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Method development and validation for the analysis of 20 hormones (including estrogens, androgens and progestagens compounds) in various aqueous matrices P. Bados, F. Combaluzier, M. Coquery, C. Miège

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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



Statgraphics®

data treatment

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.



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



Compound	Retention	TransitionsMs(Quantification and cetentionMs(Quantification and confirmation)para					S-MS meters		
	rime (mm)	Parent ion	Daughter ion	Туре	DP (V)	EP (V)	CE (V)	CXP (V)	
Cortisol	3,60	363 363	121 91	Q C	76		33 89	8 6	
Cortisol-D4	3.59	367	121	Q	66		31	21	
Corticopo	2 60	367 361	97 163	C Q	61		47 33	18 10	
Contisone	5,00	361 369	91 168	C Q			89 35	16 16	
Cortisone-D8	3,58	369	351	С	65		17 12	24 24	
Dexamethasone	4,08	393	91	C	46		87	6	
Dexamethasone-D4	4,06	397 397	377 359	Q C	45		13 17	22 24	
Testosterone	4,71	289 289	109 97	Q C	36		37 31	8 6	
Testosterone-D4	4,69	293 293	98 110	Q C	76		31 35	18 20	
Norethindrone	4,73	299	109	Q	69		37 65	9 16	
Norethindrone-D6	4,71	305	91	Q	65		68 51	16	
Androstenedione	4.92	287	97	Q	66		31	6	
Androstanadiana D7	4 00	287 294	109 100	C Q	76		35 33	8 18	
	+,3U	294 367	113 97	C Q	70	10	37 37	20 18	
Drospirenone	5,06	367 371	91 99	C	76		89 43	6 17	
Drospirenone-D4	5,05	371	91 07	ĉ	74		81 22	16	
Epitestosterone	5,07	289 289	109	C	68		33 37	19	
Epitestosterone-D5	5,05	294 294	100 113	Q C	77		35 38	17 19	
Levonorgestrel	5,18	313 313	91 109	Q C	71		63 37	16 8	
Levonorgestrel-D6	5,16	319 319	91 87	Q C	76		65 55	16 16	
Megestrol Acetate	5,71	385 385	325 267	Q C	61		21 25	20 18	
13C-Megestrol Acetate-D3	5,70	389 389	325 267	Q C	61		21 27	20 16	
Progesterone	5,73	315 315	109 97	Q C	46		35 33	8 6	
Progesterone-D9	5,70	324 324	100 113	Q C	76		35 37	18 10	
Medroxyprogesterone*	5,37	345 345	123 97	Q C	70		37 43	22 17	
Androsterone*	5,51	291 291	273 255	Q C	45		11 19	16 25	
Estriol	0,60	287 287	145 171	Q C	-110		-54 -50	-7 -15	
Estriol-D2	0,60	289	147	Q C	-95		-54	-13	
β-Estradiol	1,80	270 270	145 183	Q C	-95		-52 -54	-7 -9	
β-Estradiol-D2	1,80	273	185	Q C	-90		-58	-19	
α-Estradiol	2,20	270 270	145 183	Q	-95		-54 -46	-7 -9	
α-Estradiol-D2	2,20	273	185	Q C	-90		-54	-19	
α -Ethinylestradiol	2,50	295 295	154 159	Q C	-55		-54 -46	-7 -13	
α -Ethinylestradiol-D4	2,40	299	147	Q C	-90	-10	-54	-7	
Estrone	2,70	269 269	145 143	Q C	-85		-50 -66	-7 -25	
Estrone-D4	2,70	273	147	Q C	-90		-50	-7	
Diethylstilbestrol	3,30	267 267	251 237	Q C	-93		-32 -36	-26 -53	
Diethylstilbestrol-D8	3,22	275 271	259 255	Q C	-75		-38 -39	-145 -20	
Dienestrol	3,54	265 265	93 249	Q C	-103		-42 -42	-3 -13	
Dienestrol-D6	3,77	271 271	95 255	Q C	-90		-38 -40	-17 -21	
*Medroxyprogesterone and	nd Androsterone pro	are respectogesterone-	tively associate -D9	ed to IS te	estoste	erone	-D3 ar	nd	



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)

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) \rightarrow no data for these compounds in this matrices (\bigstar).

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

PURIFICATION OPTMISATION



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)	Compound	WWTP Influent (ng/L)	WWTP Effluent (ng/L)	River water (ng/L)	
CORT-OH	61	ND	ND	E3	169*	ND	ND	
CORT	79	ND	ND	β-E2	17	3	ND	
DEXA	3	2	ND	α-E2	2	ND	ND	
EPI-T	26	ND	ND	EE2	ND	ND	ND	
NORE	ND	ND	ND	E1	39	4	0,5	
TESTO	27	ND	ND	DES	ND	ND	ND	
ANDRO	121*	5	ND	DIEN	ND	ND	ND	
DROSPI	ND	ND	ND					
LEVO	11	ND	ND					
MEDROX	2	ND	ND					
ANDROSTER	792*	4	ND	*these concentrations are out of calibration range. We have to dilute and reanalyze them to get more accurate values.				
MEG-AC	0,3	0,2	0,1					
PROG	8	0.3	ND	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