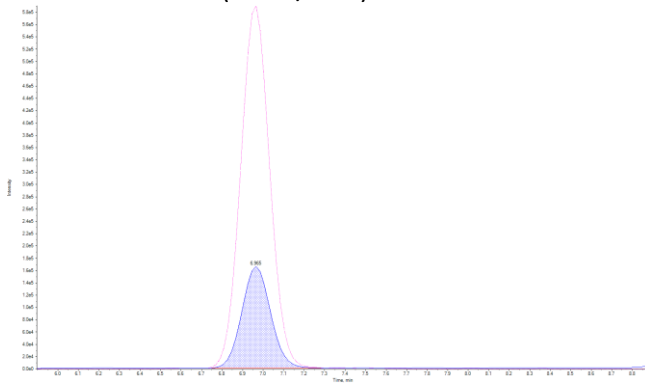
Testosterone (289.2/97.0) calibration level 3



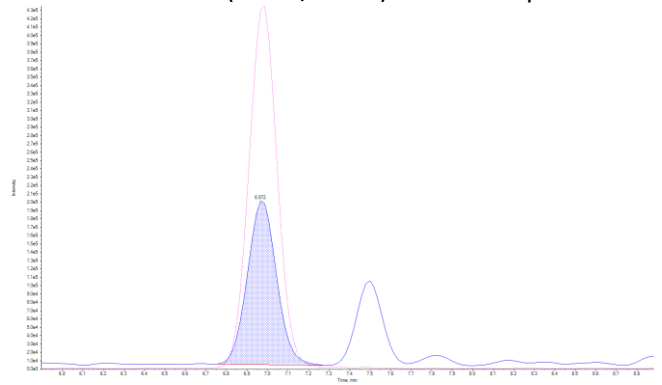
Testosterone (289.2/97.0) serum sample



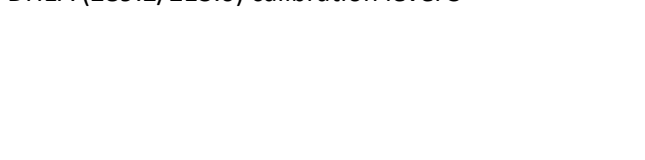
Androstenedione (287.2/97.0) calibration level 3



Androstenedione (287.2/109.0) serum sample

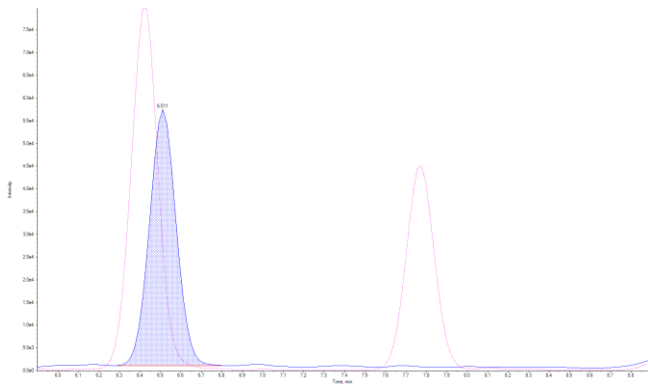


DHEA (289.2/213.0) calibration level 3

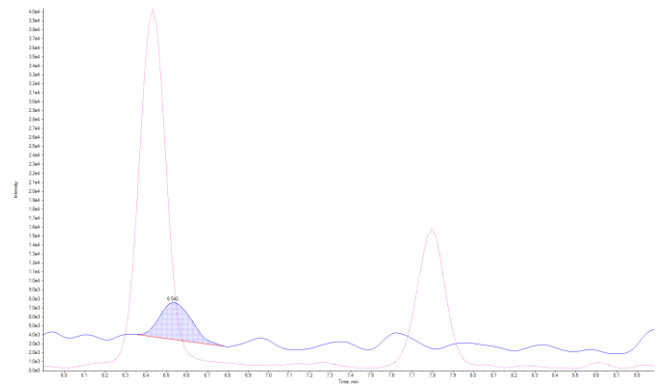


DHEA (289.2/213.0) serum sample

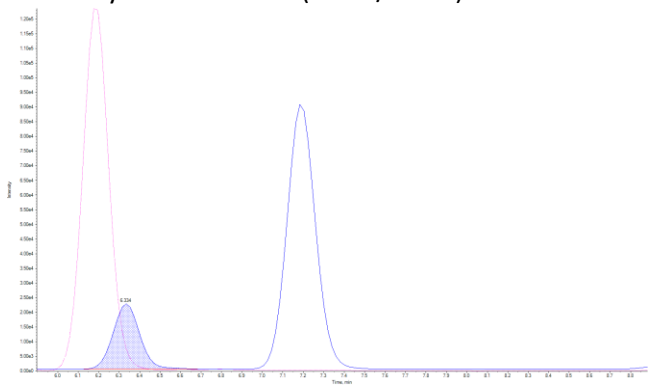




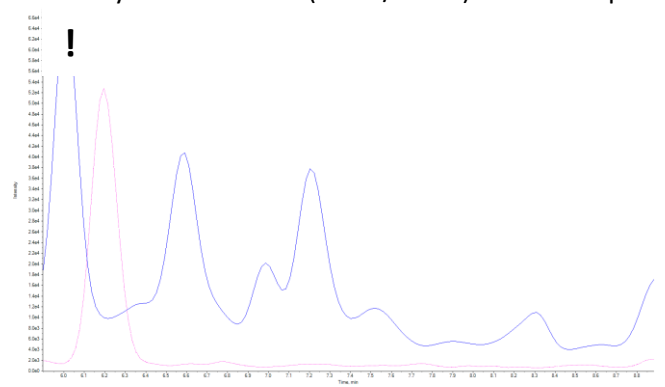
11-Deoxycorticosterone (331.2/109.0) calibration level 3



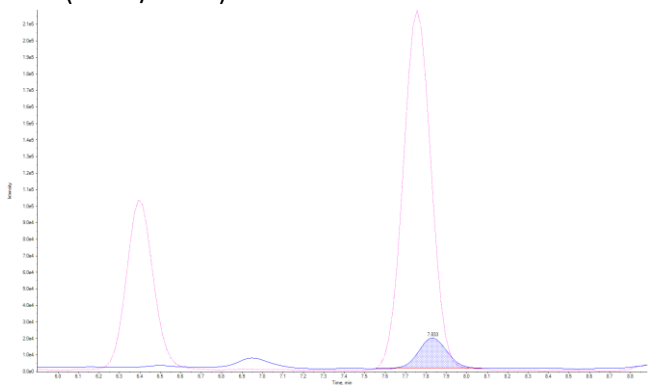
11-Deoxycorticosterone (331.2/109.0) serum sample



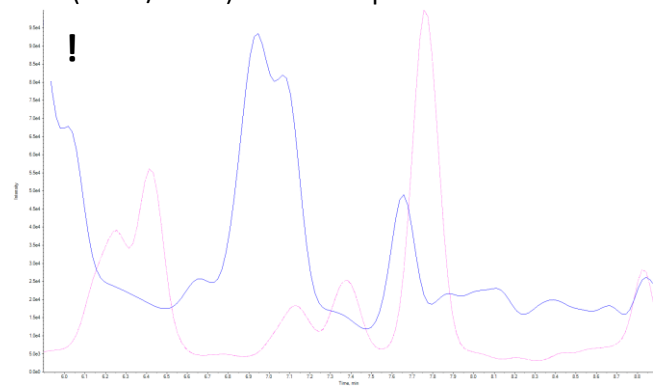
DHT (291.2/255.1) calibration level 3



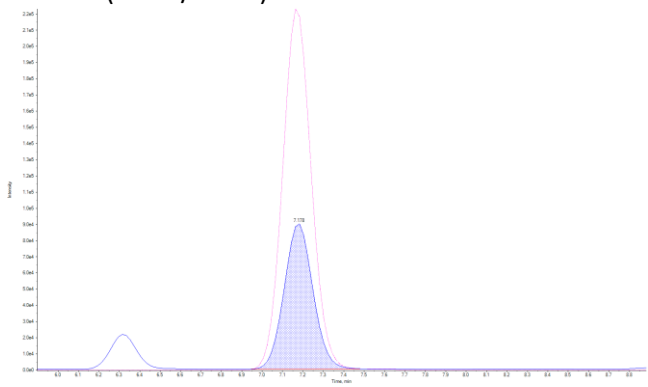
DHT (291.2/255.1) serum sample



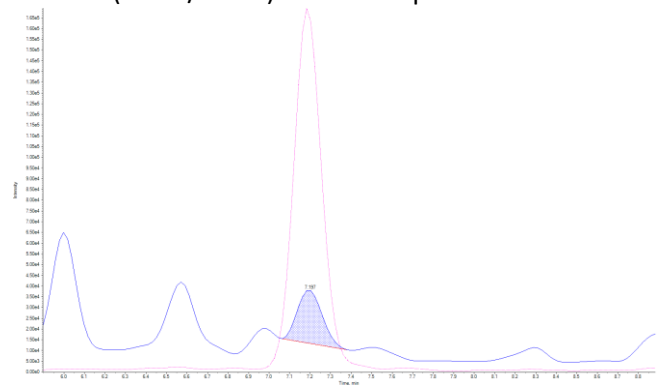
17-OHP (331.2/109.0) calibration level 3



17-OHP (331.2/109.0) serum sample



Progesterone (315.2/97.0) calibration level 3



Progesterone (315.2/97.0) serum sample

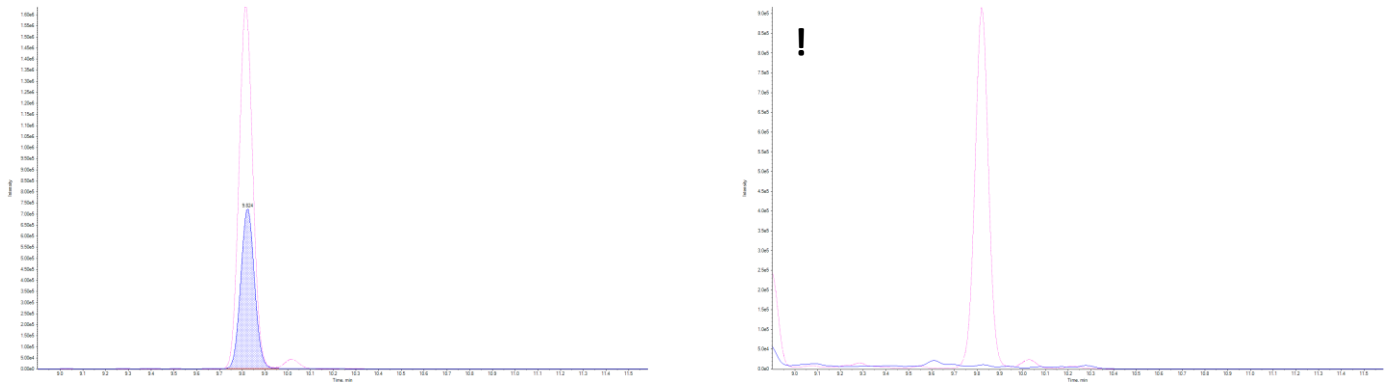


Figure A 6: MRM chromatogram of steroids (panel 2) in calibration concentration level 3 (left) and serum sample (right). MRM transition from Q1 to Q3 (m/z) is given in brackets. MRM signal of the qualifier transition is marked blue, corresponding internal standard signals are shown in pink. Steroids that did not result in reference intervals for all or one of the sex or age groups due to low concentrations or chromatographic difficulties are marked with exclamation mark. Chromatogram of estradiol shows high background but no target signal. Chromatograms of 11-deoxycortisol, DHT and 17-OHP show a high background and several undesired signals. Source of the signals is unclear, but affects the correct integration of the target signals, if present. Target signals of isobars 11-deoxycorticosterone and 17-OHP (m/z 331.2) are baseline separated from each other. 17-OHP elutes after 11-deoxycorticosterone. Isobaric internal standards DHEA-d5 and DHT-d3 (pink signals) are also baseline separated. DHEA-d5 elutes before DHT-d3.

Table A 1: Mass to charge ratio (m/z) for ions in first (Q1) and third (Q3) quadrupole and retention time of quantifier transitions.

	Q1 (m/z)	Q3 (m/z)	RT (min)
Aldosterone	359.1	331.1	4,77
Cortisol	363.1	97.1	5,86
Cortisone	361.2	163.0	6,16
Corticosterone	347.2	121.0	8,39
11-Deoxycortisol	347.2	97.0	8,76
21-Deoxycortisol	347.2	121.0	8,12
DHEA-S	369.1	98.9	2,38
Estradiol	255.1	159.1	5,04
Testosterone	289.2	97.0	5,68
Androstenedione	287.2	109.0	7,15
DHEA	289.2	213.0	6,68
11-Deoxycorticosterone	331.2	109.0	6,49
DHT	291.2	255.1	8,01
17-OHP	331.2	109.0	7,39
Progesterone	315.2	97.0	9,85

Abbreviations: DHEA-S – Dehydroepiandrosterone-sulfate; DHEA – Dehydroepiandrosterone; DHT – Dihydrotestosterone; 17-OHP – 17-Hydroxyprogesterone; Q - quadrupole

Table A 2: D/Dmax (quotient of observed deviation from target value and maximal allowed deviation from target value (D/Dmax)) of available steroids in national external quality assessment scheme for steroid hormones of RfB in the time period in which the study samples were measured. Target values were determined by reference measurement procedures. Accuracy is considered acceptable, when (D/Dmax) is equal or below 1.00.

	Q3/18-A	Q3/18-B	Q4/18-A	Q4/18-B	Q3/19-A	Q3/19-B	Q4/19-A	Q4/19-B	Q1/20-A	Q1/20-B
Aldosterone	-0,03	-0,08	-0,78	-0,45	-0,5	-0,24	-0,87	-0,33	-0,23	-0,64
Cortisol	0,35	0,35	0,15	0,18	0,2	0,06	0,14	0,09	0,09	0,11
Estradiol	0,01	0,23	0,21	-0,73	5,86	1,59	-0,63	-0,75	1,29	3,93
Progesterone	0,02	0,07	0,32	0,34	0,16	-0,11	0,17	0,06	0,24	0,23
Testosterone	0,28	0,31	0,35	0,35	0,38	0,2	0,12	0,06	0,15	0,19
DHEA-S	0,02	0,14	-0,04	0,12	0,14	0	0	0	0	0,08
17-OHP	0,01	0,03	0,01	0,07	0,14	0,09	0,01	-0,27	0,17	0,22

Abbreviations: DHEA-S – Dehydroepiandrosterone-sulfate; DHEA – Dehydroepiandrosterone; 17-OHP – 17-Hydroxyprogesterone; Q – quarter (of the year); D - deviation from target value; D_{max} - maximal allowed deviation from target value; RfB - Reference Institute for Bioanalytics, RfB, Bonn, Germany

Table A 3: Performance characteristics of the steroid kit. Quality control samples, provided by the kit manufacturer, in three different concentrations were measured multiple times and compared to the target value, provided by the manufacturer.

	QC1				QC2				QC3									
	CV 1 (%)	Δ 1 (%)	\bar{x} (ng/mL)	tv (ng/mL)	CV 2 (%)	Δ 2 (%)	CV 1 (%)	Δ 1 (%)	\bar{x} (ng/mL)	tv (ng/mL)	CV 2 (%)	Δ 2 (%)	CV 1 (%)	Δ 1 (%)	\bar{x} (ng/mL)	tv (ng/mL)	CV 2 (%)	Δ 2 (%)
Aldosterone	3.3	0.6	0.11	0.11	7.5	7.7	1.2	2	0.28	0.28	4.2	7.2	2.4	4.5	1.15	1.10	5.2	11
Cortisol	1.6	3.6	26.0	25.0	1.9	5.8	1.5	4.9	62.7	60.0	2.4	4.6	1.5	1.5	178	175	1.7	4.8
Cortisone	1.7	1.4	2.06	2.03	0.9	2.7	0.9	1.7	11.8	11.6	3.1	2.6	1	2	30.0	29.4	3.1	5.5
Corticosterone	1.9	3.8	0.830	0.797	3.2	0.8	3	1.8	4.05	3.98	4.7	3.3	2.3	3.9	27.6	28.7	4.7	2.2
11-Deoxycortisol	3.9	4.7	0.299	0.286	2.3	5.5	4.4	7	1.54	1.44	4.3	4.3	2	6.3	9.99	9.40	4.7	8.3
21-Deoxycortisol	4.4	1.9	0.109	0.111	6.9	6.3	3.6	2.2	0.432	0.423	4.2	3.9	4.9	3.8	2.42	2.33	3.9	6.5
DHEA-S	3.2	3.7	299	288	6.6	1.5	4.3	3.2	2033	1558	3.4	5.4	3.7	3.5	4246	5001	3.9	1.0
Estradiol	1.9	0.9	0.083	0.084	2.3	1.6	1.8	8.4	0.389	0.425	2.3	2.4	0.7	0.5	2.62	2.63	2.8	0.1
Testosterone	1.4	0.4	0.201	0.202	3.4	0.5	0.8	0.5	1.52	1.51	1.5	4.4	0.6	0.8	7.92	7.85	2.5	5.6
Androstenedione	1.9	1.3	0.311	0.315	3.2	4.7	1.6	1	1.25	1.26	2.6	1	1.5	3.2	9.97	10.30	2.4	3.0
DHEA	3	3	2.07	2.13	6.6	5.8	3.6	2.2	12.7	12.4	4.6	0.7	4.1	2	40.2	41.0	4	8.3
11-DOC	2.1	4.2	0.081	0.078	2.8	2.5	2.6	5.5	0.203	0.192	2.8	3	2.4	6.3	1.061	0.998	2.4	0.2
DHT	1	1.1	0.089	0.088	5.7	1.8	1.4	3.4	0.420	0.406	2.3	1.2	2.8	2.2	1.26	1.23	2.5	3.0
17-OHP	2.3	3.8	0.316	0.304	2.8	0.1	1.4	4.4	1.59	1.52	1.3	3	1.2	4.8	9.62	9.18	2.2	3.9
Progesterone	0.8	1.8	0.346	0.352	15.9	5.9	0.3	2.1	3.31	3.24	2.2	4.9	0.3	1.5	16.1	15.9	1.9	7.2

Abbreviations: QC – quality control; CV 1 – coefficient of variation of intra-day measurement (n=5); Δ 1 – discrepancy between the mean of the intra-day measurement (n=5) and the target value; \bar{x} - mean of the intra-day measurement; tv – target value for the intra-day measurement, provided from the manufacturer; CV 2 – coefficient of variation of inter-day measurements (n=12); Δ 2 – discrepancy between the mean of the inter-day measurement (n=12) and the target value; DHEA-S – Dehydroepiandrosterone-sulfate; DHEA – Dehydroepiandrosterone; 11-DOC – 11-Deoxycorticosterone; DHT – Dihydrotestosterone; 17-OHP – 17-Hydroxyprogesterone.

Table A 4: Impact of age on steroid concentrations. Steroids were grouped into 30-39 years, 40-49 years, 50-59 years and 60-90 years for males and pre- and postmenopausal for females. Groups were compared by pairwise Wilcoxon test with Bonferroni correction. P-values below 0.05 were considered significant. Tested comparisons: 1 = 30-39y and 40-49y (males), 2 = 40-49y and 50-59y (males), 3 = 50-59y and 60-90y (males), 4 = premenopausal and postmenopausal (females), 5 = males and females.

	Cortisol	Cortisone	Corticosterone	11-Deoxycortisol	DHEA-S	Testosterone	Androstenedione	DHEA	17-OHP
p (1)	1	1	1	1	0.2244	0.8857	1	0.0208	1
p (2)	1	1	1	1	0.0513	0.1571	1	0.0361	0.0087
p (3)	0.2023	1	0.0087	0.0019	<0.0001	0.0762	<0.0001	<0.0001	1
p (4)	0.3589	0.2524	0.0003	0.0189	<0.0001	0.1641	<0.0001	<0.0001	<0.0001
p (5)	0.0209	0.9120	0.1110	<0.0001	<0.0001	<0.0001	<0.0001	0.5475	<0.0001

Abbreviations: DHEA-S – Dehydroepiandrosterone-sulfate; DHEA – Dehydroepiandrosterone; 17-OHP – 17-Hydroxyprogesterone; SHBG – sex hormone binding globulin, p – p-value of pairwise Wilcoxon rank sum test with Bonferroni correction

Table A 5: Impact of BMI on selected steroid concentrations. Steroids were grouped into normal weight, overweight and obesity groups according to the WHO recommendation (normal weight = 18 – 24.9 kg/m², overweight = 25 – 29.9 kg/m², obesity > 30 kg/m²). Groups were compared using Kruskal-Wallis test followed by pairwise Wilcoxon test with Bonferroni correction (p corr). P-values below 0.05 were considered significant. Concentrations for each group are given as median (minimum-maximum) values.

Steroid	Sex male	normal weight (nmol/L) n=79	overweight (nmol/L) n=144	obese (nmol/L) n=67	BMI Group 1	BMI Group 2	p corr
Testosterone		18.21 (9.86-33.88)	15.64 (0.03-38.49)	13.24 (0.03-26.65)	normal weight	overweight	0.0027
					overweight	obesity	0.0275
					normal weight	obesity	< 0.0001
DHEA-S		4,209 (213-10,500)	3,049 (415-12,471)	2,288 (491-11,231)	normal weight	overweight	0.0339
					overweight	obesity	0.2045
					normal weight	obesity	0.0017
DHEA		11.16 (0.74-31.71)	8.81 (0.74-46.53)	7.66 (1.02-35.40)	normal weight	overweight	0.2730
					overweight	obesity	0.2930
					normal weight	obesity	0.0260
17-OHP		2.71 (1.07-7.05)	2.39 (0.72-6.90)	2.07 (0.13-5.78)	normal weight	overweight	0.2397
					overweight	obesity	0.1447
					normal weight	obesity	0.0037
Progesterone		0.16 (0.11-0.56)	0.12 (0.11-0.54)	0.11 (0.11-0.55)	normal weight	overweight	0.0587
					overweight	obesity	0.3559
					normal weight	obesity	0.0033
SHBG		57.0 (15.0-125.5)	48.0 (0.0-119.0)	53.7 (0.0-158.0)	normal weight	overweight	0.0420
					overweight	obesity	1.0000
					normal weight	obesity	0.4570
	female	n=73	n=84	n=72			
Cortisone		54.66 (35.26-78.24)	53.32 (20.64-80.73)	49.79 (29.77-83.23)	normal weight	overweight	1.0000
					overweight	obesity	0.5690
					normal weight	obesity	0.0910
Estradiol		0.015 (0.015-1.554)	0.015 (0.015-3.635)	0.015 (0.015-0.904)	normal weight	overweight	0.4900
					overweight	obesity	1.0000

				normal weight	obesity	0.2900
Androstenedione	2.8 (0.34-7.63)	1.97 (0.53-7.67)	1.85 (0.61-6.25)	normal weight	overweight	0.0600
				overweight	obesity	1.0000
				normal weight	obesity	0.0270
Progesterone	0.16 (0.01-72.12)	0.11 (0.00-42.14)	0.11 (0.00-62.36)	normal weight	overweight	0.0320
				overweight	obesity	1.0000
				normal weight	obesity	0.0390
SHBG	94.4 (0.0-191.0)	73.7 (33.7-183.0)	57.8 (29.3-181.0)	normal weight	overweight	0.0236
				overweight	obesity	0.0004
				normal weight	obesity	< 0.0001

Abbreviations: DHEA-S – Dehydroepiandrosterone-sulfate; DHEA – Dehydroepiandrosterone; 17-OHP – 17-Hydroxyprogesterone; SHBG – sex hormone binding globulin, p – p-value of pairwise Wilcoxon rank sum test with Bonferroni correction

