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

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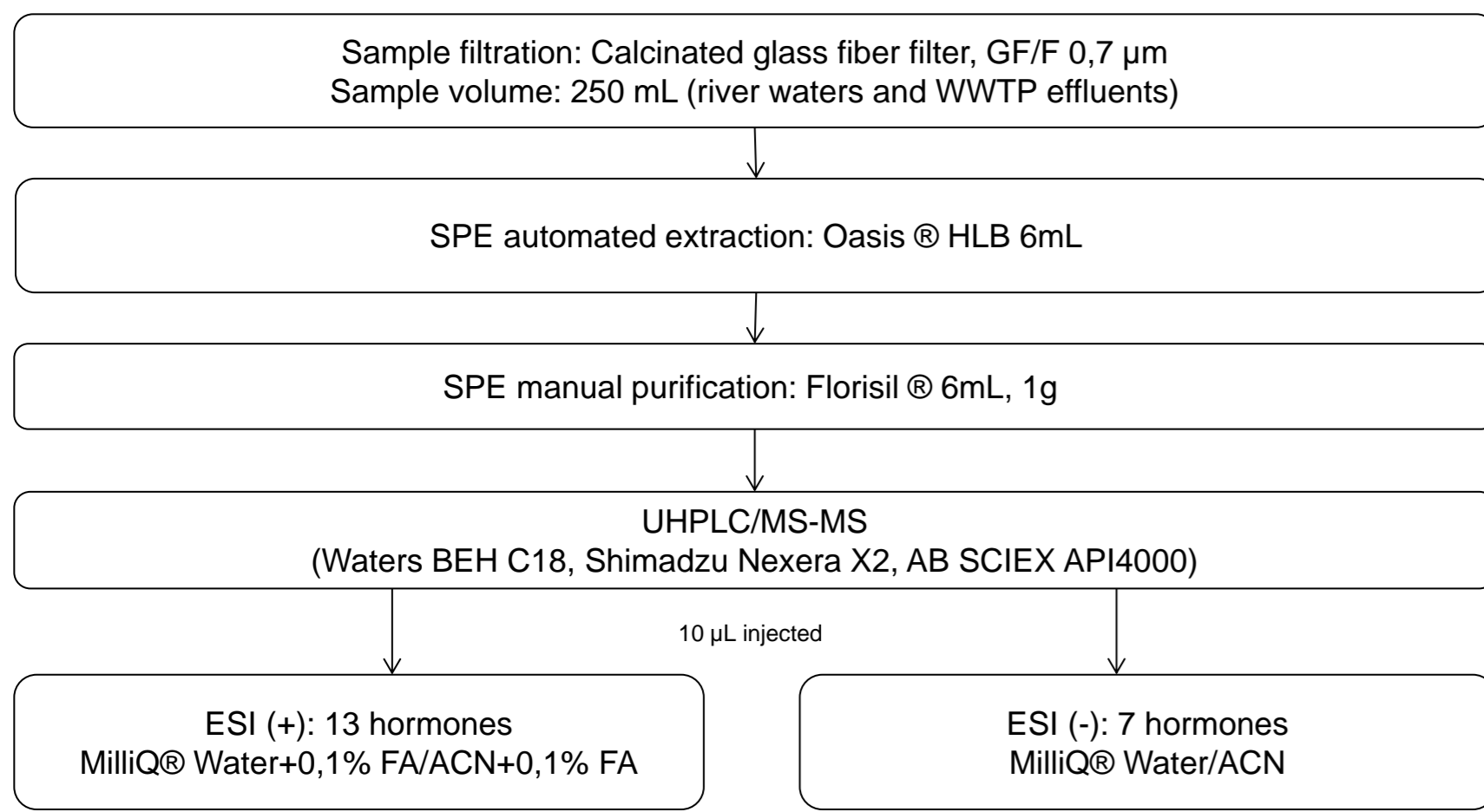
INTRODUCTION

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 synopsis

Initially based on previous estrogenic analytical method [1]

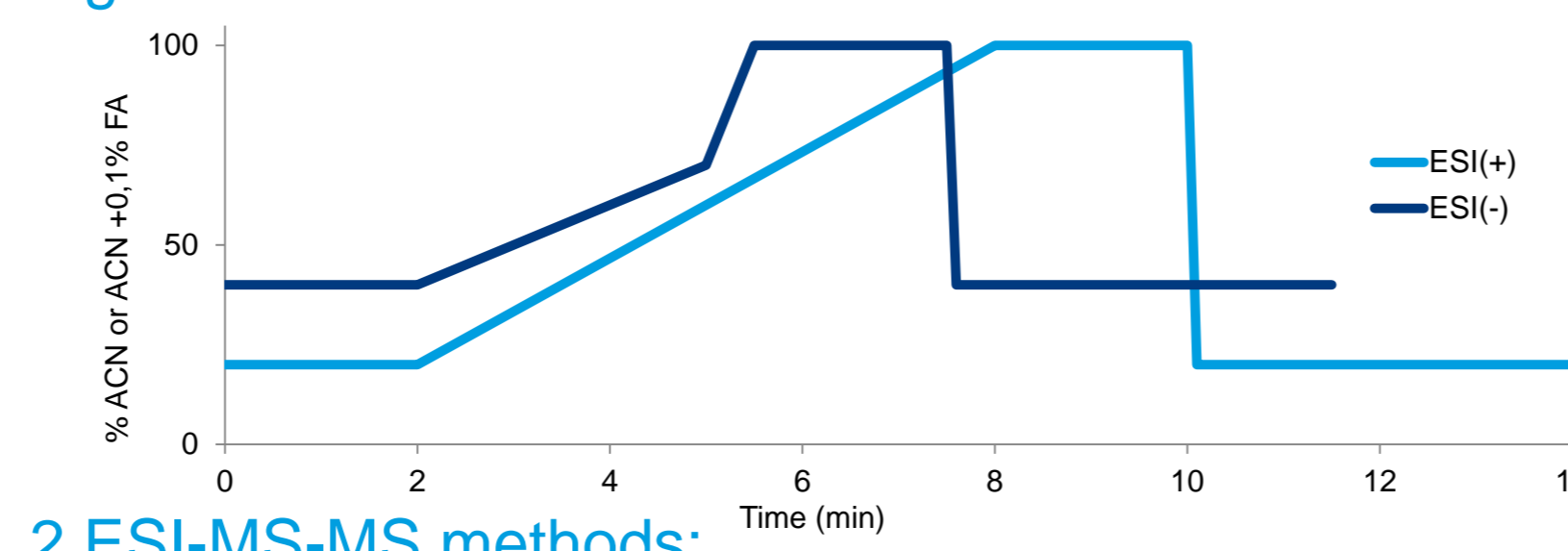


UHPLC/MS-MS parameters

Apparatus:

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

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 ¹³C) as internal standard (IS)

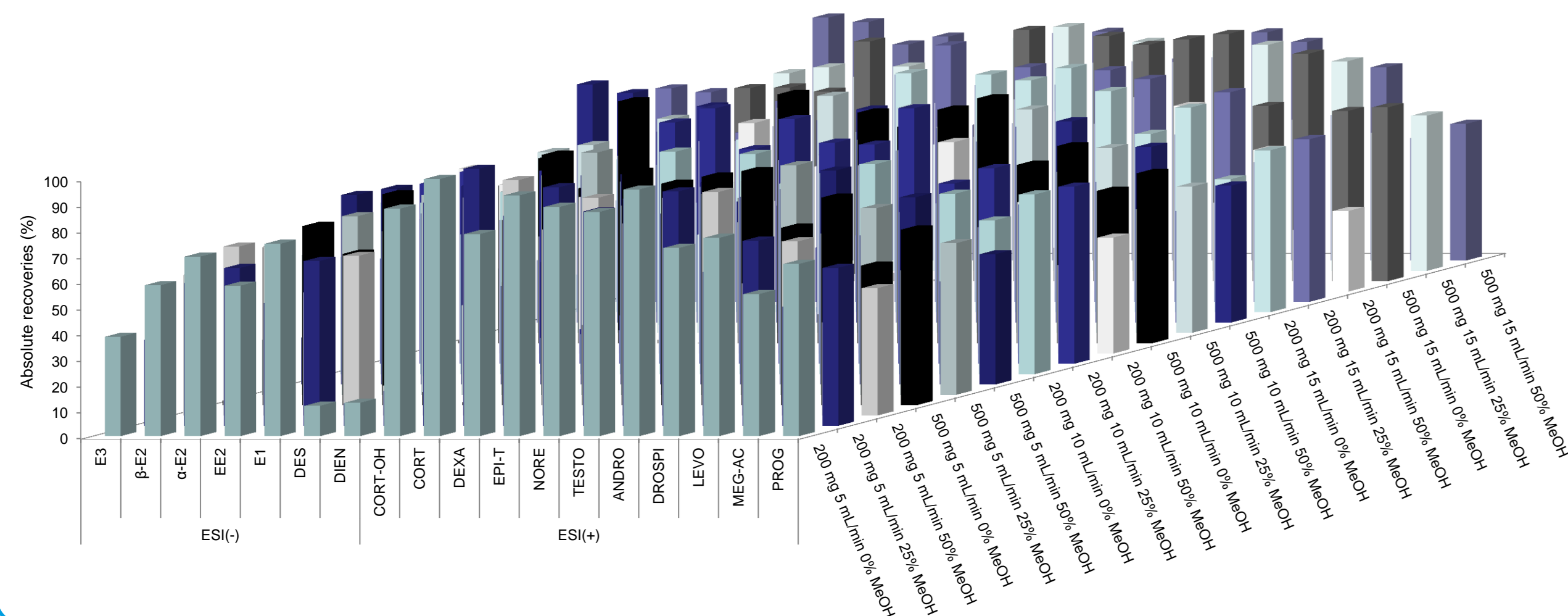
MRM acquisition parameters

Compound	Retention Time (min)	Transitions (Quantification and Confirmation)		MS-MS parameters			
		Parent Ion	Daughter Ion	Type	DP (V)	EP (V)	CE (%)
Cortisol	3.60	363	121	Q	76	33	8
Cortisol-D4	3.59	363	91	C	66	89	6
Cortisone	3.60	361	163	C	61	33	19
Cortisone-D8	3.58	361	91	C	65	35	16
Dexamethasone	4.08	393	373	Q	46	13	24
Dexamethasone-D4	4.06	393	91	C	46	87	6
Testosterone	4.71	289	109	Q	36	31	8
Testosterone-D4	4.69	289	97	C	76	31	18
Norethindrone	4.73	299	109	Q	69	37	9
Norethindrone-D6	4.71	299	91	C	65	16	16
Androstenedione	4.92	287	97	Q	66	31	6
Androstenedione-D7	4.90	284	100	Q	76	33	18
Drosiprenone	5.06	367	97	Q	76	37	18
Drosiprenone-D4	5.05	371	91	C	74	81	16
Epitestosterone	5.07	289	97	Q	68	33	17
Epitestosterone-D5	5.05	294	100	Q	77	35	17
Levonorgestrel	5.18	313	91	C	71	37	8
Levonorgestrel-D6	5.16	319	87	C	76	55	16
Megestrol Acetate	5.71	385	325	Q	61	21	20
13C-Megestrol Acetate-D3	5.70	389	325	Q	61	21	20
Progesterone	5.73	315	97	Q	46	33	6
Progesterone-D9	5.70	324	113	C	76	37	10
Medroxyprogesterone*	5.37	345	123	Q	70	37	22
Androsterone*	5.51	291	273	Q	45	11	16
Estriol	0.60	287	145	C	-110	-54	-7
Estriol-D2	0.60	289	147	C	-95	-54	-13
β-Estradiol	1.80	270	145	C	-95	-52	-7
β-Estradiol-D2	1.80	273	185	C	-90	-58	-19
α-Estradiol	2.20	270	145	C	-95	-54	-7
α-Estradiol-D2	2.20	273	185	C	-90	-54	-19
α-Ethinylestradiol	2.50	295	154	Q	-55	-54	-7
α-Ethinylestradiol-D4	2.40	299	147	Q	-10	-54	-7
Estrone	2.40	269	145	C	-85	-50	-7
Estrone-D4	2.70	269	143	C	-85	-66	-25
Diethylstilbestrol	3.30	267	251	Q	-93	-32	-26
Diethylstilbestrol-D8	3.22	271	259	Q	-75	-39	-20
Dieneol	3.54	265	249	C	-103	-42	-13
Dieneol-D6	3.77	271	255	Q	-90	-38	-17

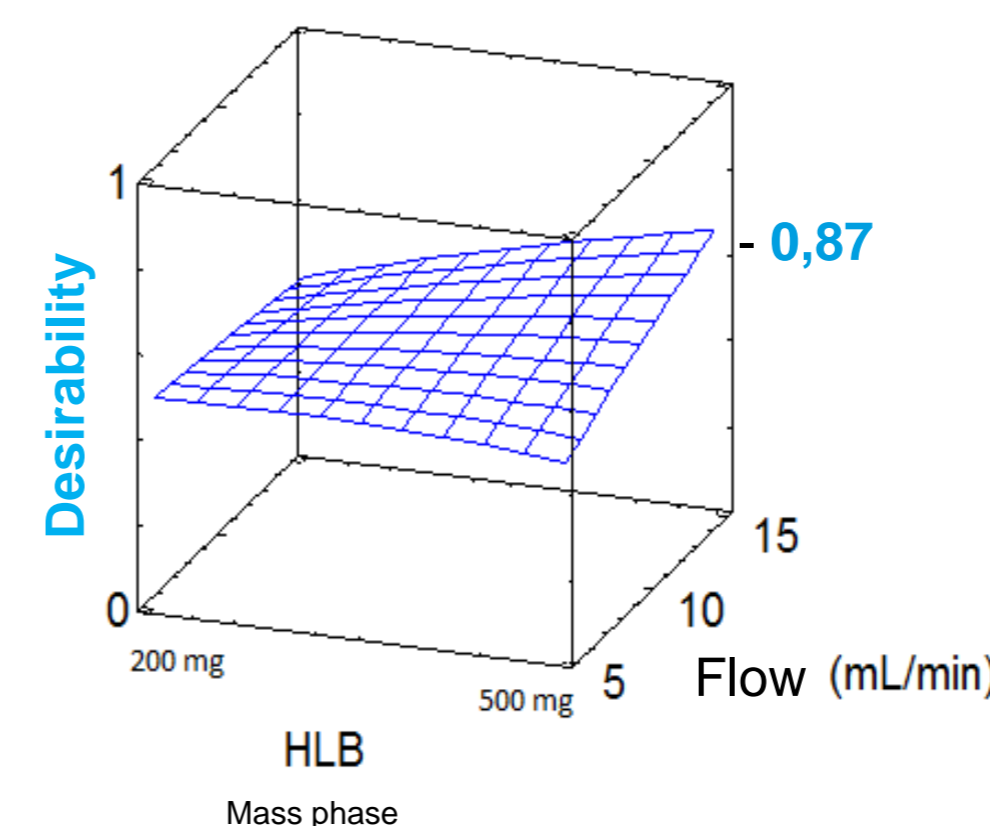
SPE OPTIMISATION

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



METHOD PERFORMANCES

Estimated limits of quantitation

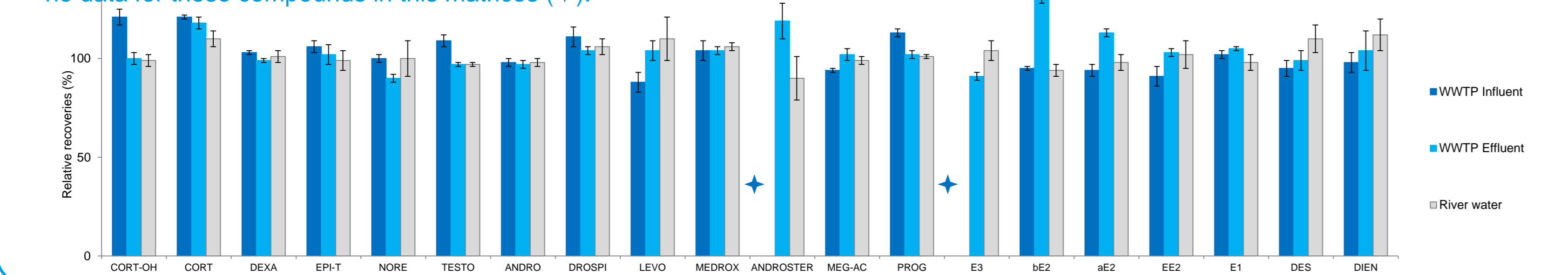
Compound	S/N* in calibration standard at 0,1 µg/L or 0,25 µg/L	Calculated instrumental LOQ (µg/L)	Estimated LOQ river water and WWTP effluent (ng/L) (CF**=1000)	Estimated LOQ in WWTP influent (ng/L) (CF**=400)
CORT-OH	62	0,02	0,02	0,04
CORT	91	0,01	0,01	0,03
DEXA	106	0,01	0,01	0,02
EPI-T	45	0,02	0,02	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

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

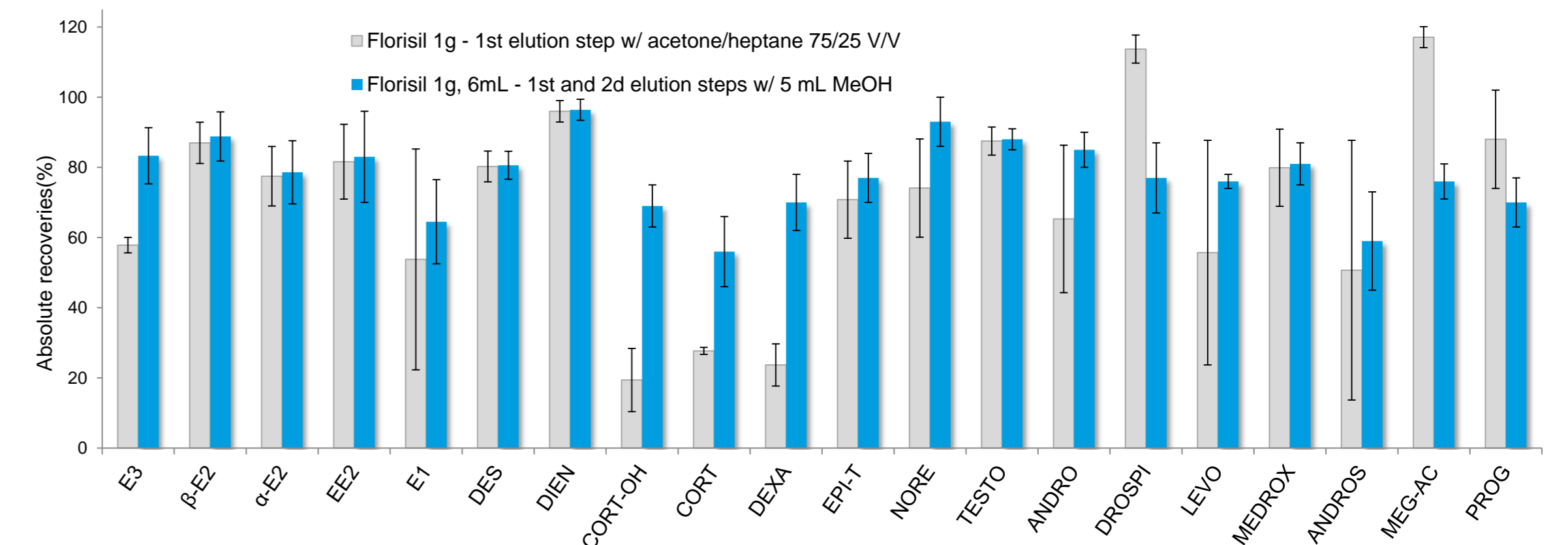
Accuracy & repeatability

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 OPTIMISATION

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)	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				
MEG-AC	0,3	0,2	0,1				
PROG	8	0,3	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).

[1] C. Miegé, 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