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Determination of steroid hormones in sediments based on quick, easy, cheap, effective, rugged, and safe (modified-QuEChERS) extraction followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

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Hormones, constantly excreted by humans and animals, are discharged into surface waters *via* various routes including municipal and industrial sewage treatment plants and also animal agriculture and aquaculture. Many analytical methods have been studied, developed and used for the determination of hormones in the aqueous phase while the study of the particulate phase (suspended solids, sediment, and soil) was less developed due to more complex pre-treatment. This paper describes a simple, rapid and effective procedure based on modified-QuEChERS to analyze targeted hormones with very different chemical structures, from river sediments. We coupled this sample preparation method with LC-MS/MS for the detection and quantification, the whole method proposed is both selective and sensitive. Except for the more hydrophobic compound, the recoveries are higher than 74%. The repeatability is comprised between 2 and 36% and the intermediate precision is comprised between 4 and 30%, depending on the compound and the level. The method enables the determination of the target analytes with limits of detection comprised between 0.03 and 0.2 ng g^{-1} and limits of quantification comprised between 0.1 and 0.6 ng g^{-1}. It is applied to the analysis of sediments collected at four points of the Bourbre river (Isère, France) and its tributary in October 2011. The results reveal the presence of estrone (up to >40 ng g^{-1}) and tamoxifen in all the samples (≥0.6 ng g^{-1}).

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1. Introduction

Various pollutants are scattered in different compartments of the environment due to the high consumption of drugs and the growing use of chemicals in everyday life and the agricultural activities in industrialized countries. The presence of environmental pollutants is measured at concentrations ranging from less than one ng L^{-1} or ng g^{-1} to dozens of g L^{-1} or mg g^{-1}, resulting in variable effects on the environment and on human health. In particular, the presence of natural and synthetic hormones in the aquatic environment has become a cause for increasing concern in recent years due to the endocrine disrupting properties of these chemicals at such a lower range of concentrations (ng L^{-1}).[1–3] The main sources of hormones in aquatic environments are their continuous discharge into surface waters by municipal and industrial sewage treatment plants that do not allow their total elimination, animal agriculture[4] and aquaculture.[5] Many data indicate the presence of hormones in surface waters,[6,7] or groundwaters.[6] As a sign of the importance attached to their presence in aquatic ecosystems, two hormones, a synthetic used contraceptive (17α-ethinylestradiol) and one natural (17β-estradiol), have been proposed to integrate the list of priority substances of the Water Framework Directive.[8]

It should be noted that analytical methods have mainly been studied, developed and used for the determination of hormones in the aqueous phase while the study of particulate phase (suspended solids, sediment, and soil) was less developed due to more complex pre-treatment. In this case, the first step required, by far the longest and delicate, is the sample preparation that combines one or more extractions and purifications. Conventionally used methods for the extraction of contaminants in solid matrices are Soxhlet, ultrasonic-assisted extraction or techniques like pressurized liquid extraction, microwave-assisted extraction and solid-phase microextraction.[9–11] An indispensable purification is then carried out by solid phase extraction (SPE), liquid–liquid extraction (LLE), gel permeation (GPC) or semi-preparative liquid chromatography.[9–12] The SPE is often preferred because it is faster, requires less solvent and has less risk of sample contamination.

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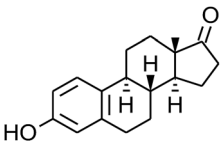
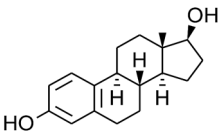
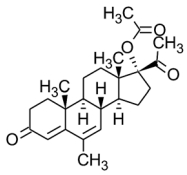
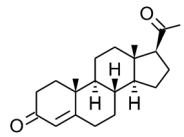
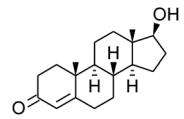
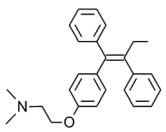
More recently a technique of extraction/partition with acetonitrile assisted by salts and usually followed by purification with dispersive SPE was developed by Anastassiades *et al.*[13] This technique is known in the field of food analysis under the trade name QuEChERS. This principle of extraction was applied to the determination of organic micropollutants in other solid matrices such as soil,[14,15] sediment[14,16] or sludge.[17]

Steps of separation and detection of compounds are based primarily on optimising coupling between gas (GC) or liquid (LC) chromatography and mass spectrometry (MS) or tandem mass spectrometry (MS/MS). The analysis of non-volatile and thermolabile compounds by GC-MS, however, requires an additional step of derivatisation,[18–20] and taking into account recent improvements in LC-MS/MS interfaces; the latter technique is more often preferred for identification and quantification of hormones in environmental matrices.[21–24]

The objective of the study paper was to develop a simple, rapid and efficient method to analyze targeted hormones from river sediments at realistic concentrations relative to environmental levels, *i.e.* less than or equal to a few nanograms per gram of dry sediment. The levels of hormones in sediments have been very little studied so far, compared to waters,[25,26] although this solid matrix represents a significant environmental compartment. Indeed substances may adsorb or accumulate and in this case the sediments may represent a kind of reservoir for chemicals and therefore represent a source of exposure to aquatic ecosystems.[27] A modified-QuEChERS extraction was developed as sample preparation followed by LC-MS/MS for the detection and quantification. The method was evaluated in terms of extraction efficiency, but also in terms of generated matrix effects. Matrix effects correspond to the perturbation of the signal due to the presence of residual components of the matrix that may promote either enhancement or more often suppression of the response in the mass spectrometer, causing a decrease of the sensitivity and errors in the results. The optimized method was finally validated.

In order to conduct these developments and comparison, hormones with different chemical structures were selected from lists of compounds known as endocrine disruptors. These molecules have partition coefficient values water/octanol (K_{ow}) comprised between 3 and 6 (Table 1), thus can be considered as moderately hydrophobic to hydrophobic and therefore likely to be found in the sediment. Thus, the list of selected compounds contains 4 natural or synthetic hormones, namely the androgen testosterone (T), the progestagen progesterone (P), two estrogens estrone (E1) and 17β-estradiol (β-E2). These compounds may be naturally excreted or may be issued from hormonal treatments as well as animal husbandry.[28,29] Two compounds were added and affiliated with synthetic hormones in the study list. The first is megestrol acetate (MegA), a derivative of progesterone used as a contraceptive when combined with an estrogen or as an appetite stimulant and endometrial or breast cancer treatment.[30] The second is tamoxifen (Tam), an anti-estrogen used as a treatment against hormone-dependent breast cancers expressing the estrogen receptor (ER+).[31] The empirical formulas, the semi-structural formulas and some other molecule properties are listed in Table 1.

Table 1 Structure and properties of the target compounds (ND: not disposable)

Molecule	Formula	Structure	log K_{ow}[32]	pK_a
E1	$C_{18}H_{22}O_2$		3.13	10.7
β-E2	$C_{18}H_{24}O_2$		4.01	10.7
MegA	$C_{24}H_{32}O_4$		3.78	ND
P	$C_{21}H_{30}O_2$		3.87	ND
T	$C_{19}H_{28}O_2$		3.32	ND
Tamo	$C_{26}H_{29}NO$		6.24	ND

2. Experimental

2.1. Chemicals and materials

The high quality analytical standards (all of purity greater than 98%) of T, P, E1, 17β-E2, MegA and Tam were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

The solvents acetonitrile (ACN), isopropanol (iPrOH) and methanol (MeOH) were of LC/MS grade and were obtained from Fluka (Saint Quentin Fallavier, France) as well as ammonium acetate (purity ≥ 99.0%) and formic acid (purity ~ 98%).

Water of endocrine disruptor quality (ED-quality) was obtained from a system MilliQ® Gradient A10 equipped with an EDS-PAK cartridge and a 0.2 μm Millipak® 40 filter from a Merck-Millipore (Saint Quentin Yvelines, France).

Individual standard stock solutions were prepared in MeOH at 200 mg L^{-1} and stored at −18 °C for six months, in the complete absence of water to avoid hydroxylation of Tam. Working standard solutions were prepared daily by appropriate dilution of standard stock solutions and stored in the dark at <4 °C. The stability of the extracts after the steps of sample preparation has not been evaluated. Each extract was stored in

a freezer at −18 °C and stored for a maximum of one week until analysis.

QuEChERS extract tubes (AOAC and EN methods) were obtained from Agilent Technologies (Massy, France). The acetate buffer contained 1.5 g of NaOAc and 6 g of $MgSO_4$, (pH = 4.8), whereas the citrate buffer contained 1 g of NaO citrate, 4 g of $MgSO_4$, 1 g of NaCl and 0.5 g of disodium citrate sesquihydrate (pH = 5–5.5).

Dispersive SPE PSA (primary and secondary amine exchange, which contained 900 mg of $MgSO_4$ and 150 mg of CUPSA) and PSA C18 (which contained 900 mg of $MgSO_4$, 150 mg of CUPSA and 150 mg of CEC18) were obtained from Carlo Erba – SDS (Val de Reuil, France). PSA/GCB (which contained 900 mg of $MgSO_4$, 150 mg of CUPSA and 15 mg of graphitized carbon black) were obtained from Macherey Nagel (Hoerd, France).

2.2. Sediment sample

For all the developments, sediment from a Rhône-Alpes river was collected. This sediment was hormone-free, and was subsequently verified. For validation, a hormone-free sediment collected from Rhône-Alpes that was previously verified by our group was used. The sediment was freeze-dried and sieved to 250 μm before extraction to obtain a homogeneous sample. For the development of the QuEChERS-based procedure, 1.5 or 2.5 g of sediment was spiked at 500 ng g^{-1} (by deposition of 750 μL or 1250 μL, respectively, of a solution containing 1 mg L^{-1} of target compounds in MeOH). The sample was then left one night under a gentle nitrogen stream before the extraction.

2.3. Sampling site

A sampling site located in the department of Isère (France) was selected to evaluate the method developed and measure the levels of the target compounds. The watershed of the Bourbre (850 km^2) is experiencing significant economic development for 40 years. It concerns 180 000 inhabitants, with a significant demographic growth of medium cities under the influence of the Lyon area. Agriculture (cattle breeding and milk field crops in the plains) occupies 75% of the basin area, while artificialized territories occupy 9%. The site consists of a river and its main tributary. Four sampling points were considered from both sides of the confluence of these two streams between the main and the tributary rivers: (1) the main river upstream of the confluence; (2) the main river downstream of the confluence; (3) the tributary upstream of the confluence; and (4) the influent at the confluence. Note that site 4 is located downstream of a small stream receiving a portion of discharges of a sewage treatment plant. For each point, it was a single sampling point, which was analyzed in duplicate.

2.4. QuEChERS-based procedure

The final conditions for the extraction step were as follows: 7.5 mL of MilliQ water and 10 mL of the mixture ACN/iPrOH (90/10) were added to the extract tube that contained 2.5 g sediment, and then the tube was shaken with a vortex device. The acetate buffer was then rapidly added, and the tube was immediately manually shaken for 30 s and swirled on a vortex mixer for 1 min. After centrifugation at 5000 rpm for 2 min, 8 mL of the ACN layer were transferred into a 12 mL glass tube containing the clean-up phase (150 mg PSA and 900 mg $MgSO_4$). After that, the clean-up tube was manually shaken for 10 s and swirled on a vortex mixer for 1 min. After this step, the extract was centrifuged again (5000 rpm for 2 min) and 6 mL of supernatant were evaporated to dryness under a gentle stream of nitrogen at a temperature of 40 °C. The dry residue was dissolved in 10 mL of 80/20 H_2O/ACN prior to LC-MS/MS analysis. All of the extractions were performed in duplicate.

2.5. Analysis by liquid chromatography-tandem mass spectrometry

The liquid chromatographic system was a 1200 RRLC from Agilent Technologies, equipped with a G1312B Binary Pump SL, a G1369A autosampler and a G1315B column heater. The separations were performed with a Kinetex XB-C18 Core Shell (100 mm × 2.1 mm) column with 1.7 μm diameter particles preceded by a KrudKatcher (0.2 mm) filter from Phenomenex (Le Pecq, France). In positive mode, the mobile phase was composed of 0.01% formic acid in ED-quality water (pH = 3.2) and ACN. In negative mode, the mobile phase was composed of 0.1 mmol L^{-1} of ammonium acetate in ED-quality water and ACN. The separations were achieved in 9 min and 7 min, respectively. In both cases, the column oven temperature was 60 °C, the flow-rate was 0.5 mL min^{-1} and the injection volume was 100 μL.

The LC system was coupled to a triple-stage quadrupole mass spectrometer 3200 QTrap (ABSciex, Les Ulis, France) with an electrospray ion (ESI) source (TurboV, ABSciex). The analytes were identified by both their chromatographic characteristics and their specific multiple reaction monitoring (MRM) fragmentation patterns. In order to optimize the MRM, full scan and product ion spectra, acquisitions in positive and negative ion modes were performed by infusion of standard solutions at 1 mg L^{-1} of each analyte. Three types of standard solutions were prepared: water/MeOH (50/50), the same mixture containing formic acid and another dilute ammonia. Data processing was performed with Analyst software (version 1.5.1). The source parameters were as follows: the ion source gas nebuliser was 50, the ion source turbo gas was 60 psi, and the ion spray voltage was 5500 and −4500 V in ESI+ and ESI−, respectively and the source temperature was 500 °C. The MS/MS condition parameters are summarised in Table 2.

2.6. Performance of the method

The validation was performed according to the recommendations of ICH guidelines.[33] Different method performance criteria were assessed, such as linearity, recoveries, matrix effects, intra- and inter-day precisions and limits of detection (LD) and quantification (LQ).

The linearity was evaluated by analysing eight sediments spiked between 0.5 and 50 ng g^{-1}. The linearity was evaluated in triplicate on three days for each compound.

To evaluate the recoveries the signal obtained for the sediment spiked at C_1: 1 ng g^{-1}, C_2: 5 or C_3: 50 ng g^{-1} (S_{spiked}) was

Table 2 Parents and product ions selected and the corresponding voltages: declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE) and collision cell exit potential (CXP)

Molecule	Parent ion m/z (Da)	DP (V)	EP (V)	CEP (V)	Product ion 1 m/z (Da)	Product ion 1 CE (V)	Product ion 1 CXP (V)	Product ion 2 m/z (Da)	Product ion 2 CE (V)	Product ion 2 CXP (V)
β-E2	271.1	−75	−4	−14	145.2	−52	0	183.1	−52	−2
E1	269.1	−70	−4.5	−14	145.1	−48	0	159.2	−48	0
Tam	372.2	51	8	20	72.0	39	4	129.1	35	4
T	289.3	46	4	22	97.1	31	4	109.1	35	4
MegA	385.3	41	7	18	325.4	19	4	267.3	23	4
P	315.4	66	7	16	97.1	31	4	109.3	31	4

compared with the signal obtained for sediment extract spiked after the sample preparation with a solution containing the target substances at the same concentration (S_{stand}):

$$\text{Recovery}(\%) = \left(\frac{S_{spiked}}{S_{stand}}\right) \times 100$$

The lowest value C_1 was chosen to be slightly higher than the quantification limit of the compounds for which the method is the least sensitive. Then the middle and high concentrations were determined to be 5 and 50 times higher than C_1, which *a priori* was to cover the range of environmental concentrations.

Matrix effects were determined at C_1, C_2 and C_3 by comparison of the signal (S_{stand}) with the signal of the standards in a solvent ($S_{solvent}$) at the same concentration:

$$\text{Matrix effect}(\%) = \left(\frac{S_{stand}}{S_{solvent}} - 1\right) \times 100$$

Thus, a negative value of the matrix effect indicates signal suppression while a positive value indicates an enhancement of the signal.

Intra- and inter-day precisions were evaluated for each compound at the levels of C_1, C_2 and C_3 in 3 replicates and on three days, respectively. They were expressed as the relative standard deviation (RSD, %) for the different concentrations.

The LD and LQ values were determined as the concentration at which the signal to noise ratio was greater than 3 and 10, respectively.

3. Results and discussion

3.1. Development of the modified-QuEChERS extraction

The analysis of complex matrices such as sediments requires efficient and selective sample preparation. Because the target molecules exhibit different characteristics and physico-chemical properties, the optimisation of the steps was challenging.

The QuEChERS[34] extraction method is based on the salting-out liquid–liquid extraction principle. In the first step of the QuEChERS-based procedure, an organic solvent allows the extraction of the analytes from the matrix and the addition buffer permits a separation of the phases. This procedure increases the transfer of the target compounds into the organic phase and improves the pH dependent stability of the substances. To determine the optimal extraction procedure, several parameters were studied: the choice of buffer, the properties and volumes of the solvent and the initial mass of the sediment.

Two standards are commonly used in the QuEChERS approach with regard to the buffer type. An acetate buffer is involved the American standard (AOAC)[35] while a citrate buffer is used in the European standard EN 15662.[36]

The recoveries obtained with both buffers were compared (Fig. 1). For all the compounds the acetate buffer resulted in better recoveries (superior to 88%) except for Tam (only 12% with acetate and 32% with citrate buffer). Therefore, an acetate based-method was chosen for the extraction.

ACN was the first solvent used for QuEChERS extraction[13] and is known to be the most efficient and widely used.[15] However, the mixture of ACN and iPrOH was previously mentioned to enhance the recoveries of various compounds from the Water Framework Directive[37] during solid phase extraction, or to exhibit a positive effect during the extraction of alkylphenols from sediments.[16] The recoveries obtained with the addition of 10% iPrOH in the organic phase are shown in Fig. 1. The effect of iPrOH on the extraction was completely different depending on the target substances. Recoveries were greatly reduced for both estrogens and were globally unchanged for progestagens and androgen while that for Tam increased substantially reaching nearly 70% in the presence of iPrOH.

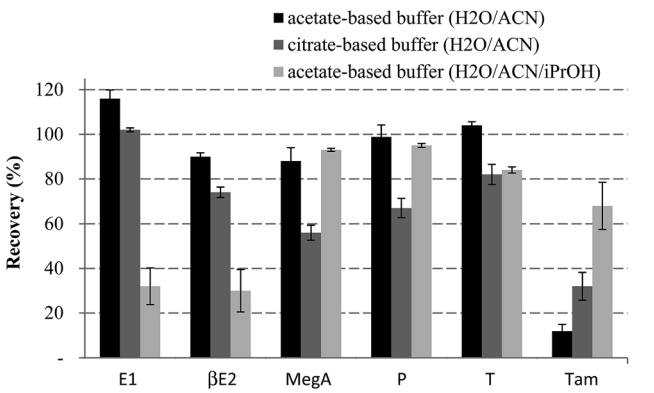


Fig. 1 Recoveries obtained during the QuEChERS extraction using different buffers (500 ng g^{-1}, 1.5 g sediment, 4.5 mL H_2O, 6 mL organic solvent, and two replicates).

This could be partly explained by the presence of co-extracted interfering substances. Given these very different results, these two options (only ACN or mixture with iPrOH) were kept for subsequent experiments.

3.2. Reduction of matrix effect

Because the sediment matrix is complex, an additional purification step was optimised to limit the presence of interfering substances. Indeed, one of the greatest drawbacks of LC-MS/MS is the perturbation of the signal by co-extracted substances from the sample matrix. These residual components can induce either ion suppression or enhancement of the analyte in the electrospray interface, leading to sources of error during the quantification.

We focused on dSPE for the development of an easy and rapid protocol. The first extract was replaced into a tube that contained the sorbent phase for purification and 900 mg $MgSO_4$ for eliminating excess water. To maintain a simple and quick analysis, we favoured dSPE phases available on the market in the format "pre-weighed". Two of them seem suited to environmental solid matrices. The first is PSA, a weak anion exchanger usually used to eliminate polar organic acids, sugars or lipids. The second is the mixture PSA/C18 which additionally allows the retention of hydrophobic molecules by means of C18 chains. To determine the optimal conditions for this cleanup step, not only the recoveries were evaluated but also the decrease of matrix effects.

In the case where the extraction was carried out with ACN as the sole organic solvent, the presence of PSA or PSA/C18 phases reduced the extraction efficiency of the hormones, except in the case of Tam with the PSA phase. Indeed, its extraction efficiency was tripled (Fig. 2).

The impact of the dispersive stage on whole method recoveries was completely different when the solvent used during extraction was the mixture ACN/iPrOH. Indeed, the recoveries obtained at the end of the purification were significantly better in the presence of a dispersive phase for estrogens and androgens; the recoveries were nearly 100%. The addition of a fraction of 10% iPrOH probably reduces the affinity of the compounds of interest in the purification steps while allowing the removal of the most hydrophilic interfering compounds from the matrix.

On the other hand, in the case of Tam, the best conditions to maintain good recovery for this hydrophobic compound were without purification.

The matrix effects are summarized in Fig. 3. It is noted that in the absence of cleaning, inhibition of the signal varied from 30 to 55% when ACN was used alone, and could reach more

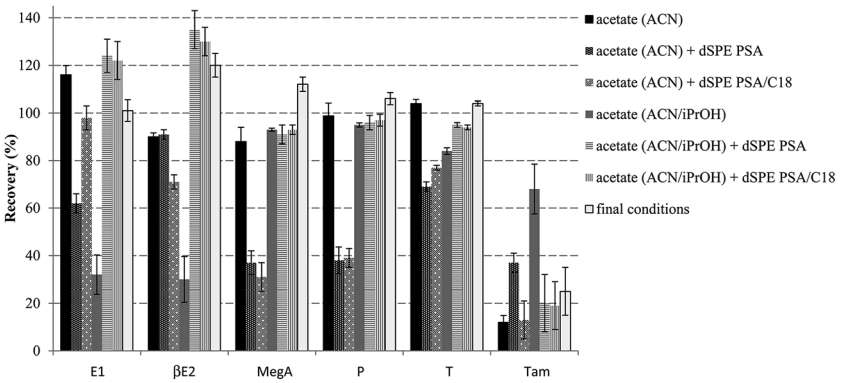


Fig. 2 Recoveries obtained during the acetate-based extraction using different solvents and the dispersive phase (500 ng g^{-1}, 1.5 g sediment, 4.5 mL H_2O, 6 mL organic solvent, and two replicates).

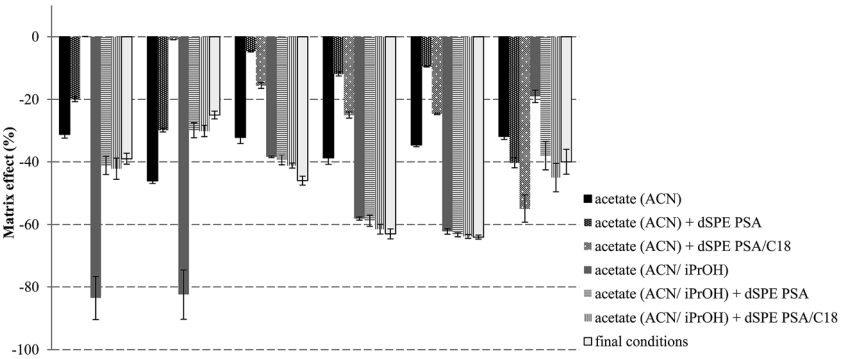


Fig. 3 Matrix effects obtained during the acetate-based extraction using different solvents and the dispersive phase (500 ng g^{-1}, 1.5 g sediment, 4.5 mL H_2O, 6 mL organic solvent, and two replicates).

than 80% in the case of estrogen extracted with a proportion of iPrOH. This confirms the fact that this surfactant is very effective for the extraction of many substances.

Fig. 3 shows that the presence of the dispersive phase did not exert the same effect on the reduction in the signal, depending on the compounds. Indeed, the matrix effects on estrogens were strongly reduced with the use of a dispersive phase regardless of the solvent used in the extraction step. Regarding androgen and progestagens, the PSA phase decreased matrix effects when ACN was used alone. In the case where the ACN/iPrOH mixture was used, none of the dispersive phases made it possible to reduce the matrix effects. Finally in the case of Tam, the effects observed were still different since the PSA and PSA/C18 phases decreased signals.

A good compromise had to be found to maintain efficient extraction while minimizing matrix effects. In resume, the most suitable conditions were the ACN/iPrOH mixture, during the extraction phase, and then the clean-up procedure using the PSA.

All attempts to optimize the extraction and clean-up steps were made using a test sample of 1.5 g of sediment spiked at 500 ng g^{-1} with each of the compound of interest. Before validating the method, an additional test was conducted to verify if this method could be applied to 2.5 g of sediment. Indeed a larger test sample could increase the method sensitivity. The protocol

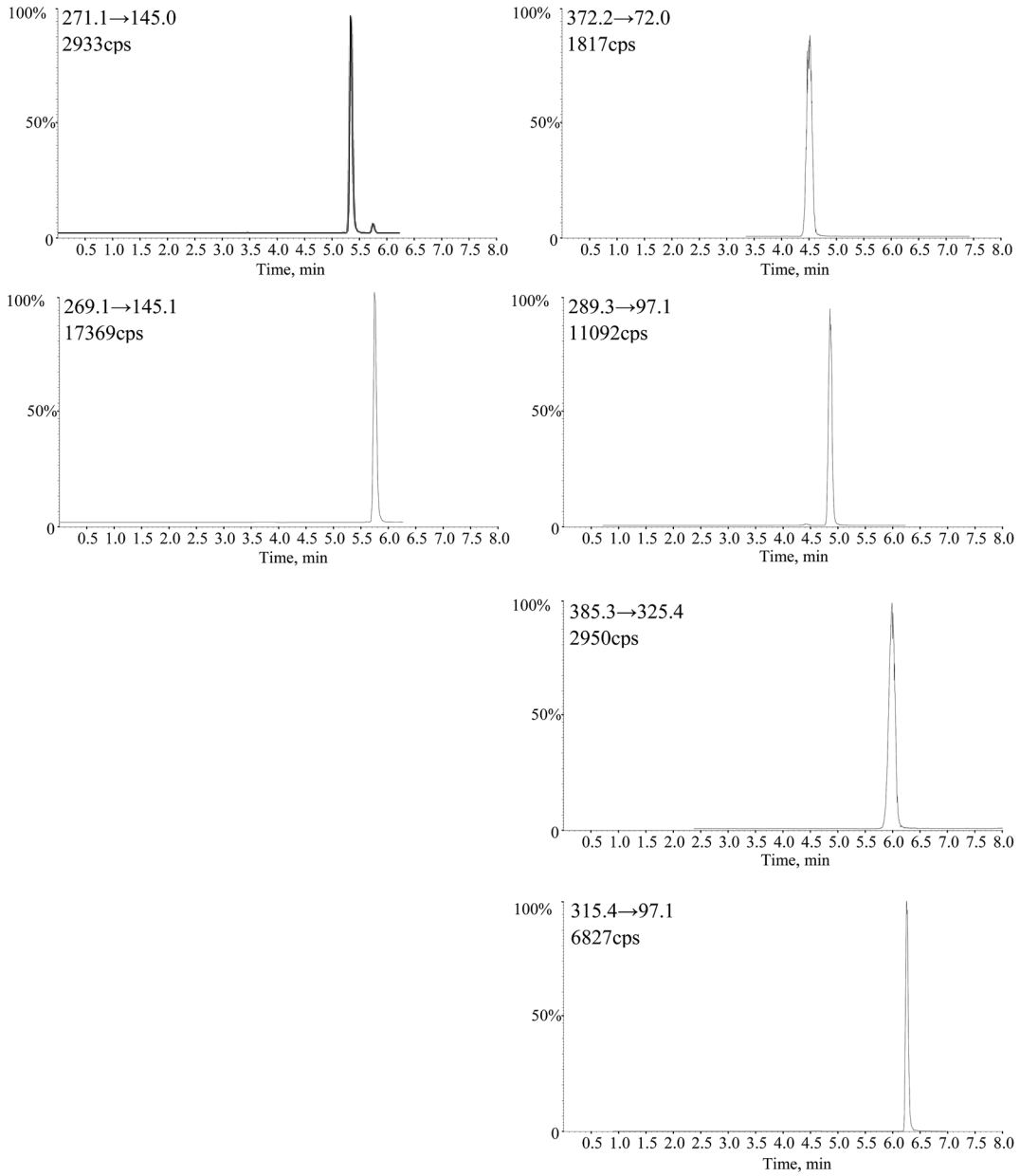


Fig. 4 Chromatograms of an extract of sediment spiked at 50 ng g^{-1} with analytical standards (on the left: β-E2, E1; on the right: Tam, T, MegA and P).

was applied using the following volumes for the extraction: 7.5 mL H_2O and 10 mL ACN/iPrOH (90/10). Recoveries and matrix effects under these conditions appear in Fig. 2 and 3, respectively. The results indicated that recoveries and matrix effects varied only a little between these two conditions. An intake of 2.5 g of the sediment test sample was used for the final protocol.

3.3. MS/MS optimization

T, P, Tam and MegA were analyzed in positive ionization mode. The choice of an aqueous phase acidified with formic acid (pH = 3.2) was done during the infusion of the compounds in the mass spectrometer. Precursor ions corresponded to the protonated molecules $[M + H]^+$. In the MS-MS spectra of T and P, main product ions were m/z 109 and m/z 97, deriving respectively from the cleavage of the rings. Tam broke down into very small fragments, at m/z 72 and m/z 45, which correspond to imines[38] (Table 2).

E1 and β-E2 were analyzed in negative ionization mode. The ionization of the precursor ions $[M - H]^-$ was enhanced by the presence of ammonia in the mobile phase. The product ions used for the identification and quantification represent common fragments at m/z 145 and 159 for E1, and m/z 145 and 183 for β-E2 (Table 2). These fragment ions are consistent with a fragmentation pathway that proceeds through ring cleavages and subsequent retrocyclization, previously described.[39] As an example, chromatograms in the negative and the positive ion modes are shown, in Fig. 4, from the analysis of analytical standards at the level of 50 ng g^{-1} in sediment.

3.4. Validation of the method

The study of the analytical performances of the method and validation of the linearity of the response were performed at the same time. The linearity was validated using high levels of concentration, ranging from 0.5 to 50 ng g^{-1} (0.5, 1, 2.5, 5, 10, 16, 25, and 50), with three standards prepared independently, during three days, leading to nine independent samples for each calibration level. The calibrations were realized with independent standard solutions prepared each day. Three independent standards were used to determine the repeatability at each calibration level. Nine replicates per level were used to calculate the intermediate precision. The day of analysis represented the unique variation during this evaluation. The accuracy of the method was assessed by the use of 5 quality control samples at concentrations corresponding to C_2.

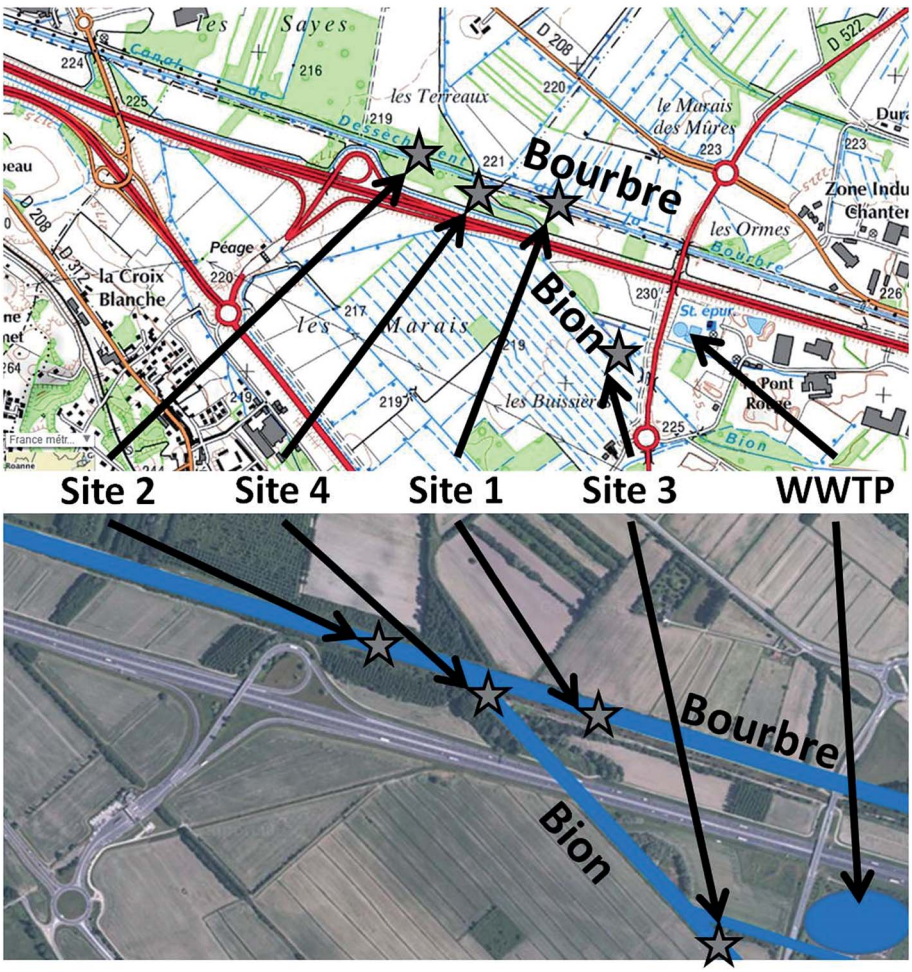


Fig. 5 Map and satellite views (from the National Geographic Institute – IGN) of the sampling sites in the department of Isère, France.

Table 3 Parameters of performance of the whole method: retention time (R_t); determination coefficient (R^2) and Fisher ratio for the linearity; recoveries, matrix effect, repeatability and intermediate precision at three levels C_1, C_2 and C_3; limits of detection and quantification

		Linearity		Recovery (%)			Matrix effect (%)			Repeatability (%)			Intermediate precision (%)				
Compound	R_t (min)	R^2	Fisher ratio	C_1	C_2	C_3	C_1	C_2	C_3	C_1	C_2	C_3	C_1	C_2	C_3	LD (ng g^{-1})	LQ (ng g^{-1})
β-E2	5.4	0.9932	0.01	120	123	103	−25	−49	−25	36	4	10	30	23	16	0.2	0.6
E1	5.7	0.9981	0.02	82	101	99	−41	−39	−39	12	5	9	19	20	5	0.15	0.5
Tam	4.5	0.9883	0.05	63	109	25	−34	−64	−40	51	12	—	—	—	—	0.2	0.6
T	4.9	0.9955	<0.01	102	104	89	−47	−64	−64	4	5	2	10	11	4	0.03	0.1
MegA	6.0	0.9924	0.05	100	112	100	−83	−78	−46	21	15	6	26	25	6	0.15	0.4
P	6.3	0.9946	0.01	74	106	102	−71	−77	−63	11	7	5	16	18	4	0.07	0.2

The results of the validation are summarized in Table 3.

Data measured during the determination of the analytical performances were well described by a linear model as shown by the determination coefficient R^2. Indeed, for all the compounds except Tam, R^2 was higher than 0.99. As a correlation coefficient close to 1 is not a reliable indicator of linearity,[40] the apparent linear relationship was also verified by statistical means, using an ANOVA approach based on the Fisher ratio. The Fisher ratio was inferior to the unit for all the target compounds which validated the choice of the linear model for the regression.

The intra-day precision was less than 15% for all the investigated compounds, at the concentrations C_2 and C_3. On the other hand, it was superior for the lower concentration C_1, since it exceeded 20% in the case of β-E2 and MegA, and reached 51% in the case of Tam. Therefore, this hydrophobic compound did not fulfill this criterion of validation.

The inter-day precision (Table 3) was less than 30% for all of the compounds except Tam which was not validated because the values were greater than 60% for all the concentrations.

All the target compounds but Tam exhibited recoveries between 74% and 123%. The efficiency was very good using this method based on QuEChERS extraction and comparable to those obtainable with more sophisticated techniques as pressurized liquid extraction,[41] ultrasounds[42,43] or supramolecular solvent.[44]

The accuracy values were comprised between 95 and 105%.

The limits of quantification were very low and ranged from 0.1 to 0.6 ng g^{-1} depending on the target compounds. Few methods achieved thresholds lower than 0.6 ng g^{-1} for steroids in sediments. Gorga *et al.*[41] reported LQs of 0.031 ng g^{-1} for E1 and 0.069 ng g^{-1} for E2, with a method based on pressurized liquid extraction followed by on-line turbulent flow chromatography-liquid chromatography-tandem mass spectrometry. Recently, it was proved that a supramolecular solvent made up of inverted hexagonal aggregates of alkanols had the potential for sample preparation during the analysis of endocrine disruptors in sediment.[44] The LQ reported for this protocol was 0.03 ng g^{-1} for E1 and 0.3 ng g^{-1} for E2. Compared to estrogens, androgens and progestagens have been less studied. The LDs are often higher than our method. They are, for example, greater than 2 ng g^{-1} for T and P after successive extractions with different solvents followed by SPE clean-up and analysis by LC-MS/MS[45] or by a method based on microwaves-SPE-derivatization-GC-MS.[46] Liu *et al.*[47] developed a sensitive method to analyze several androgens and progestagens, with LQs as low as 0.01 and 0.03 ng g^{-1} for T and P, respectively. However, the method is relatively long and solvent-consuming since it implements a triple ultrasonic-based extraction followed by SPE with a self-made silica gel cartridge. The LD and LQ values of Tam in sediments were 0.5 and 1.7 ng g^{-1}, respectively, *i.e.* about 10 times higher than ours, with a method based on PLE for the extraction.[48] To our knowledge, no limit has been reported for the determination of MegA in sediments.

Table 4 Levels of the target compounds in the sampling sites

Compound	LD (ng g^{-1})	LQ (ng g^{-1})	Site 1 (ng g^{-1})	Site 2 (ng g^{-1})	Site 3 (ng g^{-1})	Site 4 (ng g^{-1})
E1	0.15	0.50	19.0 ± 1.9	42.6 ± 16.1	1.1 ± 0.1	31.3 ± 0.7
β-E2	0.20	0.60	nd	nd	nd	nd
MegA	0.10	0.40	nd	nd	nd	nd
P	0.07	0.20	nd	nd	nd	nd
T	0.03	0.10	nd	nd	nd	nd
Tam	0.20	0.60	>LQ	>LQ	>LQ	>LQ

3.5. Application to the study of real samples

The method developed was applied to the analysis of sediments collected at different points of a river and its tributary (Fig. 5). The results are summarised in Table 4.

The presence of Tam was confirmed in the 4 samples analyzed. This substance has not often been sought in the environment, particularly in sediments. However, very recently, Tam has been quantified in sediments from an urban estuarine setting[48,49] with levels up to 11 ng g^{-1}. Our work confirms that this hydrophobic compound must be considered with interest in sediments.

E1 was quantified in all the samples. The presence of E1 in the sediments confirms earlier studies conducted in Europe on endocrine disruptors that put in evidence that this estrogen was the most frequently detected, with the range varying from 0.4 ng g^{-1} to about 12 ng g^{-1}.[50] However, the concentrations found at sites 1, 2 and 4 were slightly higher than those reported in the literature, as the level in the site 2 increased 40 ng g^{-1}. It would be inappropriate to forge conclusions or some interpretations from these few data. However it seems that the values show a pronounced presence of E1 downstream of the confluence of the river and along the bed of the river. The sediments would thus represent an interesting accumulation matrix for environmental monitoring of this estrogen.

There are several examples of effect-directed analysis (EDA) conducted in sediments that reveal the importance of estrone in explaining total estrogenic activity.[51] Thus, E1 was found to be an important estrogenic contributor to the total estrogenic activity of 15.6 ng EE2 equivalent per g (with EE2: ethinylestradiol) among the analyzed compounds in bed sediments of a polluted tributary.[52] Similarly, an EDA-like approach conducted in sediments downstream of a major sewage treatment plant discharge of United Kingdom rivers put in evidence that, together with E2, E1 (0.025–0.025 ng g^{-1}) was a major estrogenic compound.[27] These previous results, compared with the quantified data in our study, reveal that the levels quantified are likely to induce estrogenic effects on aquatic organisms.

4. Conclusions

This work presents a simple rapid and effective procedure for the determination of five hormones with very different chemical structures, in river sediments. By coupling a sample preparation based on modified-QuEChERS with LC-MS/MS analysis, the method was both selective and sensitive. This procedure enables the determination of the target analytes with limits of quantification comprised between 0.1 and 0.6 ng g^{-1}. Only the most hydrophobic compound does not fulfil all the criteria of validation. In this case, the method cannot be used quantitatively, only qualitatively. The analysis of sediments collected in various points of a confluence reveal the presence of estrone and tamoxifen.

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