PASSIVE SAMPLERS, AS SURROGATES FOR BIOLOGICAL MONITORING, TO MEASURE EMERGING (MICRO) POLLUTANTS IN THE MARINE ENVIRONMENT

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Abstract

The extensive use of organic chemicals for different applications (industry, agriculture, pharmaceutical usage, etc.) leads to low-concentration but long-term exposure of the aquatic environment to their residues. The protection of our coasts and marine waters is a long-standing part of the European Community environmental policy, which is also broader internationally regulated. Various organisations involved (a.o. OSPAR, USEPA, etc.) stress the need for a more integrated, consistent and economically favourable strategy to meet legislative and international obligations. In the context of a recently started Belgian project, we are studying a relatively new approach making use of passive samplers and hybrid high-resolution mass spectrometry coupled to liquid chromatography for analyzing known and unknown emerging organic micropollutants in the marine environment. Amongst others, attention is given to endocrine disrupting compounds (EDCs) including steroidal hormones. In this particular study, we present the development and optimization of the analytical method to measure multiple steroidal EDCs. The analytes were separated on a 1.9 µm Hypersil Gold column (10 mm x 2 mm) and quantified in full-scan by a Q-Exactive benchtop™ mass spectrometer. Chromatographic variables like mobile phase flow, acidification, and column oven temperature were optimized by injecting analytical standards. By analysing sea extracts, the mass spectrometric parameters (sheath gas, auxiliary gas, sweep gas, discharge current, capillary temperature, S-lens RF, and vaporizer temperature) were optimized. Next, 55 steroidal EDCs were successfully extracted from substitute ocean water. In a final step, this method will be validated and applied for the targeted analysis of steroidal EDCs in the marine environment.

Introduction

Endocrine disrupting compounds (EDCs) originating from industrial, rural and domestic activities are considered in an environmental context as emerging organic micropollutants (Garcia-Rodriguez *et al.* 2014, Laurenson *et al.* 2014). EDCs mainly include steroidal hormones and several non-steroidal synthetic compounds. The steroidal hormones can be subdivided in androgens, estrogens, gestagens and corticosteroids. Non-steroidal compounds are a.o. phthalates, phenols and pesticides. Most of these EDCs have been monitored in the aquatic environment with particular focus on riverine, ground, drinking and waste water. These four major water bodies are primarily limited to local anthropogenic activities and corresponding contamination (Tijani *et al.* 2013). By monitoring the marine environment, a broader view of EDC occurrence and contamination will be obtained. Since EDCs have barely been examined in the marine environment (Ronan and McHugh 2013), this work focuses on monitoring a broad range of steroidal EDCs in the North Sea environment.

Monitoring steroidal EDCs in the marine environment must deal with ultra-trace contamination levels as compared to river, ground, drinking and waste water (Zhang *et al.* 2016). Despite the expected low concentrations, the presence may have a significant effect on water organisms (Vlachogianni *et al.* 2013). As an example, EDC exposure to the Sea Urchin results in the inhibition of embryos development (Roepke *et al.* 2005). EDC studies with Zebra fish show disturbance of the sexual differentiation and reproduction (Segner 2009). Bioconcentration factors of EDCs (Arnot and Gobas 2006), listed in Table 1, show that bioaccumulation of these pollutants might be of main importance. Therefore, both the trace-level occurrence and the susceptibility of water organisms illustrate the complexity and need to develop a sensitive analytical method to monitor EDCs.

Beside a sensitive analytical method, there is also need to monitor the aquatic system over an extended period of time and obtain time weighted average concentrations. Promising tools to fulfil these needs are passive samplers. The most common passive samplers in literature are POCIS (Bartelt-Hunt *et al.* 2011, Vallejo *et al.* 2013), silicone rubbers (Naude *et al.* 2015) and Chemcatchers (Vrana *et al.* 2015). However, passive samplers have rarely been studied for steroidal EDCs in the marine environment. This work focuses on the development of a sensitive analytical method, which in the future will be implemented in monitoring using passive samplers. The analytical method is based on liquid-chromatography coupled to hybrid high-resolution mass spectrometry to monitor a broad range of steroidal EDC at ambient concentration levels.

Materials and Methods

Reagents and chemicals

The organic solvents were of optima UPLC-MS grade, obtained from Fisher Scientific (Loughborough, UK). The selected standards were obtained from Steraloids Inc (Newport, RI, USA) and Sigma Aldrich (St. Louis, MO, USA). In total, 55 of steroidal hormones were included in this study. The main characteristics of the EDC subcategories can be found in Table 1. The androgens were 5α-androstan-17α-methyl-3α,17β-diol, 5α-dihydrotestosterone, 5β-androstan-3α-17β-diol, 5β-androstan-3α-ol-11,17-dione , 11β-hydroxyandrosterone, 17α-trenbolone, 17β-trenbolone, α-boldenone, α-testosterone, β-boldenone, β-nortestosterone, β-sitosterol, β-testosterone, 1,4-androstadieen-3,17-dione, 4-androsten-6a-ol-3,17-dione, 9-nortestosterone-17-decanoate, 11-ketotestosterone, androstenedione, androsterone, epi-androsterone, ethinyltstosterone, fluoxymesterone, formebolone, methandriol, methylboldenone, methyldihydrotestosterone, methyltestosterone, norethindron, norethandrolone, stanozolol, testosterone-acetate, testosterone-17β-cypionate and trenbolone-acetate. The estrogens were α-zeralenol, β-zeralenol, β-zeranol, 17α-estradiol, 17β-estradiol, 17α-ethinylestradiol, dien-diacetate, dienoestrol, diethylstilbestrol, estradiol-17-acetate, estradiol-17-glucosiduronate, estradiol-17-sulfate, estrone, estrone-3-sulfate, equilin, gestodene, hexoestrol and mestranol. The gestagens were 5α-Pregnan-3α,20β-diol, 17α-acetoxyprogesterone, caproxyprogesterone, chlormadinon acetate, flugestone acetate, medroxyprogesterone, medroxyprogesterone acetate, megestrol, megestrol acetate, melengestrol acetate, norgestrel, pregnolone and progesterone. The corticosteroids were betamethasone, cortisol, cortisone, dexamethasone, prednisolone and prednisone. Internal standards were 17β-estradiol-d_3, hexestrol-d_4, methylprogesterone acetate-d_3 and methyltestosterone-d_3. Primary stock solutions and mixing standards were prepared in methanol ranging from 0.1 to 1000 ng μL^{-1}. The solutions were stored in dark glass bottles at -20°C. The inorganic salts for making substitute ocean water were supplied by Sigma Aldrich.

Table 1.The main characteristics of the targeted EDCs divided in their sub-classes at 25°C based on the SciFinder database (ACD/Labs n.d.).

Sub-class	Molecular Weight (g mol^{-1})	pKa	Log Kow	Solubility (mg L^{-1})	Bioconcentration factor (L kg^{-1})
Androgens	270.3 – 428.7	9.3 – 15.1	1.8 – 7.9	1.9 x 10^{-3} – 58	5.69 – 1.0 x10^6
Estrogens	270.4 – 446.7	-3.8 – 10.3	2.3 – 5.3	1.2 – 1900	1 – 6.6 x10^4
Gestagens	312.5 – 428.6	13.0 – 13.1	2.8 – 3.6	2.6 – 24	20.3 – 1.21 x10^4
Corticosteroids	358.4 – 392.5	12.1 – 12.5	1.4 – 2.0	35 – 140	7.23 – 20.7

Analytical method development: liquid chromatographic and mass spectrometric conditions

The EDCs were chromatographically separated using an ultra-high performance liquid chromatograph (U-HPLC) equipped with a degasser, autosampler, LC pump and column oven (Dionex Ultimate 300, Thermo Fisher). A Hypersil Gold column (1.9 μm, 10 mm x 2 mm, Intersciences, Louvain-La-Neuve, Belgium) was used to separate EDCs, as proven to be successful for anabolic steroids earlier (Vanhaecke et al. 2011). Furthermore, the mobile phase consisted of methanol and water acidified by formic acid. The sample injection volume was 10 μL. The detection of EDCs was carried out using a Q-Exactive™ benchtop mass spectrometer (Thermo Fisher Scientific), equipped with an atmospheric pressure chemical ionization source (APCI).

Liquid chromatographic and mass spectrometric optimization

The liquid chromatographic conditions were optimized by modifying the percentage formic acid addition, the column oven temperature, and the mobile phase flow between 0.00 – 0.10 % formic acid, 0.300 – 0.450 mL min^{-1}, and 30°C – 55°C, respectively. Subsequently, a linear L27 design was applied to statistically investigate the effect of 7 mass spectrometric parameters (Table 2) on the response of EDCs in real extracts. Each variable was assigned three levels, i.e., a low, central and high level. The reliability of the L27 design was increased by using 3 repetitive central experimental points and performing each test randomized.

Extraction

The development and optimization of the extraction was performed on substitute ocean water, prepared according to the ASTM-D1441 standard procedure. The substitute ocean water was spiked with 50 ng L^{-1} of each EDC. The samples were pre-treated by filtering through a Whatman 0.45 μm filter. The obtained filtrate was adjusted to pH 8. Thereafter, the samples were spiked with 50 ng L^{-1} 17β-estradiol-d_3, hexestrol-d_4, methylprogesterone acetate-d_3 and methyltestosterone-d_3 as internal standards. Subsequently, a divinylbenzene sorbent was conditioned and rinsed by loading respectively with methanol and milli-Q water. Next, samples were loaded under vacuum, followed by a washing step with Milli-Q water. The elution was performed vacuum-free by the suitable extraction solvent, acidified with formic acid. The extracts were vaporized to dryness under nitrogen at a temperature of 60°C, and the EDCs were reconstituted in the starting conditions of the mobile phase. Finally, the samples were centrifuged, prior to UHPLC-HRMS analysis.

Table 2.The ranges of the mass spectrometric parameters investigated by a linear L27 design on real marine extracts.

Variables	Unit	Lower Level	Central Level	High Level
Sheath gas	a.u.	15.0	32.5	50.0
Auxiliary gas	a.u.	5.0	15.0	25.0
Sweep gas	a.u.	2	4	6
Discharge current	kV	3	4	5
Capillary temperature	°C	250	300	350
S-lens RF	level	25	50	75
Vaporizer temperature	°C	250	375	500

Results and discussion

Liquid chromatographic optimization

The developed chromatographic method had a run time of 14 min. The best chromatographic separation was obtained by acidifying the mobile phase with 0.10 % formic acid, applying a flowrate of 0.45 mL min^{-1}, and setting the column oven temperature at 45 °C. The results of the chromatographic optimization are depicted in Figure 1 for one of the steroid sub-classes, namely the androgens.

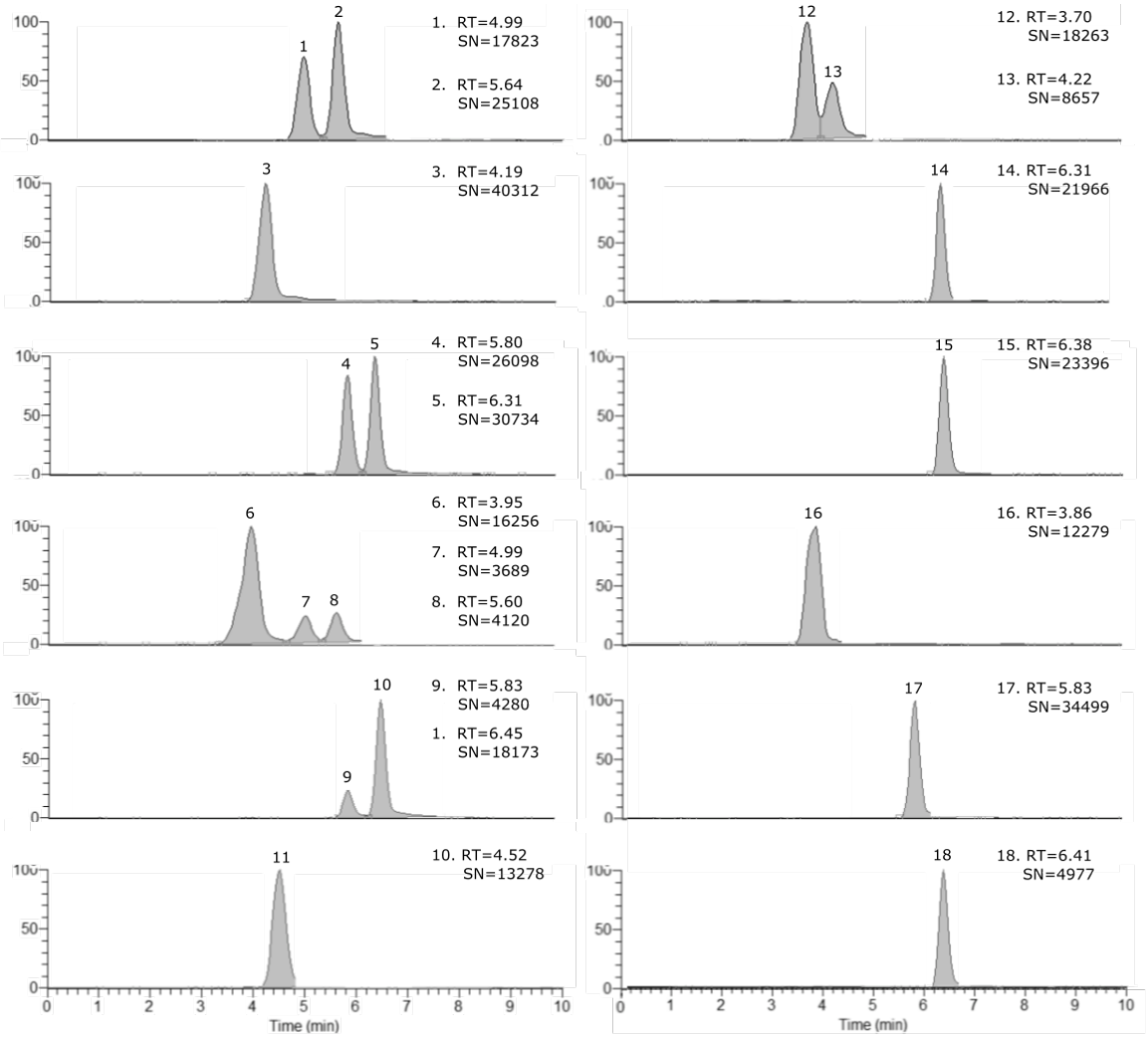

Figure 1. The result of the chromatographic optimisation for the androgens (1 ng injected on column). 1. 17β-testosterone, 2. 7α-testosterone, 3. 19- nortestosterone, 4. methyl testosterone, 5. norethandrolone, 6. formebolone, 7. androstenedione, 8. 1,4-andradien-17β-ol-3-one, 9. methandriol , 10. mestanolone, 11. methylboldenone, 12. trenbolone (α and β) , 13. testosterone acetate, 14. trenbolone actate, 15. stanozolol, 16. fluoxymesterone, 17. methandriol and 18. 5β-androstane-3α,17β-diol.

Mass spectrometric optimization

The Q-Exactive was operated in the full scan mode by alternating the polarity from negative to positive mode. Nevertheless, all the steroids were detected in the positive polarity mode. Subsequently, the EDC mass spectra – obtained with the APCI method – were mainly characterized by MH^+, MH^+-H_2O and MH^+-$2H_2O$. The loss of water in the positive APCI mode has been reported earlier for the analysis of steroids (Ma and Kim 1997). The detection was performed at a scan range of 60-900 Da and a resolution of 140000 FWHM. The effects of other instrumental mass spectrometric variables were statistically evaluated by a L27 experimental design, making use real extracts for analysis. The effect of each variable was evaluated at 95% (p=0.05) significance by an effect plot. To do so, the sum of all the normalized peak areas was taken into account as response parameter. The L27-design was performed with a determination coefficient of 0.845, which is acceptable for experimental designs. The significance of the mass spectrometric parameters is depicted in Figure 2. Five variables had a significant positive or negative effect on the total normalized chromatographic peak area, evaluated by the statistical p-value. The positive significant variables were sheath gas ($p= 4.88x10^{-8}$), auxiliary gas ($p= 2.57x10^{-1}$) and S-lens RF ($p= 5.49x10^{-6}$). The sweep gas ($p= 3.29x10^{-2}$) and capillary temperature ($p= 3.46x10^{-2}$) had a negative effect on the response. These significant parameters will be selected for future optimizing experiments, to increase the sensitivity and suppress matrix effects.

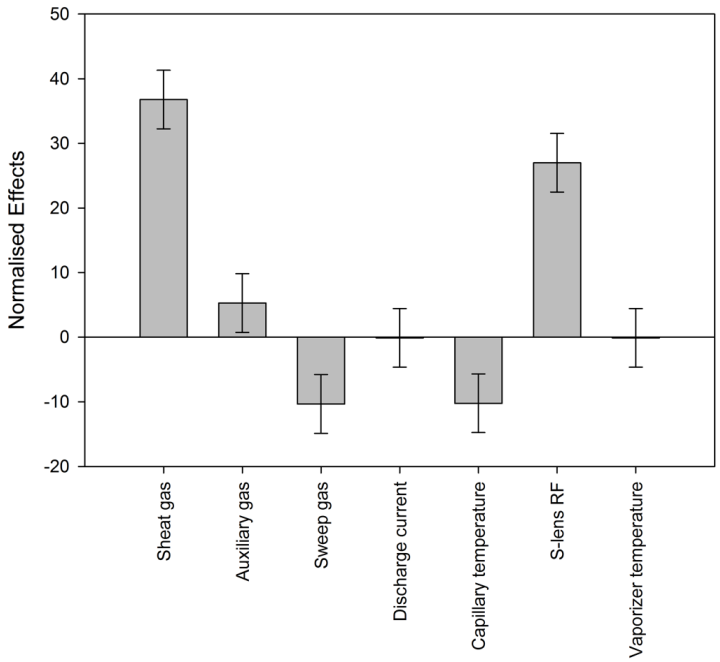

Figure 2. The experimental L27 design, used to statistically investigate the normalised effects on the MS variables at a 95% (p<0,05) significance level, show a significant effect of sheath gas, auxiliary gas, sweep gas, capillary temperature and S-lens RF.

Extraction

The newly optimised extraction method, developed for a broad range of EDCs, shows potential to extract 55 target EDCs from substitute ocean water (ASTM -D1441). The extraction must be further optimized in accordance to extract more EDCs and enhance the total signal of all detectable analytes.

Conclusions

Optimizing the chromatographic and mass spectrometric parameters lead to a sensitive and fast liquid chromatographic high-resolution mass spectrometric (LC-HRMS) method for measuring a broad range of steroidal EDCs in the marine environment. Future perspectives are further optimizing the extraction method and validating the UHPLC-HRMS method according to CD 2002/657/EC. Finally, the developed method will be an important driver to different monitoring programs in the aquatic marine environment. This can lead to international regulations to protect our coast and marine environment.

Acknowledgements

This work fits within the NewSTHEPS project (BR/143/A2/NEWSTHEPS) supported by Belgian Science Policy (BELSPO). More information and future perspectives can be found on the NewSTHEPS website; www.newstheps.be. The authors would like to express their appreciation to the technical staff of the Laboratory of Chemical Analysis for their contribution to the analysis.

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