Method development and validation for the analysis of 20 hormones (including estrogens, androgens and progestagens compounds) in various aqueous matrices

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INTRODUCTION

During the last twenty years, consumption of hormones compounds (natural hormones or synthetic analogues), mainly for human medicine, has considerably increased. Both androgens, progestogens and estrogens compounds are usually not entirely metabolized and reach aquatic environment mainly via effluents of wastewater treatment plants (WWTP). All of them can be considered as endocrine disrupting compounds because of undesirable effects in biota. Hence, a highly sensitive and selective analytical method is needed to identify and quantify these emerging contaminants in various environmental compartments, especially in surface waters and wastewaters.

MATERIAL AND METHOD

Method synoptic

Initially based on previous estrogenic analytical method [1]

Sample filtration: Calcinated glass fiber filter, GF/F 0,7 µm
Sample volume: 250 mL (river waters and WWTP effluents)

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SPE automated extraction: Oasis ® HLB 6mL

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SPE manual purification: Florisil ® 6mL, 1g

↓

UHPLC/MS-MS
(Waters BEH C18, Shimadzu Nexera X2, AB SCIEX API4000)

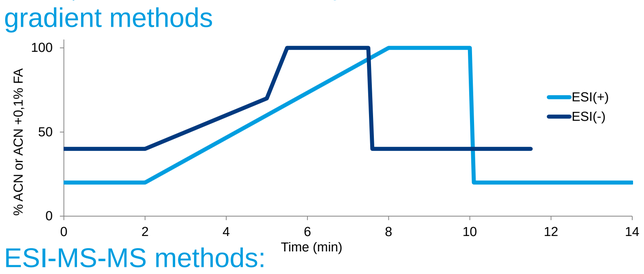
10 µL injected

ESI (+): 13 hormones
MilliQ® Water+0,1% FA/ACN+0,1% FA

ESI (-): 7 hormones
MilliQ® Water/ACN

UHPLC/MS-MS parameters

- Apparatus:
  - UHPLC: Shimadzu Nexera 2
  - Column: Waters® BEH C18 100 x 2,1 mm, 1,7 µm
  - Mass spectrometer: AB SCIEX API 4000 TQ
- 2 gradient methods



- 2 ESI-MS-MS methods:
  - 2 Ionization modes : ESI positive for androgens and progestagens, ESI negative for estrogens
  - Source parameters: see following table
  - Acquisition methods: MRM mode, parameters are listed against. Confirmation criteria are described in the EU council decision [2]

Source parameters	ESI(+)	ESI(-)
Nebulizer gas (PSI)	60	50
Turbo gas (PSI)	30	60
Curtain gas (PSI)	30	10
Collision gas (PSI)	10	8
Temperature (°C)	500	400
Ion spray voltage (V)	+4500	-4500

- Quantitation method:
  - Internal calibration
  - Use of molecular analogue labelled with stable(s) isotope(s) (D and/or ^{13}C) as internal standard (IS)

MRM acquisition parameters

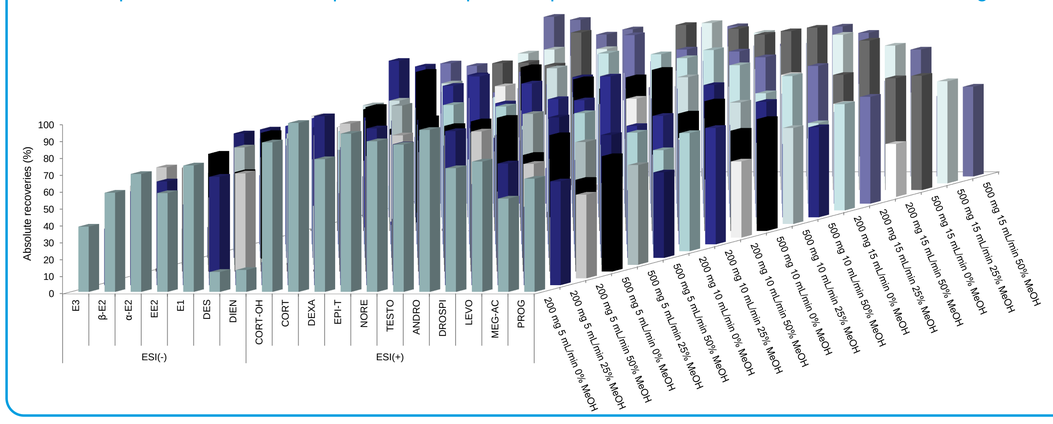
Compound	Retention Time (min)	Transitions (Quantification and Confirmation) Parent ion	Daughter ion	Type	MS-MS parameters DP (V)	EP (V)	CE (V)	CXP (V)
Cortisol	3,60	363	121	Q	76	10	33	8
		363	91	C			89	6
Cortisol-D4	3,59	367	121	Q	66		31	21
		367	97	C			47	18
Cortisone	3,60	361	163	Q	61		33	10
		361	91	C			89	16
Cortisone-D8	3,58	369	168	Q	65		35	16
		369	351	C			17	24
Dexamethasone	4,08	393	373	Q	46		13	24
		393	91	C			87	6
Dexamethasone-D4	4,06	397	377	Q	45		13	22
		397	359	C			17	24
Testosterone	4,71	289	109	Q	36		37	8
		289	97	C			31	6
Testosterone-D4	4,69	293	98	Q	76		31	18
		293	110	C			35	20
Norethindrone	4,73	299	109	Q	69		37	9
		299	91	C			65	16
Norethindrone-D6	4,71	305	91	Q	65		68	16
		305	87	C			51	16
Androstenedione	4,92	287	97	Q	66		31	6
		287	109	C			35	8
Androstenedione-D7	4,90	294	100	Q	76		33	18
		294	113	C			37	20
Drospirenone	5,06	367	97	Q	76	10	37	18
		367	91	C			89	6
Drospirenone-D4	5,05	371	99	Q	74		43	17
		371	91	C			81	16
Epitestosterone	5,07	289	97	Q	68		33	17
		289	109	C			37	19
Epitestosterone-D5	5,05	294	100	Q	77		35	17
		294	113	C			38	19
Levonorgestrel	5,18	313	91	Q	71		63	16
		313	109	C			37	8
Levonorgestrel-D6	5,16	319	91	Q	76		65	16
		319	87	C			55	16
Megestrol Acetate	5,71	385	325	Q	61		21	20
		385	267	C			25	18
13C-Megestrol Acetate-D3	5,70	389	325	Q	61		21	20
		389	267	C			27	16
Progesterone	5,73	315	109	Q	46		35	8
		315	97	C			33	6
Progesterone-D9	5,70	324	100	Q	76		35	18
		324	113	C			37	10
Medroxyprogesterone*	5,37	345	123	Q	70		37	22
		345	97	C			43	17
Androsterone*	5,51	291	273	Q	45		11	16
		291	255	C			19	25
Estriol	0,60	287	145	Q	-110		-54	-7
		287	171	C			-50	-15
Estriol-D2	0,60	289	147	Q	-95		-54	-13
				C				
β-Estradiol	1,80	270	145	Q	-95		-52	-7
		270	183	C			-54	-9
β-Estradiol-D2	1,80	273	185	Q	-90		-58	-19
				C				
α-Estradiol	2,20	270	145	Q	-95		-54	-7
		270	183	C			-46	-9
α-Estradiol-D2	2,20	273	185	Q	-90		-54	-19
				C				
α-Ethinylestradiol	2,50	295	154	Q	-55		-54	-7
		295	159	C			-46	-13
α-Ethinylestradiol-D4	2,40	299	147	Q	-90	-10	-54	-7
				C				
Estrone	2,70	269	145	Q	-85		-50	-7
		269	143	C			-66	-25
Estrone-D4	2,70	273	147	Q	-90		-50	-7
				C				
Diethylstilbestrol	3,30	267	251	Q	-93		-32	-26
		267	237	C			-36	-53
Diethylstilbestrol-D8	3,22	275	259	Q	-75		-38	-145
		271	255	C			-39	-20
Dienestrol	3,54	265	93	Q	-103		-42	-3
		265	249	C			-42	-13
Dienestrol-D6	3,77	271	95	Q	-90		-38	-17
		271	255	C			-40	-21

*Medroxyprogesterone and Androsterone are respectively associated to IS testosterone-D3 and progesterone-D9

SPE OPTIMISATION

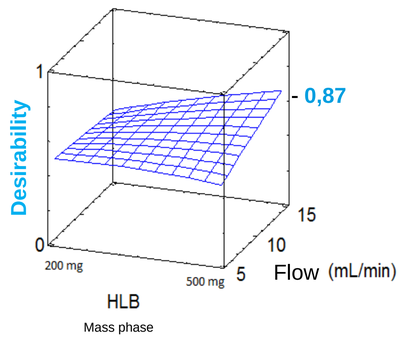
Experimental design approach

We built a full factorial experimental design. 3 parameters ranging between 2 or 3 levels lead to 18 experiments that were driven on spiked samples of Evian ® mineral. Results presented are absolute recoveries (external calibration). Data treatments have been done with Statgraphics® software (Screening experimental design, Pareto diagrams, ACPs). Best desirability (0,87) corresponding to optimum extraction parameters was attempted with sample SPE percolation flow rate of 15 mL/min, on 500 mg HLB SPE phase and with ethyl acetate/MeOH 75/25 V/V as elution solvent.



Statgraphics® data treatment

Surface response for ethyl acetate/MeOH 75/25 V/V



METHOD PERFORMANCES

Estimated limits of quantitation

Compound	S/N* in calibration standard at 0,1 µg/L or 0,25 µg/L	Calculated instrumental LOQ (µg/L)	Estimated LOQ river water and WWTP effluent (ng/L) (CF**= 1000)	Estimated LOQ in WWTP influent (ng/L) (CF**= 400)
CORT-OH	62	0,02	0,02	0,04
CORT	91	0,01	0,01	0,03
DEXA	106	0,01	0,01	0,02
EPI-T	45	0,02	0,02	0,10
NORE	40	0,03	0,03	0,10
TESTO	46	0,02	0,02	0,05
ANDRO	50	0,02	0,02	0,05
DROSPI	35	0,03	0,03	0,10
LEVO	19	0,14	0,14	0,30
MEDROX	97	0,01	0,01	0,03
ANDROSTER	10	0,10	0,10	0,20
MEG_AC	167	0,01	0,01	0,01
PROG	61	0,02	0,02	0,04
E3	31	0,10	0,10	0,20
β-E2	13	0,10	0,10	0,20
α-E2	13	0,10	0,10	0,20
EE2	11	0,20	0,20	0,60
E1	11	0,10	0,10	0,24
DES	29	0,03	0,03	0,10
DIEN	52	0,02	0,02	0,05

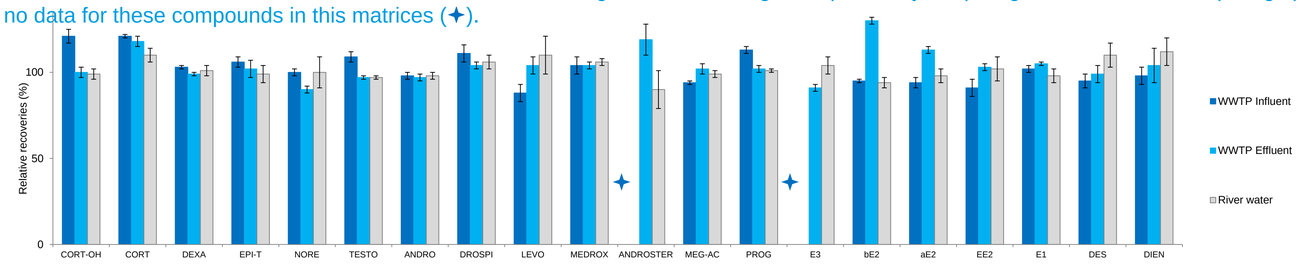
*S/N = signal to noise (peak to peak determination method)
**CF = concentration factor (sample volume is 250 mL for river water and for WWTP effluent and 100 mL fot WWTP influent)

Linearity range in waters

Compound	Correlation coefficient r^2	Concentration range for river waters and WWTP effluent (ng/L)	Concentration range for WWTP influent (ng/L)
CORT-OH	0,9995	0,02-100	0,04-100
CORT	0,9971	0,01-100	0,03-100
DEXA	0,9997	0,01-100	0,02-100
EPI-T	0,9992	0,02-100	0,10-100
NORE	0,9985	0,03-100	0,10-100
TESTO	0,9989	0,02-100	0,05-100
ANDRO	0,9991	0,02-100	0,05-100
DROSPI	0,9994	0,03-100	0,10-100
LEVO	0,9980	0,14-100	0,30-100
MEDROX	0,9987	0,01-100	0,03-100
ANDROSTER	0,9899	0,10-100	0,20-100
MEG_AC	0,9997	0,01-100	0,01-100
PROG	0,9987	0,02-100	0,04-100
E3	0,9989	0,10-100	0,20-100
β-E2	0,9995	0,10-100	0,20-100
α-E2	0,9995	0,10-100	0,20-100
EE2	0,9989	0,20-100	0,60-100
E1	0,9996	0,10-100	0,24-100
DES	0,9995	0,03-100	0,10-100
DIEN	0,9960	0,02-100	0,05-100

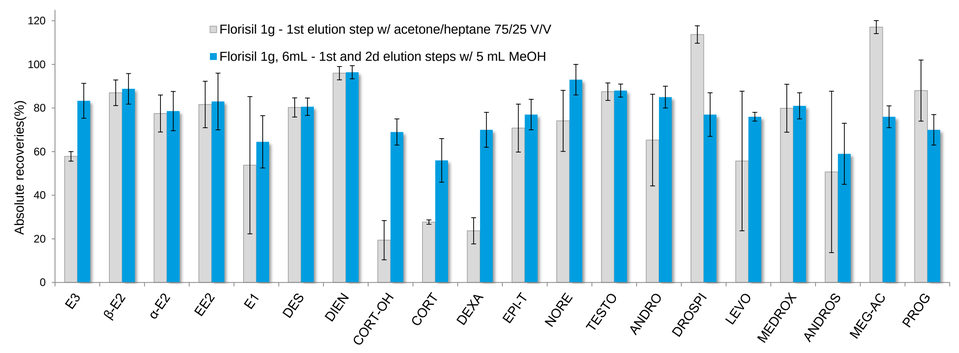
Accuracy & repeatability

For all experiments in intermediate fidelity conditions (2 operators and one duplicate, n=4. Relative recoveries (internal calibration) ranging from **88 to 130%** and relative standard deviations smaller than **11%** show good accuracy and repeatability. Native concentrations for E3 and ANDROSTER in WWTP influent are out of calibration range and are too high comparatively to spiking concentration level (80 ng/L) → no data for these compounds in this matrices (✦).



PURIFICATION OPTMISATION

Recoveries and repeatability increase with a second MeOH elution. Following results are absolute recoveries (external calibration) for n=3 experiments.



1st RESULTS

The whole analytical method has been applied on real samples. Following results show concentration levels for compounds that occur in 3 different matrices.

Compound	WWTP Influent (ng/L)	WWTP Effluent (ng/L)	River water (ng/L)
CORT-OH	61	ND	ND
CORT	79	ND	ND
DEXA	3	2	ND
EPI-T	26	ND	ND
NORE	ND	ND	ND
TESTO	27	ND	ND
ANDRO	121*	5	ND
DROSPI	ND	ND	ND
LEVO	11	ND	ND
MEDROX	2	ND	ND
ANDROSTER	792*	4	ND
MEG-AC	0,3	0,2	0,1
PROG	8	0,3	ND

Compound	WWTP Influent (ng/L)	WWTP Effluent (ng/L)	River water (ng/L)
E3	169*	ND	ND
β-E2	17	3	ND
α-E2	2	ND	ND
EE2	ND	ND	ND
E1	39	4	0,5
DES	ND	ND	ND
DIEN	ND	ND	ND

*these concentrations are out of calibration range. We have to dilute and reanalyze them to get more accurate values.
ND = Not detected

CONCLUSION & PERSPECTIVES

These preliminary results are acquired in the context of a conscientious and complete method validation process. More experiments have to be driven according to experimental design described in our reference standard (NF T90-210[3]) in order to:

- prove that linear model for calibration is the proper one (F test),
- verify LOQ on accuracy criteria by spiking various aqueous matrices,
- study accuracy and repeatability of the entire method at a lower concentration level,
- determine uncertainties at each concentration level and in various types of water (ISO 11352:2012[4]),
- control matrix effects in various types of water and show specificity of this method (T test).

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[1] C. Miege, P. Bados, C. Brosse, M. Coquery. Method validation for the analysis of estrogens (including conjugated compounds) in aqueous matrices, Trends in Analytical Chemistry, Vol. 28, No. 2, 2009
[2] 2002/657/EC: Commission Decision of 08/12/2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results
[3] AFNOR: NF T90-210 (2009) - Qualité de l'eau : Protocole d'évaluation initiale des performances d'une méthode dans un laboratoire
[4] ISO 11352:2012 - Water quality - Estimation of measurement uncertainty based on validation and quality control data