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Text S1 Mass spectrometry conditions

A, E_1, 2-MeE_2, E_2, E_1-d_2, and E_1-d_2 were detected in negative ion mode with the following parameters: capillary voltage, −1.5 kV; desolvation gas flow, 800 L/h; cone gas flow, 150 L/h; desolvation temperature, 550 °C. T-2,3,4-$^{13}C_3$, 17α-OHP-d_8, P_4-2,3,4-$^{13}C_3$, A_2-2,3,4-$^{13}C_3$, E, F, 21-DF, B, 11-DF, 21-OHP, 17α-OHP, DHT, A_2, T, CH, CH-d_7, 17α-OHP, P_4, P_5, and DHEA were detected by in positive ion mode with the following parameters: capillary voltage, 1.5 kV; desolvation gas flow, 800 L/h; cone gas flow, 50 L/h; corona needle voltage, 21 μA; source temperature, 150 °C and desolvation temperature, 550 °C. IntelliStart was used for optimization of the cone voltages and collision energies.

Text S2 Sample collection and preparation

Blood samples were obtained from *M. musculus* by puncture of the ophthalmic plexus and from *C. auratus* and *R. nigromaculata* by puncture of the cardiac vein. The blood was centrifuged at 1300 rpm for 15 min to obtain serum. The serum was clarified, freed of hemolysis and lipids, and stored frozen at −80 °C. This work was conducted in compliance with the 'Animal Research: Reporting of In Vivo Experiments' (ARRIVE) guidelines and was approved by the Laboratory Animal Welfare and Ethics Committee of Hangzhou Normal University.

A 100-μL serum sample was mixed with 5 μL stable isotope internal standard (IS), transferred to an Eppendorf tube, and fully equilibrated for 10 min. Subsequently, 10 μL 2% FA aqueous solution was added with the aim of addressing the problem of endogenous steroid hormones such as T and E_2 binding to sex hormone-binding globulins in the organism, resulting in low concentrations for detection. The samples were mixed thoroughly after each addition of reagent. Subsequently, 200 μL methyl tert-butyl ether was added for extraction, and the sample was vortexed for 5 min and then centrifuged at 5000 rpm for 10 min at room temperature. The above liquid–liquid extraction steps were repeated three times, the organic phases were combined, and the collected liquids were blown dry under nitrogen. Finally, 100 μL ACN was added for re-dissolution, filtered through polyether sulfone membrane with a 0.22-μm pore size, and then placed in an autosampler for UPLC–MS/MS analysis.

Table S1. The CAS and item number of steroid hormone standards

Steroid hormone standards	CAS	Item number
Cholesterol (CH)	57-88-5	C8667
CH-d_7	83199-47-7	700041P
Androstenedione (A_2)	63-05-8	A-075
A_2-2,3,4-$^{13}C_3$	327048-86-2	A-084
Estradiol (E_2)	50-28-2	E8875
E_2-d_2	53866-33-4	E4260
Estrone (E_1)	53-16-7	E-075
17-α-hydoxy progesterone (17α-OHP)	68-96-2	H-085
Testosterone (T)	58-22-0	T-037
Dehydroepiandrosterone (DHEA)	53-43-0	D-063
Progesterone (P_4)	57-83-0	P-069
Pregnenolone (P_5)	145-13-1	P-104
Dihydrotestosterone (DHT)	521-18-6	D-073

T-2,3,4-$^{13}C_3$	327048-83-9	T-070
17α-OHP-d_8	850023-80-2	H-096
P_4-2,3,4-$^{13}C_3$	327048-87-3	737143
Aldosterone (A)	52-39-1	S30644
Cortisone (E)	53-06-5	S47960
11-deoxycortisol (11-DF)	152-58-9	B27368
21-hydroxy progesterone (21-OHP)	64-85-7	B65163
2-methoxyestrone (2-MeE_2)	362-07-2	S46451
21-deoxycortisol (21-DF)	362-07-2	641-77-0
E_1-d_2	56588-58-0	B72569
Corticosterone (B)	50-22-6	C104537
Hydrocortisone (F)	50-23-7	H110523

Table S2 The gradient elution procedure for the separation of multiple steroid hormones

Time (min)	Flow rate (mL/min)	A (%)	B (%)	Gradient elution curve
initial	0.2	70	30	6
3.5	0.2	40	60	6
8.0	0.3	10	90	6
8.5	0.3	0	100	6
10.0	0.2	70	30	6

Table S3 The MRM parameters of multiple steroid hormones

Steroid hormone	Parent ion (m/z)	Cone voltage (V)	Daughter ion (m/z)	CE (eV)	Daughter ion (m/z)	CE (eV)
A	359.09	4	189.04*	18	331.13	14
E_2	271.02	18	145.01*	40	183.04	42
E_2-d_2	293.16	2	147.03	38	--	--
E	361.15	32	91.53	64	163.02*	22
F	363.14	32	97.17	60	121.23*	24
21-DF	347.17	26	121.32*	28	311.18	16
B	347.17	28	91.43	60	121.36*	26
11-DF	347.17	30	97.35*	24	109.33	30
P_4	315.27	30	42.94	52	109.05*	22
P_5	299.14	48	81.32*	44	159.10	20
P_4-$^{13}C_3$	318.22	26	99.81	22	--	--
A_2	287.15	38	96.95*	18	108.99	22
A_2-$^{13}C_3$	290.17	32	99.86	20	--	--
E_1	269.15	18	144.94*	40	158.93	32
2-MeE_2	301.11	2	219.08	12	286.17*	24

E_1-d_2	271.16	18	145.01	42	--	--
21-OHP	331.17	32	97.39*	22	109.37	30
17-OHP	331.21	42	96.95*	22	108.98	28
17-OHP-d_8	339.22	38	99.87	28	--	--
T	289.20	46	96.94*	18	108.98	20
DHEA	271.20	45	197.20	14	213.2*	12
DHT	291.19	45	159.10	16	255.20*	14
T-$^{13}C_3$	292.18	50	99.83	20	--	--
CH	369.18	26	80.95	44	146.90*	20
CH-d_7	376.21	20	104.93	50	161.02*	20

Table S4. Effect of different extractants on the extraction yield of steroid hormones

Steroid hormone	Efficiency of extraction (%)		
	Dichloromethane	Hexane	Methyl tert-Butyl Ether (MTBE)
A	79.7	＜50.0	87.7
E	77.1	＜50.0	91.2
F	89.3	77.8	96.6
21-DF	83.8	74.8	93.8
B	80.1	75.0	91.5
11-DF	79.8	70.8	87.0
A_2	81.3	＜50.0	85.2
E_2	68.0	＜50.0	84.2
E1	73.6	66.6	84.8
2-MeE_1	87.8	72.1	86.3
21-OHP	82.5	＜50.0	82.9
17-OHP	94.1	80.0	97.0
T	82.3	72.3	91.8
DHEA	94.4	72.8	112.3

P_4	66.7	＜50.0	83.91
DHT	76.2	51.8	88.19
P_5	87.8	＜50.0	85.10
CH	72.8	＜50.0	89.37

* If one depicts the known concentration of standards in as A, the concentration detected by the UPLC-MS/MS for standards spiked after different pretreatment as B. Efficiency of extraction can be calculated as follows: B/A ×100.

Table S5. The specificity of multiple steroid hormones

Steroid hormones	Sample	Concentration (ng/mL)	QI/QC	
			Mean	Median
A	Calibration solution	0.05-50.00	1.41	1.38
	Quality control samples	0.21-46.43	1.40	1.41
	Serum samples	0.29-10.32	1.37	1.36
E	Calibration solution	0.05-50.00	1.69	1.65
	Quality control samples	0.18-40.21	1.67	1.66
	Serum samples	0.21-8.32	1.68	1.64
F	Calibration solution	0.05-50.00	1.98	1.97
	Quality control samples	0.21-42.17	1.99	1.99
	Serum samples	0.34-4.43	2.00	1.99
21-DF	Calibration solution	0.05-50.00	1.78	1.77
	Quality control samples	0.15-39.23	1.79	1.78
	Serum samples	0.20-5.32	1.79	1.78
B	Calibration solution	0.05-50.00	1.65	1.65
	Quality control samples	0.31-39.79	1.66	1.65

	Serum samples	0.37-3.32	1.66	1.66
	Calibration solution	0.05-50.00	1.34	1.34
11-DF	Quality control samples	0.18-42.32	1.33	1.34
	Serum samples	0.32-3.32	1.34	1.35
	Calibration solution	0.05-50.00	1.42	1.42
A_2	Quality control samples	0.28-47.02	1.42	1.42
	Serum samples	0.29-5.08	1.42	1.41
	Calibration solution	0.05-50.00	1.21	1.20
E_2	Quality control samples	0.14-47.20	1.20	1.21
	Serum samples	0.77-32.32	1.21	1.21
	Calibration solution	0.05-50.00	1.31	1.31
E_1	Quality control samples	0.12-42.48	1.30	1.31
	Serum samples	0.53-18.30	1.31	1.31
	Calibration solution	0.05-50.00	1.22	1.23
2-MeE_1	Quality control samples	0.11-48.26	1.21	1.23
	Serum samples	0.52-3.24	1.22	1.23
21-OHP	Calibration solution	0.05-50.00	1.14	1.16

	Quality control samples	0.18-37.43	1.14	1.15
	Serum samples	0.08-18.29	1.14	1.16
	Calibration solution	0.05-50.00	1.33	1.34
17α-OHP	Quality control samples	0.09-39.72	1.33	1.34
	Serum samples	0.99-15.47	1.33	1.35
	Calibration solution	0.05-50.00	1.65	1.66
T	Quality control samples	0.17-48.28	1.65	1.66
	Serum samples	0.72-20.67	1.64	1.65
	Calibration solution	0.05-50.00	2.51	2.53
DHEA	Quality control samples	0.14-39.05	2.50	2.54
	Serum samples	0.48-20.37	2.51	2.53
	Calibration solution	0.05-50.00	1.22	1.21
P_4	Quality control samples	0.17-37.38	1.23	1.22
	Serum samples	0.95-22.26	1.23	1.22
	Calibration solution	0.05-50.00	2.42	2.41
DHT	Quality control samples	0.11-48.24	2.43	2.41
	Serum samples	0.15-3.57	2.43	2.41

	Calibration solution	0.05-50.00	2.55	2.56
P_5	Quality control samples	0.13-37.38	2.54	2.56
	Serum samples	0.37-2.37	2.55	2.56
	Calibration solution	50-1000.00	1.29	1.29
CH	Quality control samples	108.28-989.82	1.27	1.28
	Serum samples	382.28-899.27	1.27	1.28

Table S6. The matrix effect of multiple steroid hormones

Steroid hormones	Matrix Effect (n = 5)		
	Mean ± SD (%)		
	LQC	MQC	HQC
A	97.96 ± 6.66	93.85 ± 13.33	95.71 ± 7.62
E	91.78 ± 26.39	98.40 ± 7.99	107.34 ± 9.31
F	99.80 ± 22.57	105.46 ± 7.78	103.88 ± 2.05
21-DF	98.28 ± 22.56	98.14 ± 6.57	111.06 ± 11.37
B	86.47 ± 8.35	90.93 ± 5.98	90.53 ± 3.59
11-DF	89.29 ± 12.22	111.42 ± 3.66	109.77 ± 12.37
A_2	79.86 ± 6.69	98.93 ± 2.54	93.99 ± 9.64
E_2	99.83 ± 8.41	84.32 ± 15.46	80.98 ± 9.18
E_1	95.91 ± 9.82	104.93 ± 5.58	98.19 ± 28.53
2-MeE_2	90.15 ± 8.48	92.20 ± 8.65	86.74 ± 10.38
21-OHP	91.26 ± 9.27	93.56 ± 9.40	82.42 ± 8.75
17-OHP	89.40 ± 4.17	89.68 ± 6.61	90.68 ± 3.20
T	79.46 ± 2.69	108.12 ± 3.58	98.95 ± 6.82

DHEA	83.79 ± 11.08	98.61 ± 5.01	89.36 ± 13.64
P_4	92.25 ± 17.37	108.87 ± 3.82	107.16 ± 8.07
DHT	102.05 ± 28.56	107.60 ± 20.23	100.49 ± 21.06
P_5	100.84 ± 13.94	93.28 ± 14.37	92.61 ± 15.07
CH	102.78 ± 17.69	98.43 ± 4.79	89.31 ± 6.16

Table S7 The linearity, limit of detection and limit of quantification of multiple steroid hormones

Steroid hormone	The standard curve	r^2	LOD (ng/mL)	LLOQ (ng/mL)
A	$y = 1.003\,x + 1.834$	0.9998	0.015	0.05
E	$y = 1.743\,x + 0.773$	0.9999	0.15	0.5
F	$y = 2.374\,x + 1.383$	0.9961	0.0001	0.0005
21-DF	$y = 3.839\,x + 2.001$	0.9993	0.005	0.02
B	$y = 2.328\,x + 0.198$	0.9912	0.05	0.2
11-DF	$y = 1.023\,x + 3.384$	0.9994	0.015	0.05
A_2	$y = 3.638\,x + 2.100$	0.9999	0.0005	0.002
E_2	$y = 1.005\,x + 0.478$	0.9999	0.005	0.02
E_1	$y = 2.030\,x + 0.836$	0.9999	0.001	0.003
2-MeE_1	$y = 2.576\,x + 0.004$	0.9992	0.005	0.02
21-OHP	$y = 3.675\,x + 1.464$	0.9963	0.001	0.003
17α-OHP	$y = 1.007\,x + 0.552$	0.9996	0.005	0.02
T	$y = 1.211\,x + 0.668$	0.9991	0.01	0.03
DHEA	$y = 3.446\,x + 1.374$	0.9995	0.015	0.05
P_4	$y = 1.098\,x + 0.336$	0.9999	0.005	0.02

DHT	$y = 1.102\ x + 8.259$	0.9982	0.025	0.08
P_5	$y = 3.40\ x + 8.437$	0.9929	0.075	0.3
CH	$y = 3.373\ x + 0.226$	0.9927	0.15	0.5

Number of steroids (Internal standard included)	Serum Sample Required	LLOQ	Rate of recovery	Detection of CH	References
24	200 μL	0.01-12.5 ng/mL	88.8%-107.1%	no	[1]
13	250 μL	0.05-12.9 ng/mL	90.0%-110.0%	no	[2]
16	150 μL	0.005-0.164 ng/mL	88.7%-116.2%	no	[3]
19	290 μL	0.004-0.75 ng/mL	68.2%-135%	no	[4]
14	150 μL	0.001-0.3 nmol/L	83.0%-115.0%	no	[5]
10	/	0.75-2.1 nmol/L	86.7%-106.7%	no	[6]
8	200 μL	0.015-0.95 ng/mL	106.1%-115.7%	no	[7]
22	150 μL	0.003-0.33 nmol/L	83.0%-137.0%	no	[8]
18	200 μL	0.005-1 ng/mL	85.0%-104.0%	no	[9]
19	500 μL	0.04-1.18 ng/mL	80.0%-120.0%	no	[10]
9	200 μL	0.001-0.1 ng/mL	/	no	[11]
12	100 μL	0.1–500 ng/mL	87.0–101.0%	no	[12]
25	100 μL	0.0005-0.5 ng/mL	76.2%-113.7%	yes	This study

Table S8 Comparison of parameters of studies related to steroid detection by UPLC-MS/MS methods

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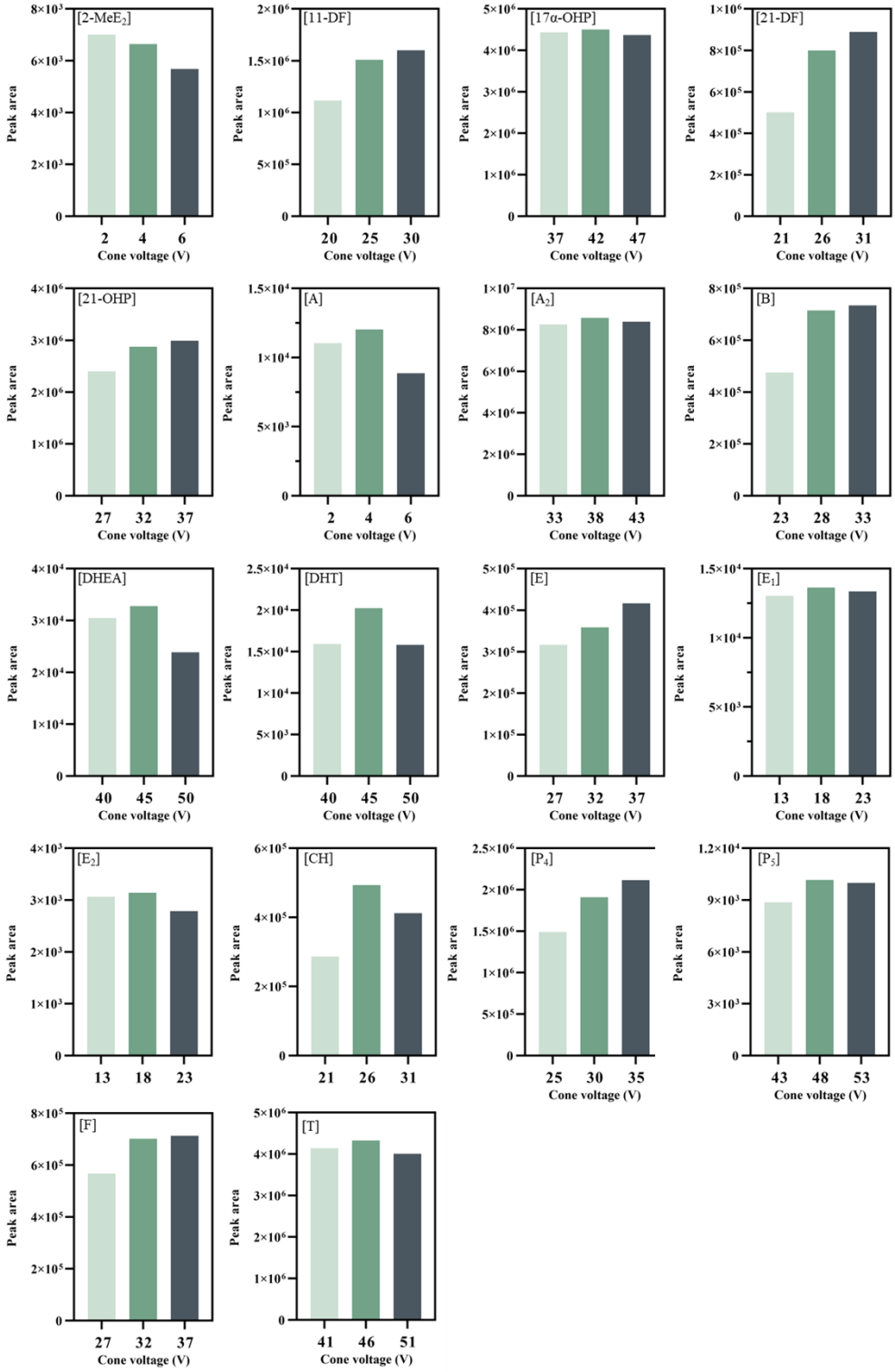

Fig.S1 Effect of different cone voltage on steroid hormone response

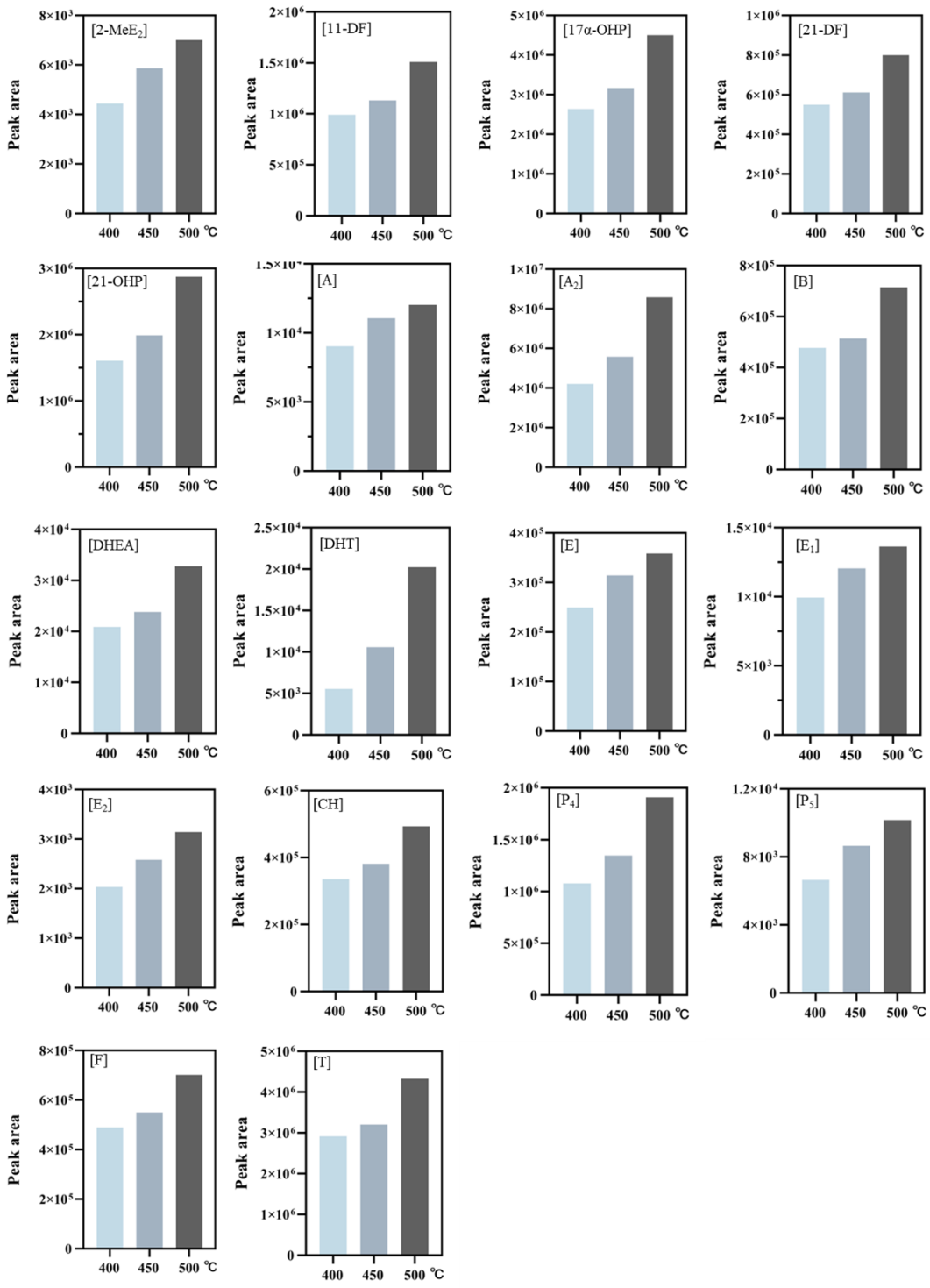

Fig.S2 Effect of different desolvation temperatures on steroid hormone response

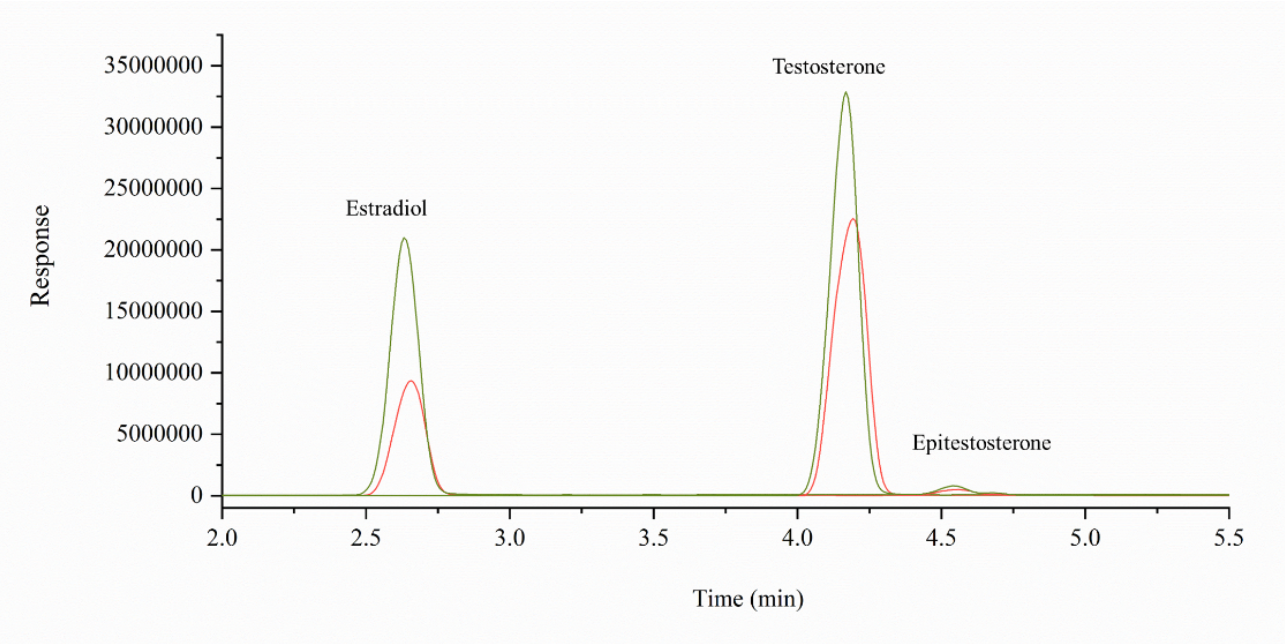

Fig. S3 The MRM chromatogram of E_2 and T with different mobile phases. 0.1 mM NH_4F + ACN (green peaks); pure water + ACN (red peaks)

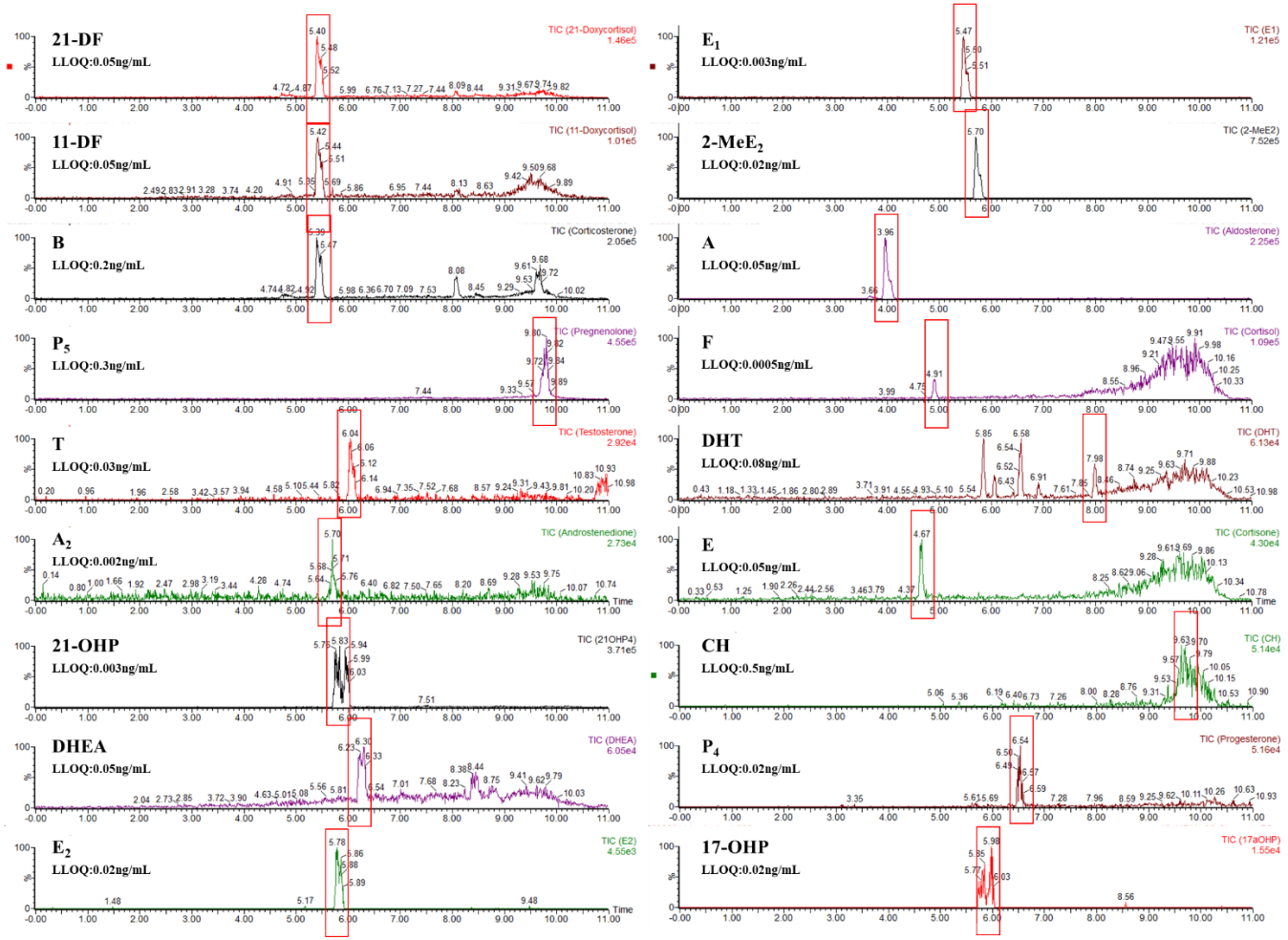

Fig.S4 LC-MS chromatograms of the 18 steroids at LLOQ included in the method.