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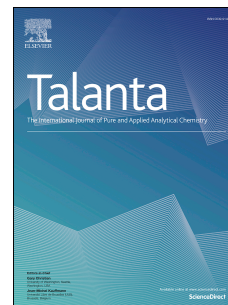


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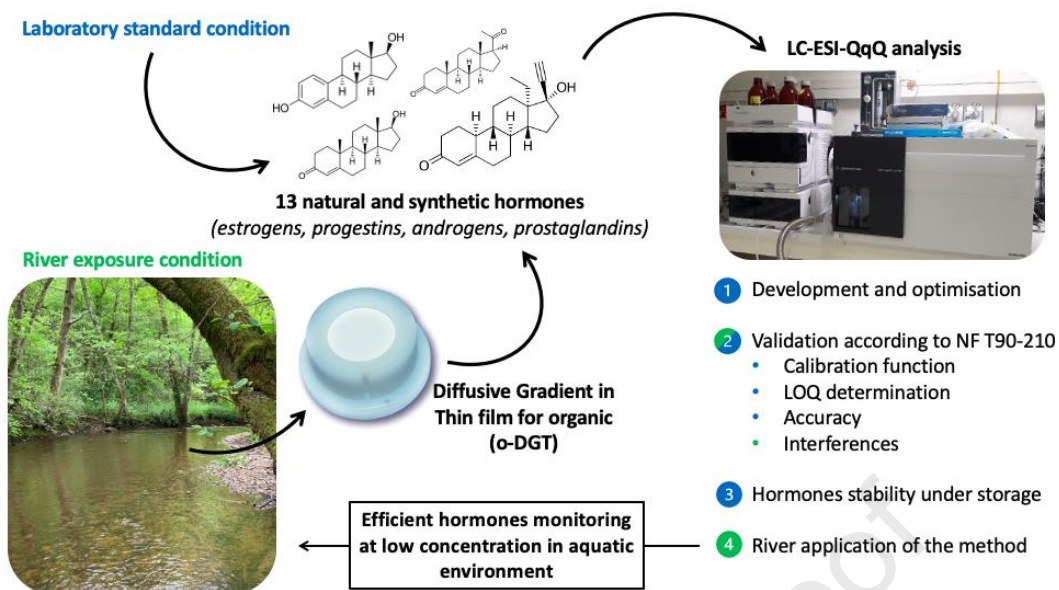
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Development of a multi-hormone analysis method by LC-MS/MS for environmental water application using diffusive gradient in thin films

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Abstract

An analysis method for four families of hormones (estrogens, progestins, androgens and prostaglandins), dedicated to an efficient water monitoring with passive sampling, was developed using a liquid chromatography tandem mass spectrometry with triple quadrupole coupling and universal electrospray ionisation. Thirteen natural and synthetic hormones in ultra-pure water could be analysed in a single run according to the French Standard NF T90-210: calibration range of 0.1 (except for 17 β -Estradiol, Estriol, Estrone and Diethylstilbestrol, from 0.5 $\mu\text{g/L}$; and Ethinylestradiol, from 1 $\mu\text{g/L}$) to 20 $\mu\text{g/L}$ with linear regressions ($R^2 \geq 0.96$), maximum accuracy deviations of 30% at intermediate fidelity for three concentration references (1, 10 and 20 $\mu\text{g/L}$) and instrumental LOQs from 0.05 to 1 $\mu\text{g/L}$. The stability of 11 hormones (10 $\mu\text{g/L}$) was studied under several storage conditions and sample evaporation. All selected hormones were stable for 60 days at -18°C, 7 days at 4°C and 7 days at 20°C but continued drying flow after evaporation should be avoided, especially for 17 α -Estradiol, Estrone and Diethylstilbestrol. Observed matrix effects using o-DGT extracts (diffusive gradient in thin-film sampler for polar organics) containing an environmental matrix varied from 24 to 92% but all matrix effects were corrected with IS use. Therefore, the developed method, coupled with o-DGT, was tested with the o-DGT deployment in rivers. Using diffusion coefficients from the literature or lab determined, the concentrations in the rivers varied for Estrone from 1.8 ng/L to 2.5 ng/L, and for Androstenedione from 0.4 to 1.1 ng/L.

Keywords: Hormone analysis, Liquid Chromatography-Electrospray Ionisation-Triple Quadrupole, matrix effects, passive sampling, o-DGT, hormones storage stability

28 Endocrine disrupting chemicals, including hormones, have a negative impact on aquatic organisms, especially
29 interfering with reproduction and development. For example, even at low concentrations in the ng/L range,
30 hormones can induce fish vitellogenin production, which has an effect on testicular growth, testis and ovaries
31 size and, consequently, on reproduction capacity and sexual differentiation [1–3]. The hormones, naturally
32 produced by humans and animals or provided by contraception and hormonal treatments, can be introduced
33 in the aquatic environment. Due to an incomplete removal in the wastewater treatment plant (WWTP) [4,5],
34 the presence of such hormones in rivers has first been demonstrated downstream of a WWTP [6,7]. Since
35 then, this has been confirmed by many authors, mainly for estrogens [8,9] and some progestins and
36 androgens [10,11]. Some studies have also demonstrated hormone contamination via agricultural practices
37 such as direct livestock excretion and runoff into pastures or fields with slurry [12–14]. These different
38 activities lead to river hormone concentrations ranging from 0 to 75 ng/L for Estrone [9,11,12], 0 to 26 ng/L
39 for Progesterone [11,15] or 0 to 8.6 ng/L for Androstenedione [11].

40 In the 1990s, as shown in Table S1, hormone analysis using a gas chromatography tandem mass spectrometer
41 (GC-MS/MS) was first developed for environmental application, with the analysis of surface water and
42 wastewater [4,7,16] with grab samples. Despite the need for compound derivatization before analysis, the
43 GC-MS/MS method is still used [17–19], mainly due to the possibility to quantify hormones from different
44 families in one run. To bypass the restrictive step of derivatization and for some advantages such as higher
45 selectivity, sensitivity and flow and also a shorter analytical time, several methods more practical by liquid
46 chromatography tandem mass spectrometer (LC-MS/MS) were developed mainly with triple quadrupole
47 coupling (QqQ) [8,20,21]. Different ionisation sources have been used: a universal analytical laboratory
48 ionisation source, the electrospray ionisation (ESI) [8,11,22]; a less universal analytical laboratory ionisation
49 source, the atmospheric pressure chemical ionisation [13,21] and an unusual analytical laboratory ionisation
50 source, the atmospheric pressure photoionisation [10,12]. These analytical methods mainly allow the
51 analysis of only three to twelve estrogens [8,12,22] or a total of five to nineteen hormones from two to three
52 families among estrogens, progestins, androgens or glucocorticoids in one run [11,13,21]. Nevertheless, the
53 methods which analyse the most hormones or families in one run require solvents being not environmentally
54 friendly (*e.g.*, toluene [10]) or an unconventional ionisation source considered less prone to matrix-induced
55 variability [10,13,14]. Moreover, to analyse more hormones via the usual LC-MS/MS method or more families
56 from environmental samples, at least two methods and runs are required [11,23].

57 In view of the low, but biologically active, concentrations of hormones in water (\approx ng/L), passive sampling
58 presents a great benefit for environmental application because of the accumulating and integrative
59 capacities [24–26]. The technique of diffusive gradient in thin film (DGT), initially developed for inorganics
60 [27], has recently been adapted for organic compounds (o-DGT), including hormones [28–31]. However,
61 these studies only concern a few hormones and mainly estrogens. Due to a better robustness to the
62 environmental conditions of deployment such as water flow, o-DGT are more and more deployed for
63 monitoring water quality, sometimes in addition to spot sampling [26]. However, in addition to hormone-
64 specific sampling, o-DGT also concentrate an environmental matrix and, potentially, a sampler-specific
65 matrix as highlighted for the polyethylene glycol release by POCIS (polar organic chemical integrative
66 sampler) polyether sulfone membranes [32]. The presence of the matrix can have a large impact on the
67 analysis and, especially, the MS ionisation by signal enhancement or suppression [33,34].

68 The aim of the present study was to propose an efficient and robustness monitoring method using passive
69 sampling approach for water monitoring of 13 natural and synthetic hormones from four families: estrogens,
70 progestins, androgens and prostaglandins. For that, the different steps were *i)* to develop and optimise an
71 easy, rapid and sensitive LC-ESI-QqQ method for a simultaneous routine analysis of the 13 hormones, *ii)* to
72 investigate the matrix effects absence or correction during o-DGT extracts analysis, *iii)* to test the hormone
73 samples stability under different storage conditions and *iv)* to investigate the developed method with the
74 sampling of hormones by o-DGT in several rivers.

75 **Experimental section**

76 Chemicals and materials

77 All reagents of LCMS grade, such as methanol (MeOH) and acetonitrile (ACN), were purchased from Carlo
78 Erba (Val de Reuil, France). Ultrapure water (UPW) was produced from a Milli-Qwater purification system
79 from Millipore (Watford, UK).

80 High-purity (> 95%) standards of 13 hormones, including six estrogens (17 α -Estradiol (α E2), 17 β -Estradiol
81 (E2), Estriol (E3), Estrone (E1), 17 α -Ethinylestradiol (EE2) and Diethylstilbestrol (DES)), four progestins
82 (Progesterone (P), Norgestrel (NG), Norethisterone (NTR) and Megestrol acetate (MGA)), two androgens
83 (Androstenedione (A) and Testosterone (T)) and one prostaglandin (Cloprostenol (C)) and internal standards
84 (IS) 17 α -Estradiol-d2 (α E2-d2), Estriol-d3 (E3-d3), Estrone-d4 (E1-d4), Progesterone-d9 (P-d9), Norgestrel-d6

85 (NG-d6), Norethisterone-d6 (NTR-d6), Megestrol acetate-d3 (MGA-d3), Androstenedione-d7 (A-d7) and
86 Testosterone-d5 (T-d5) were purchased from TechLab (Metz, France) or Cluzeau Info Labo (Sainte-Foy-La-
87 Grande, France). Stock solutions of chemicals at 100 mg/L were prepared in acetonitrile and stored at - 18°C
88 for a maximum of 1 year without observing degradation.

89 The DGT materials such as plastic holders, polycarbonate filters (pore size 0.4 µm), agarose powder and
90 Oasis® HLB powder were provided by DGT Research (Lancaster, UK), Whatman (Florham Park, USA),
91 Sigma-Aldrich (St Louis, USA) and Waters (Guyancourt, France), respectively. The syringes and syringe
92 cellulose acetate filters (pore size 0.2 µm) were obtained from Terumo (Tokyo, Japan) and Sartorius
93 (Göttingen, Germany).

94 Analysis method development by LC-MS-QqQ

95 The analysis method development was conducted using a liquid chromatography (Agilent technology 1290
96 Infinity II) mass spectrometer (Agilent technology 6470 triple Quad) with *i*) the determination of the
97 precursor/product ion transitions for each compound and the selection of optimised parameters for the ESI
98 and the transit of the precursors and the products ions and *ii*) the test of several conditions of separation
99 identified in Table 1, including different columns, column temperatures, eluents and eluent flow rates. The
100 method development was investigated for the analysis of the 13 natural and synthetic hormones presented
101 in Table 2, including six estrogens, four progestins, two androgens and one prostaglandin.

102 Analysis method validation

103 Method validation was carried out according to Guibal et al. [35] by applying the French Standard
104 NF T90-210 [36] for the study of the linear calibration, the accuracy and the limits of quantification. A
105 summary of this French Standard methodology is provided in Supporting Information. Two conditions were
106 investigated: an intermediate precision condition (change of one or more factors within the laboratory such
107 as the manipulator, the days of preparation and analysis) and a repeatability condition (same preparation
108 and analysis conditions). The internal standards (IS) were added to each standard at 5 µg/L for T-d5 and A-d5,
109 at 12.5 µg/L for MGA-d6, αE2-d2 and E1-d4, at 25 µg/L for NTR-d6, at 50 µg/L for NG-d6 and P-d9 and at
110 500 µg/L for E3-d3. The data were subjected to Grubbs and Cochran tests at α 1% to detect singular values
111 and verify the homogeneity of variances, respectively.

112

Table 1.

Tested parameters for the analytical method development.

LC parameters	Columns	InfinityLab Poroshell 120EC-C18 (2.1 x 100 mm; 2.7 μ m) EC 100/2 Nucleoshell biphenyl (2 x 100 mm; 2.7 μ m) Acquity UPLC HSS T3 (2.1 x 100 mm; 1.8 μ m)
	Eluents	UPW/MeOH UPW/ACN UPW/ACN + 0.005 to 5 mmol/L ammonium formate UPW/ACN + 0.1% formic acid
	Column temperatures	20 – 40 °C
	Flow rates	0.20 – 0.55 mL/min
	MS parameters	Sheath gas temperature (SGT) 200 – 400 °C Sheath gas flow (SGF) 8 – 12 L/min Gas temperature (GT) 150 – 350 °C Gas flow (GF) 4 – 12 L/min Nebulizer pressure (Neb) 10 – 60 psi Capillary voltage (CV) 1000 – 6000 V Nozzle voltage (NV) 0 – 2000 V

Linear calibration

Calibration linearity was investigated by a minimum five-point calibration among the standards at 0.1, 0.5, 1, 2, 5, 10, 15 and 20 μ g/L. This step was performed by five different manipulators at different analysis days with a new multi-hormone mix to consider intermediate precision condition. The adequacy and range of the linear calibration were verified by Fisher's α 1% fit test.

Instrumental limit of quantification

For each compound, the LOQ was determined by the twice injection of five solutions at an assumed LOQ_a concentration (targeted concentrations of 0.03 to 1 μ g/L) in repeatability condition. According to NF T90-210 [36], the LOQ values were validated with a 60% maximal acceptable deviation, using the following Equation 1:

$$[Average \pm 2.SD] \in [LOQ_a \pm 60\%.LOQ_a] \quad (\text{Equation 1})$$

Table 2. Hormones and internal standards (IS) details and their multiple reaction monitoring parameters. ^a datas from ChemAxon (<https://chemaxon.com/>).

Compounds	CAS number	pKa ₁ pKa ₂ ^a	ESI mode	Precursor/ product ions		Fragmentor (V)	Collision energy (V)	RT (min)	IS
Estrogens									
17 α -Estradiol (α E2)	57-91-0	<0 10.5	-	271	145 143	200	49 45	4.40	α E2-d2
17 α -Estradiol-d2 (α E2-d2)	-	-	-	273	147 145	204	45 49	4.38	-
17 β -Estradiol (E2)	50-28-2	<0 10.5	-	271	145 143	206	45 45	4.17	α E2-d2
Estriol (E3)	50-27-1	<0 10.3	-	287	171 145	194	45 49	2.53	E3-d2
Estriol-d3 (E3-d3)	-	-	-	290	148 174	194	41 53	2.52	-
Estrone(E1)	53-16-7	<0 10.3	-	269	145 143	190	41 41	5.36	E1-d4
Estrone-d4 (E1-d4)	-	-	-	273	147 145	182	41 45	5.34	-
17 α -ethinylestradiol (EE2)	57-63-6	<0 10.3	-	295	145 143	208	45 65	4.79	α E2-d2
Diethylstilbestrol (DES)	56-53-1	<0 8.6	-	267	237 251	176	25 33	5.44	E1-d4
Progestins									
Progesterone (P)	57-83-0	<0 >14	+	315	109 97	134	29 25	9.14	P-d9
Progesterone-d9 (P-d9)	-	-	+	325	100 113	148	33 25	9.01	-
Norgestrel (NG)	797-63-7	<0 >14	+	313	109 91	142	33 61	6.65	NG-d6
Norgestrel-d6 (NG-d6)	-	-	+	319	115 83			6.58	-
Megestrol acetate (MGA)	595-33-5	<0 >14	+	385	325 267	134	13 17	8.61	MGA-d3
Megestrol acetate-d3 (MGA-d3)	-	-	+	388	267 224			8.57	-
Norethisterone (NTR)	68-22-4	<0 >14	+	299	109 91	162	29 57	5.32	NTR-d6
Norethisterone-d6 (NTR-d6)	-	-	+	305	114 91	134	33 57	5.27	-
Androgens									
Androstenedione (A)	63-05-8	<0 >14	+	287	97 109	142	25 25	6.30	A-d7
Androstenedione-d7 (A-d7)	-	-	+	294	100 113	142	25 25	6.23	-
Testosterone (T)	58-22-0	<0 >14	+	289	109 97	142	29 29	5.02	T-d5
Testosterone-d5 (T-d5)	-	-	+	294	100 113	146	33 25	5.02	-
Prostaglandin									
Cloprostenol (C)	40665-92-7	<0 4.36	-	423	127 295	162	17 21	3.13	α E2-d2

Accuracy

In addition, standards at three different reference concentrations were prepared (1, 10 and 20 µg/L) in duplicate in a repeatability condition and by five different manipulators at different days according to the intermediate precision condition. Inter-days accuracy was validated with a 30% maximum acceptable deviation, as carried out in the French environmental analysis laboratory. This means that the average plus or minus twice the standard deviation (SD) must be included in a range of 30% around the reference value according to Equation 2:

$$[\textit{Average} \pm 2.SD] \in [\textit{REF} \pm 30\%.\textit{REF}] \quad (\text{Equation 2})$$

o-DGT: matrix effect and environmental application

Sampling details

The 3.14-cm² o-DGT configuration in this study consisted of a 7% Oasis[®] HLB agarose binding gel (thickness of 0.49 ± 0.01 mm; n = 3), an agarose diffusive gel (thickness of 0.75 ± 0.01 mm; n = 3) and a polycarbonate protective membrane (thickness of 0.01 mm and pore size of 0.4 µm). The gels were prepared in 1.5% agarose according to Challis et al. [28].

Field deployment

In March 2021, six o-DGT replicates were deployed in five rivers in the southwest of France for 14 days. Further information regarding the main characteristics of the watershed upstream of the sampling sites and the physico-chemical characteristics of the water is provided in Tables S2. Briefly, these rivers were characterised by a pH close to neutral (from 6.7 to 8.4); were weakly mineralised (from 63 to 141 µS/cm) with variable dissolved organic carbon contents (from 3.7 to 5.1 mg C/L) and had a flow velocity of 32 to 64 cm/s. In addition, two o-DGT field blanks were brought to the sampling sites without river exposition to evaluate the sampler contamination during the implementation.

Elution

All binding gels (30 o-DGT and 2 o-DGT field blanks) were eluted according to Challis et al. [28] by using three times 3 mL of methanol and 2 min of sonification between each methanol addition (Bioblock Scientific 210 W/TS 540, Illkirch-Graffenstaden, France). The eluates were then filtered through cellulose acetate filters

(pore size of 0.2 μm), evaporated (Biotage, Uppsala, Sweden) and reconstituted in 1 mL of eluent according to the initial chromatography conditions.

Study of matrix effects

To evaluate if the sample matrix influences the hormones analysis, a study of the specificity as recommended by NF T90-210 [36] was carried out. An average matrix was produced by mixing 800 μL of each of the 30 extracts of the o-DGT exposed to several natural rivers. This matrix was then analysed before spiking for background subtraction and after hormone spiking at 0.5, 1, 2, 5, 10, 15 and 20 $\mu\text{g/L}$. The calibration by the standard addition method in the matrix was compared with the UPW calibration, in repeatability condition, with or without internal standard addition. The percent of matrix effect (ME) was determined as follows by Equation 3 through the external and internal calibrations:

$$ME = \frac{(a' - a)}{a} \times 100 \quad (\text{Equation 3})$$

with a' and a were the calibration slope in matrix and UPW, respectively. A negative or positive ME indicated a signal suppression or enhancement, respectively. For an absolute offset from the calibration slope lower than 20%, no significant matrix effect was considered.

Determination of river concentrations

The IS were added to each sample extract. Subsequently, all samples were analysed with the method developed previously to determine the hormone amounts accumulated in the binding gel. Using the DGT equation [27] (Equation 4) and a diffusion coefficient under similar conditions (Table S3), the accumulated amount was converted to a river concentration:

$$C_{\text{DGT}} = \frac{m \Delta g}{D A t} \quad (\text{Equation 4})$$

where m was the accumulated amount, Δg was the diffusive layer thickness, D was the diffusive coefficient and A and t , respectively, were the exposure area and duration. The diffusion coefficients at 25°C (Table S3) must be corrected at the river temperature (Table S2) with Equation 5:

$$\frac{D_1 \times \eta_1}{T_1} = \frac{D_2 \times \eta_2}{T_2} \quad (\text{Equation 5})$$

179 where D_1 and D_2 were the diffusion coefficients at 25°C (T_1) and the corrected one at another temperature
180 (T_2) with η_1 and η_2 , the water viscosities at these temperature (taken from NIST chemistry WebBook).

181 Compound stability study

182 For each compound, the stability of the standard solution (10 µg/L) was evaluated under four different
183 storage conditions: storage at -18°C up to 60 days and freeze-thaw stability with three cycles ($n = 3$), storage
184 at 4°C up to 15 days ($n = 3$), storage at room temperature (20°C) with or without light exposition (clear or
185 amber vials) up to 7 days ($n = 3$). In addition, to see if the o-DGT sample matrix can affect the hormones
186 stability, the average matrix (Section above) spiked at 10 µg/L was stored at -18°C up to 60 days ($n = 3$). The
187 hormones stability to evaporation by nitrogen flux was evaluated in methanol or acetonitrile solutions and
188 this, up to 60 min of dryness beyond evaporation ($n = 5$). The experimental details are provided in the
189 Supporting Information.

190 To evaluate the hormones stability according to the different conditions, the ratios of the concentration after
191 storage or evaporation to the initial concentration were determined. For freeze-thaw stability, the ratios
192 were established between the concentration of the freeze-thaw sample and the frozen-only one. These
193 ratios were then compared to the theoretical value of 1, indicating no effect of storage or evaporation. In
194 addition of the graphical interpretation of the results, statistical tests at $\alpha 1\%$ were performed. The Student
195 and the ANOVA-LSD tests were used to identify the ratios different from 1 and to compare the ratios
196 evolution into a condition, respectively. These statistical tests assumed that the data were normally
197 distributed.

198 **Results and discussion**

199 Analysis method development

200 *MS parameters*

201 In agreement with several authors [28,38,39], estrogens were analysed in the negative ionisation mode and
202 androgens and progestins in the positive ionisation mode. The precursor/products ion transitions for each
203 compound and the selection of optimised parameters are provided in Table 2. To create a single method, an
204 ionisation mode switch between positive and negative mode was required during the analysis. The MS source
205 parameters were set with SGT and SGF of 400°C and 12 L/min respectively, GT and GF of 200°C and 6 L/min,
206 respectively, and Neb, CV and NV of 20 psi, 3000 V and 2000 V, respectively.

208 In term of chromatographic separation, the selected column was an EC 100/2 Nucleoshell biphenyl
209 (Macherey Nagel, 2 x 100 mm; 2.7 μm). To ensure adequate hormone separation, a mixture of UPW and
210 acetonitrile was used as eluents since the latter provided a better separation than methanol in agreement
211 with Chen et al. [40]. To improve and stabilise the analysis conditions, several eluent additives were tested
212 such as formic acid and ammonium formate. Alkaline additives in the eluents, such as ammonia or amines,
213 were not tested in this study due to the alkaline pKa (around 8–10) of some hormones (Table 2). The obtained
214 signals using the eluents with additives were compared with those obtained using the pure eluents. Thus,
215 the percentages of the obtained signals were calculated in relation to the use of the pure eluents.

216 Concerning the acidification of the eluents with 0.1% formic acid, a decrease of the hormone responses
217 (Figure 1) was observed compared to the use of pure eluent. Indeed, the obtained signal was less than 10%
218 under negative ionisation and varied from 15 to 25% under positive ionisation, depending on hormones. This
219 phenomenon was already shown for the esters, the parabens and the estrogens under negative ionisation
220 [40,41]. The signal decrease was too large, even if acidification could stabilise the analytical conditions by
221 promoting the acid forms of some compounds, such as Diethylstilbestrol and Cloprostenol (pKa in Table 2).

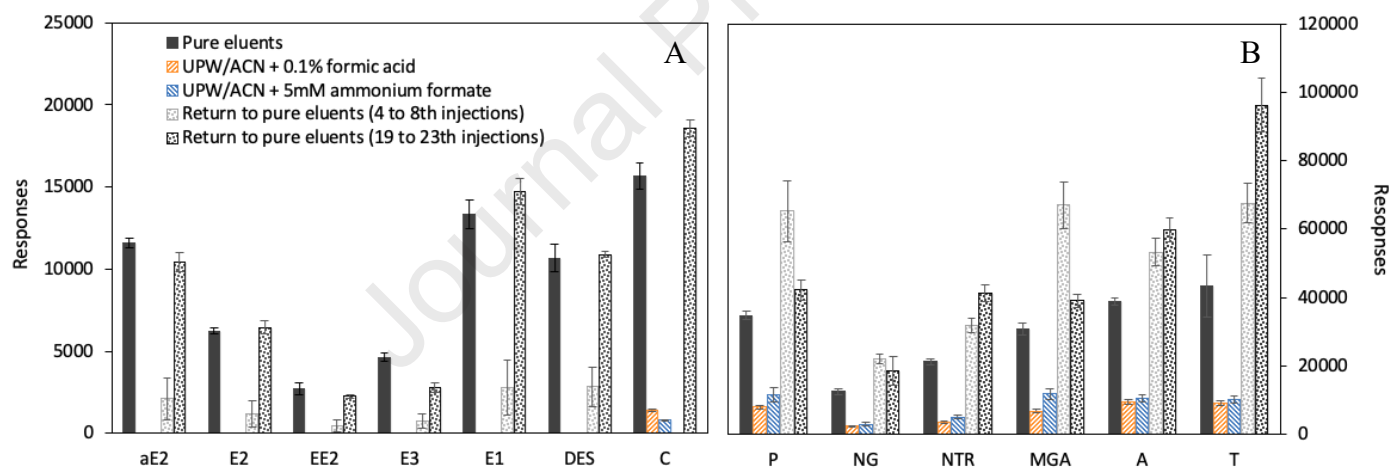
222 With the addition of ammonium formate to the eluents, the decrease of hormones signal was similar or
223 lower to that obtained with the use of formic acid (Figure 1). Indeed, under negative ionisation or positive
224 ionisation, the obtained signal was less than 5%, or between 20 and 40%, respectively. When the pure eluents
225 were reused after the eluents with 5 mmol/L of ammonium formate, the signals were increased under
226 positive ionisation up to a factor 2 compared to the initial pure eluents. However, under negative ionisation,
227 the signals were still decreased up to about 20 injections and were more widely dispersed, compared to the
228 start of the sequence. These phenomena may be related to the persistence of ammonium formate in the
229 chromatographic system. To demonstrate this, the same experiment with the addition of a smaller quantity
230 of ammonium formate in the eluents (from 0.005 to 1 mmol/L) was carried out. When the concentration of
231 ammonium formate increased, the impact on the signal was greater and the responses were then reduced
232 (Figure S1). By switching to the pure eluents again, the signals gradually increased to regain the initial one
233 after three or four injections under negative ionisation, and after one or two injections under positive
234 ionisation. As observed previously, the signals continued to increase and exceeded initial signals. If the traces

of ammonium formate were at the origin of this increase, this must correspond to a concentration of less than 0.005 mmol/L since the phenomenon was not observed at the lowest tested concentration.

Due to the issues induced by the addition of ammonium formate in the eluents, as previously underlined, and because all hormones had showed better responses with pure eluents (Figure 1), the selected eluents were only composed of UPW and acetonitrile. Various compositions of chromatographic elution gradient were tested, and the best separation was obtained with the following : starting at 20% acetonitrile up to 0.5 min, increasing to 40% from 0.5 to 1 min, further increasing to 60% from 1 to 8 min and then to 95% up to 9 min, which was maintained for 2 min; the initial conditions were recovered in 1 min. Column temperature and eluent flow were set at 35°C and 0.25 mL/min, respectively. The retention times under these conditions were listed in Table 2 and an example of the hormone separation is provided in Figure S2.

Figure 1.

Evolution of hormone responses, (A) under negative ionisation and (B) under positive ionisation, for five injections of a 10 µg/L standard with change of the UPW/ACN eluents: pure eluents (grey or spotted bars) or with 0.1% of formic acid (orange striped bar) or 5 mM of ammonium formate (blue striped bar). Hormone acronyms are defined in Table 2.



Method validation

As shown in Table 3, the study of the calibration showed that the data of calibration was linearly correlated with determination coefficients equal to or greater than 0.96 for all hormones. The Fisher's α 1% fit test revealed that the regression was validated for all hormones for concentrations range from 1 to 20 µg/L for Ethinylestradiol, from 0.5 to 20 µg/L for 17 β -Estradiol, Estriol, Estrone and Diethylstilbestrol and from 0.1 to 20 µg/L for the eight others. The instrumental LOQs varied between 0.05 and 1 µg/L depending on the

hormones, the ionisation mode and the sensitivity of the analytical method. The deviations for the accuracy were less than 28% for 1 µg/L and 18% for 10 and 20 µg/L, except for Ethinylestradiol, which was below 25% for these two concentration levels. Therefore, the accuracy was correct respecting the maximum acceptable deviation of 30% for the three concentration levels (Equation 2).

Compatibility of the analytical method with o-DGT hormone sampling

This experiment was performed with an average matrix from the different extracts containing the environmental matrix sampled by o-DGT illustrated by a chromatogram in Figure S3. The results of the investigation of the matrix effects and the suitability of IS correction is given in Table 3.

Ten hormones were impacted by the presence of an o-DGT environmental matrix. For Estrone and Diethylstilbestrol, a signal enhancement was present, leading to an increased calibration slope compared to UPW calibration of 48% and 24%, respectively. For the other hormones, the calibration slopes were reduced from 26 to 92%, indicating signal suppression. However, with the IS use, the slope deviations were lower than 20% for all impacted hormones. Therefore, the use of the IS allowed to correct the matrix effect phenomenon.

Table 3.

Results of method validation in terms of calibration, accuracy, instrumental LOQ determination and verification of o-DGT compatibility. %D, the percent of deviation. Lvl 1, 2 and 3 for 1, 10 and 20 µg/L standard concentrations. *ns*, not concerned because no matrix effect (ME). Hormone acronyms are defined in Table 2.

	Linear calibration		Instrumental LOQ (µg/L)	Accuracy (%D)			o-DGT matrix effect	
	Range	r ²		Lvl 1	Lvl 2	Lvl 3	Presence (%ME)	IS correction efficiency (remaining %ME)
Estrogens								
αE2	0.1-20	0.99	0.1	21	19	9	No effect	ns
E2	0.5-20	0.99	0.2	20	20	10	No effect	ns
E3	0.5-20	0.99	0.5	27	16	18	Suppression of 90%	Yes
E1	0.5-20	0.99	0.2	27	11	12	Enhancement of 48%	Yes
EE2	1-20	0.96	1	23	25	21	No effect	ns
DES	0.5-20	0.99	0.5	27	13	18	Enhancement of 24%	Yes
Progestins								
P	0.1-20	0.99	0.05	25	17	10	Suppression of 48%	Yes
NG	0.1-20	0.99	0.1	28	15	10	Suppression of 58%	Yes
MGA	0.1-20	0.99	0.05	15	15	6	Suppression of 78%	Yes
NTR	0.1-20	0.97	0.1	28	17	17	Suppression of 92%	Yes
Androgens								
A	0.1-20	0.99	0.05	10	9	6	Suppression of 57%	Yes
T	0.1-20	0.99	0.05	20	13	10	Suppression of 91%	Yes
Prostaglandin								
C	0.1-20	0.98	0.1	21	15	12	Suppression of 26%	Yes

Concerning the stability of hormones during the -18°C storage of the o-DGT sample matrix presented in Figure S5, the ratio variation of few hormones was around one. Therefore, the matrix presence didn't modify the hormones stability compared to the storage of a standard (Table 4) and 17α -Estradiol, Esthinyloestradiol, Estriol, Progesterone, Megestrol acetate and Testosterone were stable up to 60 days. For the others, a decreased of the ratio were observed (*e.g.*, Norethisterone) up to a factor of 2, signifying a potential degradation of these hormones. To avoid any stability issues, the o-DGT samples were not stored more than 7 days before analysis ensuring less than 20% degradation under the conditions tested for all studied hormones.

The Table 4 also presents the conclusions concerning the evaporation of the standard solutions in methanol or acetonitrile. Since the ratios of concentrations were around 1 (Figure S4), most hormones were considered stable during the evaporation and also during 60 min of maintaining drying flow after evaporation of the acetonitrile or methanol solutions. When drying flow was continued after evaporation of the methanol solution, an instability was observed for three hormones with ratios different from 1. Indeed, 17α -Estradiol, Estrone and Diethylstilbestrol cannot be considered as stable beyond 15, 30 and 30 min, respectively. Due to the instability of these compounds (17α -Estradiol, Estrone and Diethylstilbestrol), special care needs to be taken to avoid maintaining drying flow after evaporation.

Environmental application

Hormones were not detected in the o-DGT blanks, indicating that there was no significant contamination of o-DGT by the hormones during the implementation. The application of the analytical method indicated the presence of two hormones in the o-DGT extracts obtained for the studied rivers. The chromatograms revealing the presence of these hormones are provided in Figure S6.

The first hormone, Estrone (E1), was detected in the five selected rivers but only quantified in the six extracts of River 1 and one extract of River 4 and 5. The second one, Androstenedione, was also detected in all five selected rivers and quantified in all extracts except one of River 2. In some cases, some o-DGT replicas had a hormones concentration in the eluate lower than the LOQ, whereas the other replicates were quantified. In fact, the majority of the Estrone and Androstenedione concentrations measured in the extracts were close to the LOQ, with 1 to 3 and 1 to 7 times the LOQ, respectively. The presence of these compounds in rivers has already been reported by Liu et al., Matthiessen et al. and Tremblay et al. [11,12,19], among others. Other hormones were absent or not detected because of low o-DGT extract concentrations.

Table 5.

Average (\pm SD) river concentrations of Estrone and Androstenedione in ng/L in five rivers ($n = 6$). ^a only one quantified replica. ^b four quantified replicas.

	Estrone	Androstenedione
River 1	2.5 \pm 0.9	0.6 \pm 0.2
River 2	< 1.2	0.4 \pm 0.1 ^b
River 3	< 1.2	0.6 \pm 0.3
River 4	2.0 ^a	0.5 \pm 0.2
River 5	1.8 ^a	1.1 \pm 0.6

Following this exposure, Estrone and Androstenedione accumulated amount in the o-DGT binding gel were determined and converted to the average concentration during the deployment time using the Equation 4. The Estrone and Androstenedione concentrations in the studied rivers varied from 1.8 to 2.5 ng/L and from 0.4 to 1.1 ng/L, respectively (Table 5). These river concentrations were consistent with the concentrations found in another context. For example, Matthiessen et al. and Tremblay et al. [12,19] found average estrone concentrations of 1.67 ± 2.26 ng/L ($n = 23$ exposures) and 1.13 ± 0.86 ng/L ($n = 11$ exposures), respectively, in rivers close to agricultural estates. Liu et al. [11] measured 6.0 ± 0.6 ng/L and 8.1 ± 0.7 ng/L upstream of a wastewater treatment plant for Estrone and Androstenedione, respectively.

Conclusions

Our study showed that the analysis of 13 natural or synthetic hormones from four different families (six estrogens, four progestins, two androgens and one prostaglandin) can be performed and validated by the French Standard NF T90-210 [36] in one run and under standard analytical conditions, using LC MS/MS. The hormones analysis method is applicable from 0.1 to 20 μ g/L (except for few compounds such as 17 β -Estradiol, Estriol, Estrone and Diethylstilbestrol, which can be analysed from 0.5 to 20 μ g/L, or for Ethinylestradiol, from 1 to 20 μ g/L) with linear regression (determination coefficient ≥ 0.96). The maximum acceptable deviation of 30% is respected for an intermediate precision condition for three concentration levels (1, 10 and 20 μ g/L). The instrumental LOQs range from 0.05 to 1 μ g/L, depending on the compounds. During the analysis of environmental passive sampling matrix from the o-DGT extract, a signal suppression or enhancement was observed for 10 out of the 13 hormones, leading to a matrix effect of 24 to 92%, but this could be corrected by IS use for all impacted hormones, with a matrix effect lower than 20%.

The stability study revealed that a 10 μ g/L standard can be stored for 60 days at -18°C, 7 days at 4°C and 7 days at 20°C for all studied hormones. The 60 days freeze/thaw cycles have no additional effect on the

346 stability of the standards. The stability of certain hormones being lowered in o-DGT matrices, these samples
347 must therefore be analysed before 7 days of storage at -18°C. For evaporation, all hormones are stable in
348 methanol or acetonitrile, but maintaining drying flow after evaporation of a methanol solution should be
349 avoided beyond 15, 30 and 30 min, respectively for 17 α -Estradiol, Estrone and Diethylstilbestrol.

350 Finally, the developed hormone analysis method coupled with passive sampling (o-DGT), is suitable for the
351 analysis of four different hormone families (estrogens, progestins, androgens and prostaglandins) occurring
352 at the ng/L level in freshwater using standard analytical materials and general laboratory practices.

353 **Supporting Information**

354 Further experimental details, procedures and results for the various development experiments are provided
355 in the supporting information.

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487

- New analysis method for 13 natural and synthetic hormones in one LC-MS/MS run
- Stability study of hormones under several storage and sample processing conditions
- Analytical method and o-DGT sampling combination for river monitoring improvement
- Check/correction of matrix effects induced by a real environmental o-DGT deployment

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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