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Analysis of Environmental Protection Agency priority endocrine disruptor hormones and bisphenol A in tap, surface and wastewater by online concentration liquid chromatography tandem mass spectrometry

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ABSTRACT

The list of endocrine disrupting compounds (EDCs) defined under U.S. EPA Method 539 was recently expanded to include additional hormones and bisphenol A (BPA). Here, we validated a fast and robust alternative method compliant with Method 539.1 requirements in diverse water matrixes (i.e., ultra-pure water, tap water, surface water, and wastewater influent and effluent). Automated large volume injection solid phase extraction (SPE) coupled on-line to ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) was investigated for this purpose. The surveyed molecules included 13 EPA-priority hormones (testosterone, progesterone, medroxyprogesterone, levonorgestrel, norethindrone, androstenedione, estrone, β-estradiol, α-estradiol, equilin, equilenin, ethinylestradiol, estriol) and BPA. Combinations of ionization source and mobile phases were optimized for improved sensitivity. Suitable chromatographic performances were obtained and the implementation of an on-line SPE washing step consecutive to sample loading was investigated. On-line SPE extraction efficiencies in acceptable ranges (64–79%) and detection limits in the order of nanogram per liter or sub-nanogram per liter were obtained. The linearity range extended over 2–3 orders of magnitude, with determination coefficients (R^2) typically > 0.9980. Robust precision and trueness complying with acceptance criteria (70–130%) were obtained for the scope of analytes/matrix combinations. Limited internal standard variations were also observed across samples (±18%), well within the ±50% acceptance criterion. The method was successfully applied to field-collected samples in Canada and summed EDC concentrations were reported in the range of 0.80–2.8 ng L^{-1}, 6.8–19 ng L^{-1}, 260–790 ng L^{-1}, and 37–360 ng L^{-1} in tap water, surface water, effluent and influent wastewater samples, respectively.

1. Introduction

Multiple contaminants are disseminated in aquatic environments, some of which are not entirely eliminated by water treatment plants [1–5]. Concerns about their potential impacts have motivated the creation of drinking water and environmental waters guidelines for the protection of aquatic resources, as well as the implementation of surveillance activities to monitor the quality of tap water and surface water bodies [6,7]. Anthropogenic compounds such as pharmaceuticals and personal care products (PPCP), pesticides, chemicals for specialty applications (e.g., halogenated flame retardants, perfluoroalkyl substances), and synthetic hormones, among others, are produced in large quantities and may end up in surface waters and treated waters, including drinking water [8–17].

Synthetic hormones such as ethinylestradiol or levonorgestrel (which are parts of contraceptive pills) have been used for decades. In the province of Québec (Canada), which represents a population of *circa* 8 million people, 128 million birth control pills and 107 million hormone replacement therapy (medication for menopausal women) doses are prescribed each year [18,19]. Estrogens are also used as a treatment for certain types of cancer [20]. Androgens represent another notable use of hormones, due to their potential to promote muscle mass and physical strength in humans [16]. In light of their massive use as supplements and the variety of other potential sources, including natural production and release by humans

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and animals, hormones and their degradation metabolites are now ubiquitous in aquatic environments [15,21–29]. Hormones such as estrone (E1), estradiol (E2), and ethinylestradiol (EE2) bear the potential to elicit endocrine-related effects in non-targeted organisms [30–36], including developmental [31–36] and behavioral [37] alterations. At the population level, chronic exposure to low part-per-trillion concentrations ($ng{\cdot}L^{-1}$) may contribute to the decline in total number of individuals, for instance in fish [30]. In humans, exposure to hormones could be a factor in the decrease of the number of spermatozoids that has been observed in the last decades [38]. The large panel of potential disruptions in humans and wildlife make the study of these compounds relevant from low ng L^{-1} concentrations [39,40].

Widely used analytical methods for the determination of hormones in water include, notably, liquid chromatography coupled to diode array detection (DAD) [41] or mass spectrometry (MS) [42], and gas chromatography coupled to MS [17,29,43]. Such methods are typically preceded by an off-line solid phase extraction step to reach a suitable concentration factor [44]. Derivatization procedures have also been described both for LC- and GC-based methods [45–48]. Despite their acknowledgeable advantages with regard to improved method limits of detection (LOD), comprehensive sample pretreatment steps (i.e., off-line SPE and derivatization) may be resource-intensive to implement. Another caveat relates to the possibility of matrix effects which may require sample-specific adjustments [49]. Recent analytical workflows tend to favor the use of on-line pre-concentration of a limited sample volume (1–10 mL) coupled to high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) [16,50,51]. While the pre-exisiting on-line SPE methods paved the way toward higher throughput analysis, some critical knowledge gaps remain to be bridged.

For instance, some previous studies have determined LODs and validation parameters in neat reagent water [51,52]. However, the declared performances may not necessarily correspond to those involving more complex water matrixes. In addition, the methodologies employed to determine the LODs may vary considerably between studies, which makes it difficult to draw definite conclusions. The list of endocrine disrupting compounds (EDCs) defined under U.S. EPA Method 539 was recently expanded to include four additional hormones and bisphenol A (BPA) [53]. Monitoring of hormones in Canada is also the subject of continued interest [51]. The rationale for the present study is borne out from the need to achieve adequate sensitivity and quantification performances for an extended range of compounds but using a simplified analytical workflow. To the authors' best knowledge, obtaining analytical performances complying with those stated in Method 539.1 (i.e., in terms of method reporting limits and quality control requirements) has not been previously demonstrated through on-line SPE – LC–MS/MS.

Here, we set out to provide a fast, robust, and sensitive method for the simultaneous analysis of U.S. EPA-priority EDCs (including bisphenol A and 13 hormones) in diverse water matrices. On-line SPE coupled to ultra-high-performance liquid chromatography triple quadrupole mass spectrometry (UHPLC-MS/MS) was investigated for this purpose. The specific aims of this study were the following: i) develop an on-line method capable of capturing an extended range of hormones and BPA with appropriate sensitivity; ii) provide an appropriate quantification strategy to ensure accurate and robust results for each matrix type; iii) verify the compliance of the newly-developed method with regard to acceptability criteria (including internal standard [IS] recoveries); and iv) demonstrate the applicability of the method to a range of field-collected water samples. Regardless of matrix type, the herein described method requires a common sample intake of 10 mL and minimal sample pretreatment (filtration and isotope-labelled IS addition) prior to on-line SPE – UHPLC-MS/MS analysis. Method development, validation and application were conducted on a range of real water matrices (i.e., tap water, surface water, and both influent and effluent wastewater). This is the first study to demonstrate analytical performances compliant with EPA method 539.1 for such an extended range of analyte/matrix combinations. The occurrence of the targeted EDCs was assessed through a regional survey of samples from the provinces of Ontario and Québec (Canada).

2. Materials and methods

2.1. Chemicals and standards

Native standards of estrone (E1), α-estradiol (α-E2), β-estradiol (β-E2), estriol (E3), ethinylestradiol (EE2), equilin (EQUI), equilenin (EQUIL), androstenedione (ANDRO), levonorgestrel (LEVO), norethindrone (NOR), testosterone (TESTO), progesterone (PROG), medroxyprogesterone (MPROG), and mestranol (MEST) were all purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) at a purity of 97% or higher. Bisphenol A (BPA; purity 97%) was also purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). The structures of the targeted compounds are provided in the Supporting Information (Fig. S1). Isotopically-labelled internal standards (purity ≥98%), progesterone-$^{13}C_3$, mestranol-d_4, androstenedione-d_3, estradiol-$^{13}C_6$ and bisphenol A-$^{13}C_{12}$ were acquired from Cambridge Isotope Laboratories, Inc. (Andover, MA, U.S.A.). Water, acetonitrile (ACN), methanol (MeOH), and isopropanol (iPrOH) of HPLC-grade quality were obtained from Fischer Scientific (Whitby, ON, Canada). Sodium chloride (NaCl, purity >99.5%), 2-mercaptopyridine-*N*-Oxide (Omadine salt, purity 99%), ammonium fluoride (purity >98%) and formic acid (HCOOH, purity >95%) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

2.2. Sample collection and pre-treatment

Water samples were collected in pre-cleaned 250-mL amber glass bottles previously amended with preservative agent (2-mercaptopyridine, 70 mg L^{-1}) and salt (sodium chloride, 116 mg L^{-1}) used as a dechlorinating agent and an antibacterial growth agent respectively as proposed earlier [53]. Sample collection in each of the studied matrixes was operated as follows. The tap water was left to flow for 5 min, after which the sample container was filled with the site tap water and sealed. At each surface water or wastewater sampling location, the corresponding container was filled with the site surface water or wastewater, and the bottle was finally sealed. Immediately after sample collection, the bottle was vigorously shaken to homogenize the solution and kept on ice in a cooling box (temperature < 4 °C). Upon reception at the laboratory, samples were filtered on glass fiber filters (GFF, 0.3 μm, Sterlitech Corporation, Kent, WA, U.S.A.) and stored at −20 °C until analysis, which occurred no later than 28 days from the sampling date [53].

Real water samples from each matrix of interest were collected from the following locations: tap water samples were collected at Université de Montréal, surface water samples were collected from the shores of the Saint-Zéphirin River, and wastewater both influents and effluents sampled from wastewater treatment plants (WWTP) located in the Greater Montreal area. After the initial validation had been performed and an appropriate quantification strategy was devised, additional samples were collected for each matrix type to verify the influence of individual sample variation on the *relative* matrix effects. Such samples were collected in 2017–2018 from the following locations: tap water samples were collected from domestic homes in Montréal, Brossard, and Candiac, surface water samples were collected from the shores of the Des Prairies River (Montréal), St. Lawrence River (Boucherville Islands), and Yamaska River (Yamaska), while wastewater samples were col-

lected from three distinct WWTPs from the province of Québec (Canada).

Tap water samples were collected at 11 locations in the provinces of Québec and Ontario (Canada) from domestic homes in December 2016 and January 2017, respectively. For the former, five localities were surveyed (Repentigny, Mont-Saint-Hilaire, Saint-Hyacinthe, Sorel-Tracy, and Granby), while six different localities were selected for the latter (Carleton, Lindsay, Orangeville, Ottawa, Perth, and Toronto). Surface water (river) samples were collected in Québec (Canada) in March 2017 from five different locations: Richelieu River (upstream Chambly), Chateauguay River (Châteauguay), Mille Iles River (Terrebonne), and Des Prairies River (Repentigny and Montréal). At each location, a duplicate set of samples (n = 2) was collected. Wastewater samples (influent and effluent) were collected in the greater Montreal area (Québec, Canada) from 6 distinct WWTP in November or February 2017, respectively.

2.3. Sample preparation and instrumental analysis

Water samples were passed through 0.3-μm glass fiber filters [42]. Subsequently, isotope-labelled internal standards were added to each sample for a final concentration of 50 ng L^{-1}. Each sample was prepared in a glass volumetric flask and an aliquot of filtered sample was then transferred to an amber glass injection vial for on-line SPE – UHPLC-MS/MS analysis.

The instrumental setup consisted of a sample delivery system, a dual switching-column array, and an UHPLC–MS/MS system [54]. The sample delivery system comprised an HTC Thermopal autosampler from CTC analytics AG (Zwingen, Switzerland), used for a 10-mL in-loop sample injection, and an Accela 600 quaternary pump (Thermo Finnigan, San Jose, CA, U.S.A.) used for sample loading onto the on-line SPE column. The online system was composed of two-position six-port and ten-port valves to perform the column switching process (VICIs Valco Instruments Co. Inc., Houston, TX, U.S.A.). On-line SPE was achieved using a set of two Hypersil Gold aQ C18 columns (20 mm x 2 mm, 12 μm particle size) connected in series. An Accela 1250 quaternary pump (Thermo Finnigan, San Jose, CA, U.S.A.) was used for sample elution from the SPE column and separation onto the analytical column. Chromatographic separation was performed with a Hypersil Gold C18 column (100 mm x 2.1 mm, 1.9 μm particle size). The analytical column was thermostated at 50 °C. Analyte ionization was achieved using a heated electrospray ionisation (heated-ESI) source in fast polarity-switching mode. The TSQ Quantiva triple quadrupole mass spectrometer (Thermo Scientific, Waltham, MA, U.S.A.) was operated in selected reaction monitoring mode.

The 10-mL sample loop was filled in excess with the sample. The gradient program comprised three sequential steps: i) the on-line SPE loading and washing step; ii) the elution of analytes and separation onto the analytical column; and iii) the conditioning of the analytical column and on-line SPE column prior to the following injection. The gradient was adjusted to achieve the separation of α- and β-estradiol isomers. The analytical mobile phases (with the heated-ESI source) were HPLC-water (A), methanol (B) and an aqueous solution of ammonium fluoride (NH_4F) at a concentration of 1 mM, (C). Further details are provided in the SI (Table S1 and Fig. S2). The injection syringe and six-port injection valve were washed between each run as detailed elsewhere [54]. The overall procedure allowed the detection of all compounds within a single run of 15.5 min.

For the optimization of compound-dependent MS parameters, each compound was infused at a concentration of 5 mg L^{-1} in positive (PI) or negative (NI) mode depending on expected polarity ionization. Source parameters with the retained mobile phase were as follows: sheath gas (60 arbitrary unit), auxiliary gas (15 arbitrary unit), sweep gas (0 arbitrary unit), ion spray voltage (+3 kV or −3 kV, polarity switching), capillary temperature (350 °C), vaporizer temperature (400 °C). The scan time was set at 20 ms, yielding at least 10 points per chromatographic peak. The first and third quadrupoles (Q1 and Q3) were set at unit resolution (0.7 Da FWHM). The collision gas pressure in the collision cell (q2) was fixed at 1.5 mTorr. Compound-dependent MS/MS parameters with the finally retained method are provided in the SI (Table S2).

2.4. Method validation

In this study, optimization or validation tests were performed in different water matrixes (HPLC-grade water, tap water, surface water and wastewater) supplemented with target analytes and internal standards. Validation was conducted to evaluate the method performance in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), extraction recovery, precision, matrix effects, and whole-process efficiency (i.e., whole-method trueness [55]).

Calibration curves comprised six to eight calibration levels, a linear fit with inverse weighting (1/x) being applied to generate the regression line. The spiked levels ranged from 0.010 ng·L^{-1} to 200 ng·L^{-1} in HPLC water and tap water, and from 0.10 ng·L^{-1} to 200 ng·L^{-1} in surface water and wastewater. The linearity range and determination coefficients (R^2) were both examined.

The LOD and the LOQ determination was based on an analyte signal to noise ratio greater than three ($S/N \geq 3$) and greater than ten ($S/N \geq 10$), respectively [55,56]. Other validation parameters including trueness and precision, matrix effects, and recoveries were evaluated as per the procedure described in SI.

2.5. Quality assurance / quality control

After the initial validation, the compliance of the method with quality assurance and quality control requirements was routinely monitored. Analyte identification was based on compliance of retention times with those in the spiked reference, observation of both quantification and confirmation transitions, and the conservation of the ratio linked with the latter transitions.

Field blanks were performed by filling collection bottles (previously amended with preservative agent and salt) on site with HPLC-grade water; such samples were then treated in parallel to the real field samples. For each batch of samples, procedural blanks were included and consisted of HPLC-grade water filtered through GFF and added with internal standards prior to on-line SPE – UHPLC-MS/MS analysis. In each sequence, injection blank samples (i.e., HPLC-water aliquots directly submitted to on-line SPE – UHPLC-MS/MS) were regularly analyzed. No carryover was observed in the four matrices and analytes were not observed in blanks except BPA which was present both in procedural and injection blanks—albeit at low and reproducible levels. The concentrations reported for BPA in real field samples were therefore corrected by the procedural blank contribution of the corresponding sequence.

For each analytical sequence, matrix-matched calibration curves were run for the corresponding sample types. Once the calibration curve had been run, intermediate-level continued verification standards (CVS) were regularly injected to control the chromatographic retention times, analyte absolute areas, and analyte to IS area ratios. The performance endpoints of CVS were compliant with acceptance criteria specified in Method 539.1. An illustration of the control charts established during the present study is enclosed in the SI (Table S3).

The variability of internal standards among individual samples was also monitored in terms of absolute area and retention time. Overall acceptable variations were noted (SI Tables S4–S5), with

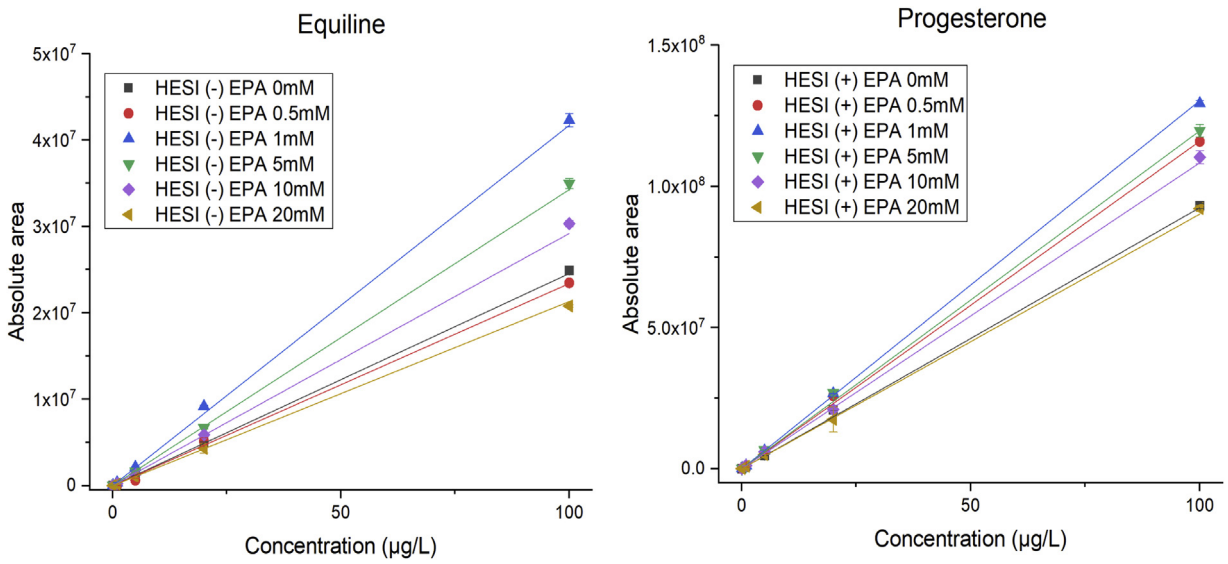

Fig. 1. Direct injection UHPLC-MS/MS sensitivity depending on different concentrations of NH_4F salt. Compounds analyzed in negative mode follow the same trend as equilin (left panel), and those analyzed in positive mode follow the same trend as progesterone (right panel).

suitable recoveries across samples compared to the reference (i.e., the average value derived from the calibration curve [53]) (SI Table S6). The relative recovery of IS absolute areas was maintained within ±20%, which is compliant with the performance criterion stated in U.S. EPA method 539.1.

3. Results and discussion

3.1. Instrumental optimization

While various analytical techniques exist for the detection of hormones, those involving UHPLC-MS/MS are increasingly used, with couplings such as APPI (atmospheric pressure photo ionization), APCI (atmospheric pressure chemical ionization), and heated-ESI. According to the literature, most studies mention the use of an (heated) ESI source for the detection of these compounds. Limits of detection reached with this source can be lower than those obtained with APCI or APPI [57,58].

In the present study, APCI and heated-ESI were both examined for their performance. The UHPLC method was modified from a previous work [42] and from U.S. EPA method 539.1 for the determination of hormones and BPA in drinking water samples [53]. The different conditions tested during the optimization allowed the determination of the best performance among APCI or heated-ESI sources. Target analytes were first infused to select suitable operating MS parameters (collision energy, tube lens, sheath gas, auxiliary gas, and corona/capillary voltage) for each condition (Section 2.3). The best sensitivity was also determined upon examination of either acidic (#1) or neutral (#2) analytical mobile phases, using direct injection UHPLC-MS/MS.

For the heated-ESI or APCI source optimization, mobile phase condition #1 involved using MeOH and H_2O (50:50, both with 0.1% FA v/v), while mobile phase condition #2 involved using MeOH (A), H_2O (B) and NH_4F (20 mM) (45/50/5; v/v/v). ESI parameters were previously described in Section 2.3. APCI parameters with mobile phase condition #1 and #2 were investigated; the optimal signal intensities were obtained when applying the following parameters: sheath gas (50 arbitrary unit), auxiliary gas (5 arbitrary unit), sweep gas (0 arbitrary unit), corona discharge (4 kV), capillary temperature (350 °C), vaporizer temperature (500 °C).

Our results indicate that the source type is indeed a critical robustness factor to optimize to achieve the most desirable sensitivity (unpublished data). Heated-ESI appeared as the best choice both for positive mode and negative mode analytes. Analyte absolute areas were, on average, 13-fold higher with heated-ESI compared to those achieved with APCI. As regards the mobile phase compositions, mobile phase condition #2 (neutral mobile phase) allowed the best LOD in terms of absolute area for all compounds except for mestranol. The latter analyte showed inverse trends concerning the LOD since the best performances were obtained when combining the APCI source and mobile phase condition #1 (SI Fig. S3). Overall, the heated-ESI source combined with mobile phase condition #2 provided the best conditions to analyze the targeted EDCs.

The effect of using the polarity switching mode (*versus* separate injections for each mode) was also studied (Fig. S4). The loss of sensitivity observed for all analytes was limited when using polarity switching (on average, - 12%), which led to the final selection of the heated-ESI source operated in polarity switching mode and using mobile phase condition #2.

The concentration of mobile phase modifier was adapted from the U.S. EPA method 539.1 [53], using initially a concentration of 20 mM NH_4F. It was hypothesized that the concentration of salt could also affect the LOD of the method. Several conditions were tested from 0.50 mM to 20 mM. An illustration of the results obtained for equilin and progesterone is provided in Fig. 1.

The overall trend observed for equilin and progesterone was the same for the majority of positive and negative mode compounds. With a decrease of salt concentration, sensitivity generally increased from 20 mM to 1.0 mM but decreased at lower values (0.5 mM). The opposite was observed in the particular case of mestranol as shown in the SI (Fig. S5); the relatively lower solubility of mestranol in water compared to other compounds could be a factor for the different trend observed [59,60]. A NH_4F concentration of 1 mM was finally selected for the improvement it yielded for most analytes (increase of 1.25–1.50 × in terms of signal intensity); this also seemed a reasonable compromise, further considering the fact that mestranol is not currently included under the priority hormones listed by Method 539.1.

To further improve method LODs, on-line pre-concentration coupled to UHPLC-MS/MS was investigated. The choice of the on-line SPE sorbent type was made in accordance with the literature and considering the range of commercially available products. For this purpose, a set of two on-line SPE C18 columns (Hyper-

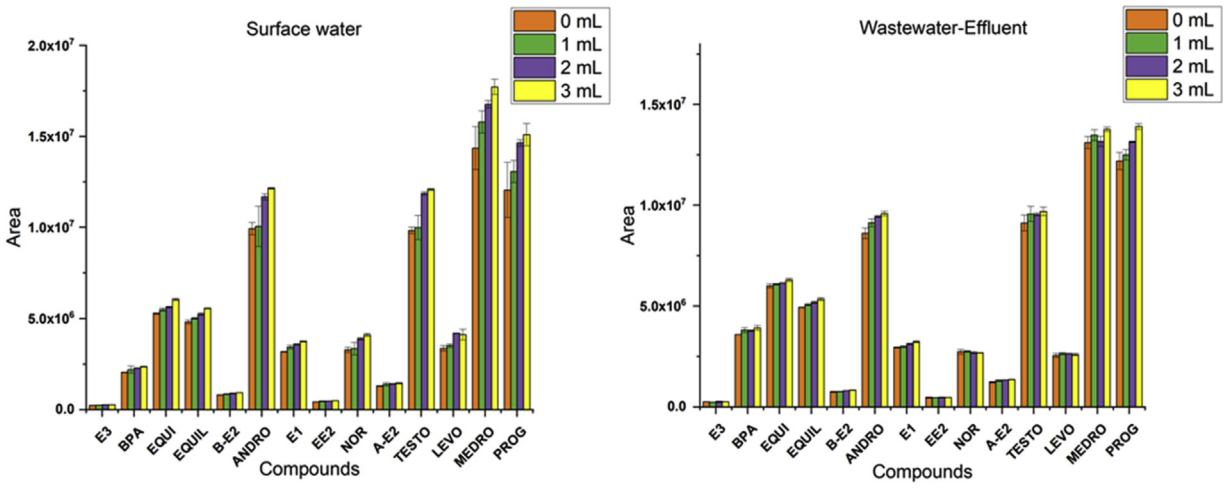

Fig. 2. Influence of the on-line SPE wash volume (mL) on analyte absolute area for the 14 targeted EDCs analyzed by on-line SPE – UHPLC-MS/MS, illustrated for surface water (left) and wastewater effluent (right).

sil Gold aQ) were connected in series (see also Section 2.4). The use of a 20-mL injection volume was readily discarded due to the typically lower extraction efficiencies [61] and problematic robustness—including progressive back-pressure increase along the LC–MS batch sequence (unpublished data). Based on a previous study [61], a 10-mL sample loading volume could be used for optimal sensitivity. Loading a 10-mL water sample could, however, lead to co-extraction of increased matrix components and salt. Consequently, the possibility of adding a washing step of the on-line SPE column after the sample loading and before the back-flush elution was examined for each matrix type. The washing step had a significant effect, the analyte absolute area generally increasing with increasing wash volume. The magnitude of the increase became less pronounced between 2 mL and 3 mL, as shown in Fig. 2 for surface water and wastewater. An optimal wash volume of 2 mL was therefore applied for all matrix types. This also seemed a reasonable compromise when considering total method running time, solvent consumption, and analytical performance obtained in the subsequent method validation.

3.2. Validation of the on-line SPE – UHPLC-MS/MS method

3.2.1. On-line SPE recovery

On-line SPE absolute extraction efficiencies were first evaluated, in order to estimate the proportion of analytes that may be lost during the pre-concentration step, although the losses would not affect the overall process efficiency (or whole-method trueness) if the internal standardization and quantification approach are designed appropriately. The extraction efficiency of the on-line SPE sorbent ranged from 64% to 79%, which may be deemed acceptable for an on-line SPE method [17] (Fig. S6). These results are also in agreement with previous on-line SPE methods that reported recoveries between 60% and 100% of various hormones [16,50,61]. Even though a complete extraction recovery could not be attained, it should be highlighted again that the recovery losses were integrated within the quantification procedure, since samples and matrix-matched calibration levels were both submitted to the on-line SPE – UHPLC-MS/MS analysis, ensuring an accurate analysis (see also Section 3.2.4).

3.2.2. Matrix effects and quantification procedure

Matrix effects were evaluated for the newly-developed on-line SPE – UHPLC-MS/MS workflow. The presence of matrix co-occurring compounds can influence the on-line SPE retention and UHPLC chromatographic performances. Matrix effects may also affect the ionization efficiency through signal enhancement or suppression. Matrix effects are not completely understood, but it is hypothesized that co-extracted contaminants, including fulvic or humic acids present in water, could play a role in the observed signal [53,62].

Raw matrix effects evaluated upon a comparison with the response in reagent water and based on absolute areas, indicated non-negligible (>20%) ion suppression or enhancement (unpublished data). Such absolute matrix effects may not always be fully compensated by internal standard correction. Net matrix effects determined on the basis of analyte to isotope-labelled IS area ratios are shown in Table S7. According to the matrix effect definition used in the present study (see also the SI), positive values indicate a net analyte to IS signal enhancement compared to the ratio in the matrix-free HPLC-water, while negative values indicate signal suppression. In the case of tap water, matrix effects ranged from - 3% for medroxyprogesterone to - 35% for bisphenol A at the low QC level, while matrix effects were rather low at the high spike level (Table S7). In surface water, net matrix effects ranged from −1% for α-estradiol to −28% for estriol at the low spike level, and between −1% for α-estradiol to −24% for testosterone at the high spike level. In the case of wastewater, net matrix effects were generally more prevalent, as could be anticipated from the higher matrix complexity together with a relatively high sample loading volume. Net matrix effects in wastewater effluent ranged from −6% for α-estradiol to +47% for bisphenol A at the low spike level, and from −2% for β-estradiol to +39% for equilin at the high spike level. Net matrix effects in wastewater influent were between -63% and +39% (Table S7).

Based on the above, it was preferred to adopt a matrix-matched calibration approach rather than a matrix-free (HPLC-water) approach that could lead to non-negligible underestimation (when signal suppression is reported) or overestimation (when signal enhancement is reported) of the true concentration in the sample. For each matrix type, a subset of each field sample of the same type was pooled to create a composite sample for addition of natives (at $\geq$ 5 levels) and internal standards (concentration set at 50 ng L^{-1}). The resulting matrix-matched calibration curves were constructed by plotting the analyte to IS response ratio as a function of concentration. The concentration in field samples was then determined by dividing the analyte to IS ratio in the sample by the slope of the matrix-matched calibration curve [42].

Relative matrix effects were evaluated to determine the response variations between different batches or samples within a same matrix type. The determination of this parameter has received due attention in bioanalytical analyses where it is essential to measure the b̈atch-to-batchänd/or s̈ample-to-sampleḿatrix effects in urine, milk, or blood plasma samples [63,64] due to biological variability, which may not be fully accounted for by a matrix-matched calibration. Here, the relative matrix effects were calculated by comparing the slope of the standard additions (with IS correction) calibration lines established for at least 3 individual samples from the same matrix type but from different locations, and the matrix-matched calibration curve reference used for quantification (also with IS correction). Relative matrix effects ranged from -10% to +9% in tap water and from -11% to +9% in surface water. Even in the case of wastewater, the results obtained were conclusive with relative matrix effects between -15% and +8% in the case of influents and between -3% and +25% in the case of effluents (Table S8). Overall, relative matrix effects were generally limited within ±20% in most cases, reflecting the robustness of the presented method. The use of a matrix-matched calibration combined with internal standards thus provided an effective quantification strategy, avoiding the need for detailed standard additions to each individual sample (a strategy which is certainly rigorous but also resource-intensive to implement for large sample series).

3.2.3. Linearity performance and LOD/LOQ

Analytical figures of merit were evaluated, and included linearity range, determination coefficients, limits of detection (LOD), and limits of quantification (LOQ).

The linearity range extended over 0.15–200 ng L^{-1}. This range is adequate with levels found in the environment which are generally in the vicinity of nanogram per liter levels. For matrix-free HPLC water, tap water, and surface water the herein developed method presented suitable determination coefficients (R^2 always > 0.9940 and in most cases > 0.9990). For wastewater matrixes, R^2 was also determined with values higher than 0.9970 in most cases.

The determined LOD and LOQ are provided in Table 1. Limits of detection and quantification in matrix-free water ranged from 0.050 ng L^{-1} to 1.0 ng L^{-1} and from 0.15 ng L^{-1} to 3.0 ng L^{-1}, respectively. In the case of tap water, LODs ranged from 0.10 ng L^{-1} to 0.70 ng L^{-1} and LOQs from 0.30 ng L^{-1} to 2.1 ng L^{-1}. In surface water, LODs and LOQs ranged from 0.40 ng L^{-1} to 2.5 ng L^{-1} and from 1.2 ng L^{-1} to 7.5 ng L^{-1}, respectively. LODs for wastewater matrixes (influent and effluent) ranged from 1.0 ng L^{-1} to 5.0 ng L^{-1} and from 0.50 ng L^{-1} to 4.0 ng L^{-1} for wastewater influent and wastewater effluent, respectively. Such LOD/LOQ performance is improved compared to values reported in influent and effluent water matrixes [42]. The herein reported LOD/LOQ performance is also comparable with that determined by Duong et al. in the case of surface water [65], and was improved compared to Naldi et al. for the other two water matrices [51]. Certain studies showed comparable LOD and LOQ performances but to the authors' best knowledge no study has exclusively focused on estrogens, progestogens, androgens, and other EDCs in multiple matrix types with an extended list of compounds [14,18,16,50].

The authors would also like to reiterate the importance of potential LOD/LOQ methodological issues. In the present study, limits of detection were determined upon the visual measurement of the signal to noise ratio (S/N) at concentration close to the LOD (S/N typically measured ~ 3–5) and deriving the actual LOD for an S/N = 3. However, other ways to determine the LOD parameter have been reported in the literature. In one study [66], the LOD of EE2 was determined by using the value generated by the software for a sample analyzed at 5 ng L^{-1}. Accordingly, the estimated LOD for EE2 was 0.035 ng L^{-1} in surface water. The referred value of S/N: 444 automatically determined was the key to get the limit of detection previously mentioned. By applying the same method of LOD determination in a blank surface water matrix and at the same spike concentration (i.e., 5 ng L^{-1}), we obtained a limit of detection of 0.024 ng L^{-1} that was based on a S/N: 633 (Fig. S7). Using this procedure, the performance of the method can be artificially increased but may not be representative of the true sensitivity (i.e., would lead to report lower LODs). Considering the need for accurate and reproducible results between studies, we feel it would be better to avoid deriving LODs based on S/N values differing in several orders of magnitude from the LOD cut-off value of S/N = 3. Similarly, the determination of LOQ via the S/N method should imply the analysis of low-level samples to obtain representative S/N values for both quantification and confirmatory MS/MS transitions. Additionally, the conditions for appropriate precision and low bias should already be satisfied at the LOQ, as previously highlighted in precision profile and trueness profile approaches [67].

3.2.4. Precision and trueness

Regardless of matrix type, suitable intra-day and inter-day precisions were typically obtained (Table 2), compliant with the acceptability criterion of ± 30% [53]. In matrix-free HPLC water, intra-day precision ranged from 1.9% to 12% for QC1 and from 0.60% to 12% for QC2, and inter-day precision from 1.3% to 18% for QC1 and from 0.50% to 19% for QC2. The method also performed satisfactorily in the case of tap water as intra-day precision was always <4.3% for QC1 and <3.2% for QC2, and inter-day precision <20% for QC1 and <16% for QC2. Comparable results were found for the surface water and wastewater (influent and effluent) matrices, which still met the

Table 1
Summary of R^2, LODs, and LOQs for the 14 targeted EDCs in the different matrices assayed (TW: tap water; SW: surface water; Inf: wastewater influent; Eff: wastewater effluent).

	LOD (ng/L)					LOQ (ng/L)					R^2				
	HPLC water	TW	SW	Inf	Eff	HPLC water	TW	SW	Inf	Eff	HPLC water	TW	SW	Inf	Eff
E3	0.30	0.40	1.3	2.5	1.7	0.90	1.2	3.9	7.5	5.1	0.9987	0.9996	0.9986	0.9994	0.9922
BPA	1.0	0.50	1.2	5.0	2.0	3.0	1.5	3.5	15	6.0	0.9943	0.9999	0.9930	0.9990	0.9983
EQUI	0.10	0.15	0.70	1.0	3.0	0.30	0.45	2.1	3.0	9.0	0.9995	0.9991	0.9992	0.9995	0.9925
EQUIL	0.050	0.10	0.40	3.3	1.0	0.15	0.30	1.2	9.9	3.0	1.0000	0.9994	0.9992	0.9997	0.9930
ANDRO	0.10	0.15	0.50	4.8	2.0	0.30	0.45	1.5	14	6.0	0.9999	0.9994	0.9997	0.9993	0.9992
β-E2	0.40	0.60	0.80	3.6	1.0	1.2	1.8	2.4	11	3.0	0.9998	0.9976	0.9998	0.9996	0.9972
E1	0.10	0.30	0.60	3.7	0.50	0.30	0.90	1.8	11	1.5	0.9999	0.9992	0.9996	0.9996	0.9970
EE2	0.30	0.70	1.4	4.6	4.0	0.90	2.1	4.2	14	12	0.9993	0.9997	0.9997	0.9994	0.9929
NOR	0.15	0.30	1.5	4.8	4.0	0.45	0.90	4.5	14	12	0.9999	0.9982	0.9998	0.9993	0.9983
α-E2	0.070	0.25	0.50	5.0	1.0	0.21	0.75	1.5	15	3.0	0.9992	0.9994	0.9999	0.9992	0.9984
TESTO	0.15	0.30	0.70	4.1	2.0	0.45	0.90	2.1	12	6.0	0.9997	0.9992	0.9990	0.9995	0.9972
LEVO	0.30	0.50	2.5	5.0	3.0	0.90	1.5	7.5	15	9.0	0.9993	0.9996	0.9992	0.9991	0.9980
MEDRO	0.10	0.40	0.70	1.0	2.0	0.30	1.2	2.1	3.0	6.0	0.9997	0.9998	0.9998	0.9996	0.9906
PROG	0.20	0.50	1.0	5.0	0.70	0.60	1.5	3.0	15	2.1	0.9991	0.9997	0.9999	0.9990	0.9973

Table 2
Intra-day and inter-day precision (RSD, %) for HPLC water, tap water (TW), surface water (SW), wastewater influent (Inf) and wastewater effluent (Eff), at two quality control levels (see also the SI for details).

	Intra-day precision (n = 5)										Inter-day precision (n = 15)									
	QC1 (%)					QC2 (%)					QC1 (%)					QC2 (%)				
	HPLC water	TW	SW	Inf	Eff	HPLC water	TW	SW	Inf	Eff	HPLC water	TW	SW	Inf	Eff	HPLC water	TW	SW	Inf	Eff
E3	4.0	0.40	3.0	9.0	8.0	8.0	3.0	9.0	1.2	7.0	18	18	4.0	1.0	15	1.1	8.0	7.0	0.40	11
BPA	12	2.0	1.4	5.0	2.0	2.0	0.90	1.1	2.0	1.2	7.0	1.5	16	5.0	33	5.0	1.5	8.0	3.0	0.10
EQUIL	2.0	2.0	3.0	1.6	4.0	6.0	3.0	3.0	1.1	4.0	6.0	20	16	5.0	6.0	10	16	4.0	0.30	8.0
EQUI	1.9	1.0	1.2	3.0	5.0	4.0	2.0	3.0	0.90	1.4	2.0	5.0	9.0	1.0	3.0	2.0	6.0	9.0	1.4	0.60
ANDRO	2.8	0.10	2.0	6.0	1.0	0.60	1.0	0.60	0.60	2.0	3.0	6.0	5.0	0.10	4.0	1.1	2.0	3.0	0.40	0.90
β-E2	3.0	2.0	3.0	1.3	1.5	0.90	0.30	0.40	1.0	1.2	1.3	7.0	8.0	0.60	7.0	0.50	2.0	0.70	3.0	3.0
E1	3.0	1.0	4.0	4.0	4.0	2.0	3.0	2.0	0.90	1.7	5.0	7.0	1.2	0.30	5.0	6.0	3.0	8.0	4.0	9.0
EE2	5.0	1.0	1.5	11	19	3.0	2.0	5.0	2.0	4.0	5.0	9.0	12	5.0	4.0	2.0	2.0	1.2	1.4	1.8
NOR	10	0.70	4.0	4.0	9.0	1.5	3.0	4.0	0.90	5.0	13	18	3.0	0.50	7.0	8.0	8.0	4.0	3.0	6.0
α-E2	3.0	3.0	2.0	3.0	7.0	1.7	3.0	0.90	1.0	5.0	1.3	11	6.0	2.0	19	4.0	5.0	7.0	1.8	2.0
TESTO	12	0.30	1.7	7.0	11	6.0	1.4	4.0	0.60	3.0	15	6.0	14	1.1	0.20	19	1.7	6.0	2.0	2.0
LEVO	12	4.0	1.0	6.0	16	8.0	3.0	9.0	1.0	6.0	13	8.0	5.0	9.0	11	5.0	6.0	9.0	1.9	1.2
MEDRO	9.0	1.0	4.0	4.0	15	12	2.0	3.0	1.5	2.0	7.0	7.0	0.70	3.0	10	10	9.0	5.0	3.0	15
PROG	5.0	0.50	1.9	3.0	1.5	1.4	1.0	1.2	0.60	1.2	12	6.0	5.0	0.50	12	3.0	3.0	2.0	0.50	2.0

Table 3
Trueness (%) in HPLC water, tap water (TW), surface water (SW), wastewater influent (Inf), and wastewater effluent (Eff), determined at two spike levels (see also the SI for details).

	Trueness (%)									
	QC1 (%)					QC2 (%)				
	HPLC water	TW	SW	Inf	Eff	HPLC water	TW	SW	Inf	Eff
E3	83	80	75	97	97	96	89	90	96	94
BPA	96	96	86	85	87	92	99	99	90	88
EQUIL	90	86	85	90	77	87	94	96	91	78
EQUI	90	88	86	84	93	92	98	91	95	74
ANDRO	100	82	83	96	92	97	99	90	98	95
β-E2	91	85	75	85	85	99	100	91	95	72
E1	89	87	94	100	90	96	96	96	91	96
EE2	85	86	85	90	74	97	96	96	97	78
NOR	99	89	89	93	77	89	96	96	97	91
α-E2	90	83	80	86	87	96	95	88	96	89
TESTO	93	99	81	97	97	97	93	88	99	93
LEVO	98	89	94	96	75	98	99	86	95	86
MEDRO	87	95	90	97	89	97	90	96	96	93
PROG	99	98	82	95	84	93	98	98	99	84

acceptability criterion for precision (Table 2). The results obtained were also in agreement with the values reported in previous studies [17,50].

Whole-method trueness was determined for each matrix type (Table 3) and proved satisfactory (overall range: 72–100%). For matrix-free water, whole-method trueness ranged from 83% to 100% at QC1 and from 87% to 100% at QC2. Such results are in agreement with the acceptability criterion of ± 30% (i.e., trueness range = 70–130%) usually set in U.S. EPA methods [53]. For tap water, trueness values were equally acceptable, as trueness ranged from 80% to 99% at QC1 and from 89% to 100% at QC2. Similar results were found for the surface water matrix (Table 3). Even in the case of the complex wastewater matrix effluent, trueness remained suitable, with values ranging between 74% and 97% at QC1 and between 72% and 96% at QC2. Such performances are comparable to or better than values reported in similar studies on hormones [50,61].

3.3. Method demonstration to water samples from Eastern Canada

Water samples representative of the different matrixes were analyzed. Raw sewage, treated wastewater samples and surface water were collected from Quebec sites (see also Section 2.2) while tap water samples were collected from Quebec and Ontario locations (Canada). In raw sewage samples from the Greater Montreal area (Quebec, Canada), estriol (E3), bisphenol A (BPA), equilin (EQUI), androstenedione (ANDRO), β-estradiol (β-E2), estrone (E1), α-estradiol (α-E2), testosterone (TESTO), and levonorgestrel (LEVO) were variously quantified. The individual levels ranged from 4.6 ng L^{-1} to 300 ng L^{-1}. When considering the sum of 14 targeted EDC, concentrations ranged from 37 ng L^{-1} to 360 ng L^{-1}. The most recurrent compounds were natural hormones except for the synthetic levonorgestrel that was found in most of the sites with levels ranging from 26 ng L^{-1} to 300 ng L^{-1} (Table 4). Levels found for these analytes are comparable to those detected in other studies and confirm the non-negligible quantities at which these compounds can be found [14,18,16]. Determined levels are consistent with previous results coming from raw sewage samples from Canada that mentioned concentrations ranging from 26 ng L^{-1} to 495 ng L^{-1} for E3 and from 22 ng L^{-1} to 170 ng L^{-1} for LEVO [18,68]. Bisphenol A (BPA) was found at concentrations ranging from 24 ng L^{-1} to 41 ng L^{-1} for sites C and D.

Table 5 shows the quantified hormones in treated (effluent) wastewater samples from Quebec, Canada. Estriol (E3), bisphenol A (BPA), equilenin (EQUIL), β-estradiol (β-E2), estrone (E1), norethindrone (NOR), testosterone (TESTO), and progesterone (PROG) were variously reported. Individual concentrations of EDCs ranged from 1.7 ng L^{-1} to 240 ng L^{-1}, while cumulative concentrations ranged from 260 ng L^{-1} to 790 ng L^{-1}. Bisphenol A (BPA) was particularly ubiquitous (occurrence at each studied site) with a con-

Table 4
Individual concentrations and total concentration (∑EDC) of the targeted endocrine disruptors in raw sewage (WWTP influent) measured at selected WWTP in the province of Quebec (Canada). Concentrations are expressed in ng L^{-1}.

Raw sewage (Wastewater influent)						
	Site A	Site B	Site C	Site D	Site E	Site F
E3	ND	ND	ND	92 ± 9.0	ND	ND
BPA	ND	ND	41 ± 4.2	24 ± 2.8	ND	ND
EQUI	ND	ND	ND	ND	ND	4.6 ± 0.10
ANDRO	74 ± 5.2	ND	21 ± 2.4	ND	10 ± 3.4	8.3 ± 1.8
β-E2	ND	ND	ND	15 ± 1.3	ND	6.3 ± 1.7
E1	ND	ND	ND	21 ± 2.8	ND	ND
α-E2	ND	ND	ND	< LOQ	ND	ND
TESTO	13 ± 0.30	< LOQ	ND	ND	ND	14 ± 0.50
LEVO	145 ± 43	73 ± 3.7	299 ± 17	ND	27 ± 1.8	26 ± 1.7
PROG	ND	< LOQ	< LOQ	ND	ND	ND
∑EDC	**232**	**73**	**361**	**153**	**37**	**59**

Table 5
Individual concentrations and total concentration (∑EDC) of the targeted endocrine disruptors in the treated effluent of selected WWTPs in the province of Quebec (Canada). Concentrations are expressed in ng L^{-1}.

Wastewater effluent					
	Site A	Site B	Site C	Site D	Site E
E3	113 ± 1.6	95 ± 8.7	75 ± 11	ND	241 ± 22
BPA	134 ± 3.8	60 ± 4.6	234 ± 10	189 ± 4.8	211 ± 0.90
EQUIL	4.7 ± 0.50	8.6 ± 0.60	6.4 ± 0.20	ND	7.0 ± 0.70
β-E2	122 ± 4.5	45 ± 0.30	43 ± 0.20	31 ± 0.70	94 ± 4.3
E1	14 ± 0.10	15 ± 0.10	14 ± 0.30	44 ± 0.50	33 ± 1.5
NOR	ND	ND	2 ± 0.20	ND	132 ± 2.2
α-E2	< LOQ	ND	ND	ND	ND
TESTO	42 ± 4.6	41 ± 3.6	23 ± 0.80	ND	59 ± 2.5
PROG	7.3 ± 0.30	8.6 ± 0.20	14 ± 0.70	< LOQ	13 ± 1.4
∑EDC	**437**	**273**	**434**	**264**	**790**

centration range of 60 ng L^{-1} to 234 ng L^{-1} (Table 5), similar with those reported in wastewater treatment plants from China [69]. Some compounds such as progesterone, β-estradiol and testosterone were present at respective levels ranging from 4.0 ng L^{-1} to 25 ng L^{-1}, from 0.20 ng L^{-1} to 157 ng L^{-1} and from 0.20 ng L^{-1} to 124 ng L^{-1}, in samples from Quebec (Montreal) or China (Beijing) [68,70], with potential endocrine-disrupting impacts [71]. Most of the compounds found in these wastewater samples were natural or endogenous hormones while only one site showed a significant concentration of the synthetic hormone norethindrone (Site E).

It must be emphasized that this study focused on dissolved EDCs measured after filtration. Interaction with the suspended particulate phase will be critical to fully explain the fate within the treatment plants (and environmental fate beyond, as it is released). Furthermore, a significant proportion of hormones excreted by humans occurred as conjugated form such as glucuronide or sulfate conjugates (which would not be observed by this method). Such conjugated forms will nevertheless be relatively quickly deconjugated in the environment and within water treatment plants. This can result in an apparent increase in (free) hormone concentrations within the water as it passes through the wastewater treatment plant [51,68,72–74].

Several samples were collected across the province of Quebec in order to determine levels of targeted endocrine disruptors that can be found in rivers that potentially serve as drinking water sources (Table 6). Only two compounds were systematically quantified across the 5 surveyed sites: bisphenol A and norethindrone. Estrone, β-estradiol and medroxyprogesterone were solely quantified at 1/5 site while androstenedione, α-estradiol, testosterone and progesterone were variously detected but remained < LOQ. Bisphenol A was the main contributor to the sum of 14 targeted EDCs in the case of surface water samples with concentrations ranging from 3.9 ng L^{-1} to 17 ng L^{-1} (i.e., contributions ranging from 55% to 89% of the sum of 14 EDCs). The BPA concentrations reported in our work were in the same range as those reported by Jin et al. for lakes and rivers from China (4.2 ng L^{-1} to 141 ng L^{-1}) [75]. In the latter study, the occurrence of BPA was 100%, similar to the findings from the present study. The second most abundant target analyte was norethindrone, with concentrations ranging from 1.7 ng L^{-1} to 2.7 ng L^{-1} (Table 6). In a previous study, Ciofi et al. found comparable levels of estrogens in rivers with concentrations ranging from 0.53 ng L^{-1} to 4.5 ng L^{-1} for water courses in Italy, while Duong et al. found relatively higher levels in South Korea ranging from 3.2 ng L^{-1} to 130 ng L^{-1} [65,76].

Estriol (E3), β-estradiol (β-E2), norethindrone (NOR), and progesterone (PROG) were variously detected in tap water from Quebec province (Table 7). Total concentrations of target EDCs in tap water were < LOQ, with one exception at 1.2 ng L^{-1}. These levels are similar to those detected in other studies and confirm the background concentrations for this type of compounds [43,51]. Similar levels of EDCs were found in tap water samples from Ontario (Table 7), albeit a smaller range of compounds could be detected. Results showed the presence of bisphenol A (BPA) at three different locations but at concentrations < LOQ. Androstenedione (ANDRO) was found at only two locations while testosterone (TESTO) was detected or quantified at three locations as shown in Table 7. Among the studied compounds, levels ranged from 0.81 ng L^{-1} (ANDRO) to 2.8 ng L^{-1} (β-E2).

3.4. Contribution of the study to the state-of-art

Some pre-existing analytical methods for endocrine disruptors performed satisfactorily in clean water [51,52], but their transferability to multiple environmental matrices and an extended range of compounds had not been investigated. The herein study describes an alternative analytical method to analyze the expanded

Table 6
Individual concentrations and total concentration (∑EDC) of the targeted endocrine disruptors in surface water at different locations in the province of Quebec (Canada). Concentrations are expressed in ng L^{-1}.

Surface water					
	Chateauguay River (Chateauguay)	Richelieu River (upstream Chambly)	Des Prairies River (Repentigny)	Mille Iles River (Terrebonne)	Des Prairies River (Montréal)
BPA	5.1 ± 0.18	3.9 ± 1.0	17 ± 1.9	7.4 ± 0.51	5.8 ± 0.76
ANDRO	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
β-E2	ND	ND	ND	ND	1.7 ± 0.010
E1	< LOQ	0.50 ± 0.37	< LOQ	< LOQ	< LOQ
EE2	ND	ND	ND	ND	< LOQ
NOR	1.7 ± 0.050	2.7 ± 0.17	2.1 ± 0.20	2.1 ± 0.030	2.3 ± 0.050
α-E2	ND	< LOQ	ND	< LOQ	ND
MEDRO	ND	ND	0.40	ND	ND
PROG	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
∑EDC	**6.8**	**7.1**	**19**	**9.5**	**9.8**

Table 7
Individual concentrations and total concentration (∑EDC) of the targeted endocrine disruptors in different tap water samples from selected municipalities in two Canadian provinces (Quebec and Ontario). Concentrations are expressed in ng L^{-1}.

Tap water (Québec)					
	Repentigny	Mont-Saint-Hilaire	Saint-Hyacinthe	Sorel-Tracy	Granby
E3	<LOQ	1.2 ± 0.37	<LOQ	ND	ND
β-E2	ND	ND	ND	ND	<LOQ
NOR	<LOQ	<LOQ	ND	<LOQ	<LOQ
PROG	ND	ND	<LOQ	ND	ND
∑EDC	**< LOQ**	**1.2**	**< LOQ**	**< LOQ**	**< LOQ**

Tap water (Ontario)						
	Carleton	Lindsay	Orangeville	Ottawa	Perth	Toronto
BPA	ND	<LOQ	<LOQ	ND	ND	<LOQ
β-E2	ND	ND	ND	2.8	ND	ND
ANDRO	1.0 ± 0.020	0.81 ± 0.070	ND	ND	ND	ND
NOR	ND	<LOQ	ND	ND	ND	ND
TESTO	<LOQ	ND	ND	<LOQ	ND	2.1
∑EDC	**1.0**	**0.81**	**< LOQ**	**2.8**	**< LOQ**	**2.1**

scope of EPA-priority EDCs, based on on-line SPE–UHPLC-MS/MS. Using this faster approach, we demonstrated performances equivalent to those stated in Method 539-1 (for drinking water), and further confirmed its applicability to other sample types.

A substantial number of matrices and validation endpoints were therefore ascertained in this study. One of the advantages of the present work consists in the applicability of the method to four different matrices with an extended range of targeted compounds, including those from the list of recently updated EPA Method 539.1. Previous methods may differ from this in terms of performance and/or range of targeted analytes. A first study can achieve lower detection limits but for a small number of compounds and a high sample volume (250 mL) requiring off-line sample preparation and longer instrumental analysis time [77]. Another study was based on an on-line SPE approach and the use of an injection volume similar to that of our study, but using a different source technology [42] that did not yield the same LOD performance.

The developed method used a relatively low sample volume (10 mL) compared to off-line methods that tend to use larger amounts of sample from 250 mL to 1 L [77,78]. The developed method represents a compromise between analysis time, higher sample throughput capabilities, sample volume, and simplicity for the analysis of BPA, androgenic, progestagenic and estrogenic steroid hormones in a single run. The LODs and LOQs were sufficiently low to detect and quantify these EDCs in tap water, surface water and wastewater samples (Section 3.3). The final method showed suitable chromatographic performances (see also SI Fig. S8). The asymmetry factor generally ranged between 1.2–1.35 and the tailing factor between 1.1–1.2. These values are within acceptable range according to EU Commission and US Pharmacopeia [79–81]. The method was compliant with QA/QC requirements, including trueness of CVC standards and internal standard recoveries (SI Table S6), making it eligible for the analysis of long sample series.

4. Conclusions

A fast, sensitive and robust on-line SPE – UHPLC-MS/MS workflow was evaluated for the analysis of U.S. EPA-priority endocrine disruptors. The suitable selectivity and sensitivity of the present analytical method allowed for the detection of bisphenol A, estrogens, progestagens, androgens and steroids, at or below ng·L^{-1} levels in environmental samples. The method is based on a simple pre-filtration of the samples through GFF, followed by the addition of isotope-labelled internal standards and direct analysis by on-line SPE – UHPLC-MS/MS. Compared to off-line SPE based methods, this represents a considerable improvement in total analysis time. For the first time, the compliance to EPA method 539.1 performance criteria was validated for an extended scope of EDCs and matrix combinations (tap water, surface water, and wastewater influents and effluents). The method performed satisfactorily in terms of trueness (bias generally comprised within ± 10%), linearity (R^2 >0.9980 in most cases), and internal standard variations across samples (±18%).

Some of the targeted endocrine-disrupting compounds (EDCs) were detected in the different types of field-collected sam-

ples, confirming their ubiquitous presence in aquatic ecosystems. Bisphenol A, β-estradiol, norethindrone, testosterone, androstenedione, ethinylestradiol, and estriol were detected in tap water samples (<LOD–2.8 ng L^{-1}). Bisphenol A, estrone, norethindrone, and β-estradiol were detected in surface water samples (<LOD–17 ng L^{-1}). Estriol, Bisphenol A, equilin, androstenedione, β-Estradiol, estrone, testosterone, and levonorgestrel were variously detected in raw sewage samples (<LOD–300 ng L^{-1}), while estriol, bisphenol A, equilenin, β-estradiol, estrone, norethindrone, α-estradiol, testosterone, and progesterone were found in treated wastewater effluents (<LOD–240 ng L^{-1}).

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Appendix A. Supplementary data

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