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Optimization of large volume injection-programmable temperature vaporization-gas chromatography–mass spectrometry analysis for the determination of estrogenic compounds in environmental samples

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ABSTRACT

Large volume injection-programmable temperature vaporization-gas chromatography–mass spectrometry (LVI-PTV-GC–MS) was optimized for the determination of estrone (E1), 17β-estradiol (E2), 17α-ethynyl estradiol (EE2), mestranol (MeEE2) and estriol (E3) for their determination in environmental samples (estuarine water, wastewater, fish bile and fish homogenate) after derivatization with 25 μL (BSTFA + 1% TMCS) and 125 μL of pyridine. Experimental designs such as Plackett–Burman (PBD) and central composite designs (CCDs) were used to optimize the LVI-PTV variables (cryo-focusing temperature, vent time, vent flow, vent pressure, injection volume, purge flow to split vent, splitless time and injection speed). Optimized conditions were as follows: 45 μL of n-hexane extract are injected at 60 °C and 6 μL/s with a vent flow and a vent pressure of 50 mL/min and 7.7 psi, respectively, during 5 min; then the split valve is closed for 1.5 min and afterwards the injector is cleaned at 100 mL/min before the next injection. The method was applied to the determination of estrogenic compounds in environmental samples such as estuarine water, wastewater, and fish homogenate and bile. Limits of detection (0.04–0.15 ng/L for water samples, 0.04–0.67 ng/g for fish bile and 0.1–7.5 ng for fish homogenate) obtained were approx. ten times lower than those obtained by means of a common split/splitless inlet.

1. Introduction

The development of analytical methods for analytes such as hormones and steroids is of increased interest due to their possible adverse effects as endocrine disrupters in the aquatic environment, especially in fishes and amphibians [1–3]. These effects can cause feminization in male fishes (imposex) or to promote abnormal reproductive processes [4–6]. Therefore, in 2003, fourteen steroids and hormones were included in the list of emerging pollutants of concern (EPOCs) within the European Water Framework Directive (WFD, 2009) [7].

Estrone (E1), 17β-estradiol (E2), 17α-ethynyl estradiol (EE2), mestranol (MeEE2) and estriol (E3) are some of the analytes included in the previously mentioned list. Natural hormones, E1, E2 and E3, are synthesized by all species and sexes. Humans, livestocks and wildlife are the main sources of those compounds and the concentration excreted varies during the different stages of life [8–11]. Synthetic hormones such as EE2 and MeEE2 are supplied to females as contraceptives or used in different medical treatments [9].

These compounds are excreted in urine or excrement as glucuronide, glucoside or sulfate derivatives [12,13], they are partially deconjugated in sewer systems or in wastewater treatment plants (WWTPs) and finally accumulated in sludges and effluents [14–16]. Within this scenario, fish are exposed to doses of such analytes that are accumulated in bile preferentially in the glucuronide form [5,17–20].

Estrogens, such as those mentioned above, can be analyzed using different techniques such as gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS), tandem systems of mass spectrometry (LC–MS/MS or GC–MS/MS), liquid chromatography with fluorescence detector (LC-FLD), or even by means of immunoassay techniques [21–25]. When GC is used, derivatization of the compounds is a necessary step in order to improve selectivity and sensitivity. Usually, this last step is carried out in heater blocks, sand baths or ovens [26–30] for 30–90 min at high temperatures (60–75 °C). However, this step can be accelerated using other energy sources such as microwave ovens [31] or ultrasound baths [32,33]. Although ultrasonic probes are mostly used with extraction purposes [34–36], it can be also used for derivatization [37] or even using other systems such as ultrasonic cup boosters [38]. The latter are small ultrasonic baths (<15 mL) which can handle low sample volumes (<1.5 mL). Besides,

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since samples are introduced in safe lock microtubes, cross contamination is avoided.

The splitless inlet is the most commonly used device in GC when trace analysis is accomplished. However, it has several deficiencies since it is a hot vaporizing device. In order to improve it, in the late 1970s, the programmable-temperature vaporization (PTV) inlet was developed based on a splitless inlet. During the injection this inlet is kept cool and, using a temperature controlled program, it is heated up quickly to transfer the sample into the column. One of the most interesting aspects of PTV injection is the ability to perform large volume injection (LVI) using the cold split/splitless solvent vent technique [39]. In this sense, LVI-PTV can improve the sensitivity in several orders of magnitude in comparison with common splitless inlets. LVI-PTV has already been used for the determination of several pollutants [40–42], including some estrogenic compounds [43] but with no previous derivatization. In this work, LVI-PTV-GC–MS conditions were optimized for the determination of estrogenic compounds (E1, E2, EE2, MeEE2 and E3) in environmental samples (estuarine water, wastewater, fish homogenate and fish bile). Variables such as cryo-focusing temperature, vent time, vent flow, vent pressure, injection volume, purge flow to split vent, splitless time and injection speed were evaluated by means of design of experiments. Limits of detection (LODs) obtained were compared with those obtained in a common split/splitless inlet.

2. Experimental

2.1. Reagents and materials

Estrone (E1, 99.5%), mestranol (MeEE2, 99.4%), 17α-ethynylestradiol (EE2, 99.4%) and estriol (E3, 99.7%) were obtained from Riedel-de HaënSeelze, Germany. 17β-estradiol (E2, Sigma Reference Standard, Steinheim, Germany), and 17β-estradiol-16, 16, 17-d_3 (E2-d_3, 98%, Steinheim, Germany) were also purchased. All of them were dissolved individually in anhydrous methanol from Sigma and Aldrich, respectively (Alfa Aesar, 99.9%, Karlsruhe, Germany) at ∼2000 mg/L concentration and the standard solutions were stored at 4 °C in the dark. 3 μg/L to 150 mg/L standards were prepared in ethyl acetate (HPLC, Lab Scan analytical science, 99.8%, Dublin, Ireland) and stored in amber vials at −20 °C.

Acetone (HPLC grade) was supplied by LabScan (Dublin, Ireland) and n-hexane (HPLC grade) and acetic acid (99.7%) were obtained from Panreac (Reixac, Barcelona, Spain).

β-Glucuronidase, type VII-A, from *Escherichia coli* (4974.48 unit), β-glucosidase from almonds (102.8 unit) and sulphatase from *Aerobacter aerogenes* (12.25 unit/mL) were obtained from Sigma (Steinheim, Germany), dissolved in Milli-Q water (<0.05 μS/cm, Milli Q model 185, Millipore, Bedford, MA, USA) and divided in 250 μL aliquots, which were kept at −20 °C in closed amber vials until use.

Potasium di-hydrogenphosphate (RFE, USP-NF, BP, Ph.Eur., 100%, Panreac, Reixac, Barcelona, Spain) and di-ammoniun hydrogenphosphate (99%, Merck, Darmstadt, Germany) were used to prepare 0.1 mol/L buffer solution (pH = 6).

Anhydrous pyridine (99.8%) was obtained from Sigma–Aldrich (Steinheim, Germany) and the derivatization reagent, BSTFA + 1% TMCS (Sylon BFT, 99:1) from Supelco (Walton-on-Thomas, UK).

200-mg (6 mL) Oasis HLB cartridges were obtained from Waters (Milford, MA, USA) and 1-g and 5-g Florisil® cartridges from Supelco (Walton-on-Thames, UK).

2.2. Sampling

Estuarine water samples were collected from Zorrotza (Basque Country, Spain) and influent and effluent water samples were collected from WWTPs of both Gernika and Bakio (Basque Country, Spain) in October of 2009 in pre-cleaned glass bottles, transported to the laboratory in cooled boxes, filtered through 0.45 μm filters (Whatman, cellulose nitrate membrane filters, Dassel, Germany) and analyzed within 48 h.

Biles were obtained from thicklip grey mullets (*Chelon labrosus*) fished in the estuary of Urdaibai near the WWTPs of Gernika (Biscay, North of Spain).

Zebrafish (*Danio rerio*) homogenate was prepared eliminating the tail and the fins of each zebrafish. Samples of each experimental group were homogenized adding 20% ultra pure water in a Potter S homogenizer (B. Braun, Melsungen, Germany) and held in an ice-water cooled bath with 4–5 strokes.

2.3. Water sample pre-concentration

In the case of estuarine and wastewater samples, target analytes were pre-concentrated using SPE according to Hernando et al. [44]. Briefly, a 100-mL aliquot of the water sample, which contained E2-d_3 at ∼10 ng/L, was loaded into a 200-mg Oasis HLB cartridge, which had been previously conditioned with 5 mL of ethyl acetate, 5 mL of methanol and 5 mL of Milli Q water. Then, the cartridge was washed with 5 mL of a Milli Q-water:methanol mixture (95:5, v/v) and dried under vacuum for 15 min. Finally, the analytes were eluted using ethyl acetate (two portions of 4 mL). The extract was finally evaporated to dryness under a gentle stream of nitrogen in a Turbovap LV Evaporator (Zymark, Hopkinton, USA) previous to the derivatization step.

In the case of wastewater samples, the ethyl acetate extract was further cleaned-up using 1-g Florisil® cartridge as described by Guitart et al. [42]. The extract (8 mL of ethyl acetate) was evaporated to approx. 100 μL under a gentle stream of nitrogen and passed through a previously activated (5 mL n-hexane and 5 mL ethyl acetate) 1-g Florisil® cartridges. Finally, the analytes were eluted with 8 mL of a mixture of dichloromethane:ethyl acetate:methanol (40:40:20, v/v) and evaporated to dryness under a gentle stream of nitrogen previous to derivatization.

2.4. Fish homogenate, ultrasonic extraction and clean-up

The extraction was carried out according to the results obtained in a previous work [45]. Thus, approx. 0.03–0.1 g of fish homogenate were weighed and transferred to a Teflon lined extraction vessel. 2 ng of E2-d_3 and 5 mL of acetone were added and the mixture was exposed to ultrasonic irradiation (Sonopuls HD 2070, 20 kHz, 70 W, Bandelin electronic GmbH & Co. KG, Berlin, Germany) under 45% power for 2 min and 5 cycles, with the titanium tip of the probe (MS73, diameter 3 mm, Bandelin, Berlin, Germany) immersed 1 cm. The supernatant was filtered though PTFE filters (25 mm, 5 μm, Waters) and the extract concentrated to ∼0.5 mL using nitrogen blow-down evaporation after the addition of ∼1 mL of n-hexane. The concentrated extract was then submitted to a SPE clean-up.

5-g Florisil® cartridge was conditioned with 10 mL of n-hexane. Afterwards the extract was loaded and eluted with 15 mL of ethyl acetate. Finally, the extract was evaporated to dryness under a gentle stream of nitrogen previous transfer to 2 mL of amber vial and derivatized as explained below (see Section 2.6).

2.5. Ultrasonic hydrolysis and SPE clean-up of fish bile

Biles were obtained from thicklip grey mullets (*Chelon labrosus*) and stored at −80 °C until analysis.

The ultrasonic hydrolysis was previously optimized [46]. In this sense, 100 μL of fish bile were accurately weighed and 1.5 mL of phosphate buffer (0.1 mol/L, pH 6.0), 800 μL of Milli-Q water, 2 ng of E2-d_3 and 200 μL of corresponding enzymes (1000 units/mL for

β-glucuronidase, 2 units/mL for sulfatase and 20 units/mL for β-glucosidase) were added to a 10 mL glass vial. The titanium microtip coupled was immersed 1 cm in the mixture and the enzymatic hydrolysis was carried out at 10% of amplitude and 1 cycle during 20 min. Then, 300 μL of acetic acid and 2 mL of Milli-Q water were added previous to the SPE clean-up step.

The hydrolyzed bile was loaded onto a 200-mg Oasis HBL cartridge, which had been previously conditioned with 5 mL of MeOH and 5 mL of 1% (v/v) acetic acid solution in Milli-Q water. The cartridge was rinsed with 2 mL of Milli-Q water, dried under vacuum for 10 min and the target analytes were eluted in 8 mL of ethyl acetate. Finally, the extract was evaporated to dryness under a gentle stream of nitrogen.

2.6. Derivatization

The extracts were re-dissolved in 125 μL of pyridine and 25 μL of BSTFA + 1% TMCS in 2-mL amber vials, shaken in a vortex and sonicated at 80% of power and 9 cycles for 10 min in a Bandelin HD 2070 ultrasound cup booster (Berlin, Germany) [38]. The pyridine extract was evaporated to dryness under a gentle stream of nitrogen and reconstituted in 120 μL of n-hexane, which allowed 45 μL injection of the samples without bubble introduction in the PTV inlet.

2.7. LVI-PTV-GC–MS analysis

LVI of the extracts was performed in a CIS 4 PTV inlet (Gerstel GmbK & Co. KG, Mülheim an der Ruhr, Germany), which contains a septumless head and an empty baffled deactived glass liner kept cool using liquid nitrogen. A 45-μL aliquot of sample extract was injected using a 100-μL syringe in a MPS2 autosampler (Gerstel) at 60 °C, while the vent valve was opened for 5 min, at a flow rate of 50 mL/min and a vent pressure of 7.7 psi. Afterwards, the vent valve was closed for 1.5 min and the PTV temperature was increased to 300 °C at a 12 °C/s rate and held at 300 °C for 3 min. Finally, the inlet was further cleaned at a purge flow of 100 mL/min before further injections.

The derivatized analytes were introduced into a 6890 gas chromatograph (Agilent Technologies, Avondale, USA) equipped with an Agilent 5975 electron impact ionization mass spectrometer and with a HP5 MS (30 m × 0.25 mm × 0.25 μm) capillary column. The following oven temperature program was used for the separation of the analytes: 50 °C (6 min), temperature increase at 15 °C/min to 200 °C, a second increase of 1.5 °C/min up to 240 °C followed by a 20 °C/min increase up to 300 °C, where it was finally held for 2 min. Helium (99.9995%, Carburos Metálicos, Barcelona, Spain) was used as carrier gas at a constant flow rate of 1 mL/min. The transfer line temperature was maintained at 310 °C, and the ion source and quadrupole at 230 °C and 150 °C, respectively. Measurements were performed both in the scan (50–525 m/z) and in the SIM (Selected Ion Monitoring) modes. The following m/z fragment ions were monitored in the SIM mode: 342/327 (E1), 416/285 (E2), 419 (E2-d_3), 367/382 (MeEE2), 425/440 (EE2), 345/504 (E3). The first ion was used as quantifier and the second one as qualifier.

3. Results and discussion

3.1. Technical problems and improvement of blanks

In a first approach, all the derivatization experiments were carried out under sonication of the analytes in 500-μL amber Eppendorf safe lock microtubes and without evaporation of the pyridine used during derivatization with BSTFA + 1% TMCS in the ultrasound cup booster. However, large peaks overlaid the signals of EE2 and MeEE2 and overpressure and failure of the EPC (Electronic Pressure Control) unit were observed.

On the one hand, Eppendorf microtubes were substituted by amber glass vials during derivatization and, new tips for micropipettes from another supplier were obtained. As a result, all the previous undesirable chromatographic peaks were eliminated. Although an unknown peak was still observed, chromatographic separation from target analytes was possible.

On the other hand, pyridine was evaporated and the analytes were re-dissolved in 120 μL of n-hexane. No further overpressure or failure of the EPC unit was observed.

3.2. Optimization of the LVI-PTV parameters

Eight parameters of the LVI-PTV system were optimized: cryo-focusing temperature (T_{CIS}, °C), vent time (t_{vent}, min), vent flow (F_{vent}, mL/min), vent pressure (P_{vent}, psi), injection volume (V_{inj}, μL), purge flow to split vent (F_{purge}, mL/min), splitless time ($t_{splitless}$, min) and injection speed (v_{inj}, μL/s). An experimental design approach was chosen since maximum information can be obtained with minimum number of experiments and interactions among variables are also considered.

First, a Plackett–Burman design was performed to establish which variables had a significant influence in the responses. The ranges studied were: cryo-focusing temperature (40–80 °C), vent time (0.4–5 min), vent flow (50–100 mL/min), vent pressure (2–7.7 psi), injection volume (25–45 μL), purge flow to split vent (30–100 mL/min), splitless time (0.5–1.5 min) and injection speed (2–6 μL/s). A Plackett–Burman design was built (see Table 1) and the responses were defined as the ratio of the chromatographic peak area and chromatographic peak width, in order to maximize peak area and minimize peak width. The design matrix and the responses obtained for the analytes of interest are also included in Table 1.

The effect of the variables studied was defined according to the p-values obtained (p-value ≥0.05 no significant, p-value 0.01–0.05 significant, p-value 0.005–0.01 highly significant and p-value < 0.005 very highly significant) for a 95% confidence level. According to the results obtained (Table 2) all the variables studied had a significant effect on one or another analyte. However, the apparently least significant vent pressure, vent time, vent flow, purge flow to split vent and splitless time variables were fixed. Due to its negative effect, vent flow was fixed at the lowest value studied (50 mL/min), while vent pressure, purge flow to split vent, splitless time and vent time were set at the highest values studied (7.7 psi, 100 mL/min, 1.5 min and 5 min, respectively) since, when significant, they slowed a positive effect. In the case of splitless time, although significant for all the analytes, we decided not to study it further since, according to the experience of our research group [41], higher values cause contamination when repeated injections are performed. In the case of purge flow high values also prevent form inlet contamination. The rest of the variables were further evaluated by means of a CCD in order to obtain the response surfaces. Thus, the variables were studied in the following ranges: T_{CIS} (40–80 °C), V_{inj} (25–45 μL), and v_{inj} (2–6 μL/s).

The design matrix and responses (peak area/peak width ratio) are included in Table 3. The data was fitted to Eq. (1), where Y is the response, x_A, x_B and x_C correspond to the T_{CIS}, the V_{inj} and the v_{inj}, respectively, and Bi, Bij and Bii are the fitting parameters.

$$Y = B_0 + B_A x_A + B_B x_B + B_C x_C + B_{AB} x_A x_B + B_{AC} x_A x_C + B_{BC} x_B x_C + B_{AA} x_A^2 + B_{BB} x_B^2 + B_{CC} x_C^2 \quad (1)$$

B-coefficients with a p-value lower than 0.05 were considered as significant and were further used in order to build the

Table 1
Design matrix and responses, defined as the peak area/peak width ratio ($\times 10^6$), obtained for the Plackett–Burman design.

P_{vent} (psi)	T_{CIS} (°C)	t_{vent} (min)	F_{vent} (mL/min)	F_{purge} (mL/min)	V_{inj} (μL)	$t_{splitless}$ (min)	v_{inj} (μL/s)	E1	E2	E2-d_3	MeEE2	EE2	E3
7.70	80	0.4	100	100	45.0	0.5	2	0.55	1.58	1.09	12.55	3.84	1.36
2.00	80	5.0	50	100	45.0	1.5	2	1.48	3.77	2.52	15.58	5.29	1.47
7.70	40	5.0	100	30	45.0	1.5	6	2.40	6.50	4.19	24.29	8.24	2.83
2.00	80	0.4	100	100	20.0	1.5	6	0.32	0.94	0.66	8.13	2.71	1.01
2.00	40	5.0	50	100	45.0	0.5	6	1.97	4.88	3.18	18.56	7.23	2.11
2.00	40	0.4	100	30	45.0	1.5	2	0.61	2.03	1.41	15.27	4.16	1.65
7.70	40	0.4	50	100	45.0	1.5	6	0.89	4.19	2.79	27.82	6.76	2.12
7.70	80	0.4	50	30	45.0	0.5	6	0.63	2.29	1.53	15.76	5.02	1.65
7.70	80	5.0	50	30	20.0	1.5	2	0.61	1.67	1.16	7.10	2.17	0.73
2.00	80	5.0	100	30	20.0	0.5	6	0.89	2.17	1.43	7.44	3.22	0.95
7.70	40	5.0	100	100	20.0	0.5	2	0.66	1.66	1.14	5.87	2.49	0.74
2.00	40	0.4	50	30	20.0	0.5	2	0.16	0.70	0.50	5.67	1.89	0.70
4.85	60	2.7	75	65	32.5	1.0	4	0.92	1.97	1.25	10.84	3.85	1.06
4.85	60	2.7	75	65	32.5	1.0	4	1.07	2.07	1.44	12.14	4.22	1.70
4.85	60	2.7	75	65	32.5	1.0	4	1.01	2.07	1.37	11.37	4.27	1.30

Table 2
Results obtained after the linear regression analysis of the Plackett–Burman design. Significance testing method: center.

Variables	E1	E2	E2-d_3	MeEE2	EE2	E3
P_{vent}	NS	++	+	++	+	NS
T_{CIS}	−	−−−	−−	−−	−−	−
t_{vent}	++	+++	++	NS	+	NS
F_{vent}	NS	−−	−	−	−	NS
F_{purge}	NS	+	NS	+	+	NS
V_{inj}	++	+++	++	++	++	++
$t_{splitless}$	+	++	++	++	+	+
v_{inj}	++	+++	++	++	++	+

NS = not significant (p-value: ≥0.05), + = positive effect (p-value: 0.01–0.05), ++ = high positive effect (p-value: 0.005–0.01), +++ = very high positive effect (p-value: <0.005), − = negative effect (p-value: 0.01–0.05), −− = high negative effect (p-value: 0.005–0.01), −−− = very high negative effect (p-value: <0.005).

response surfaces. According to the results obtained T_{CIS} was not significant at the studied range and was fitted at 60 °C in order to minimize the N_2 consumption used during the cooling of the PTV. The influence of V_{inj} and v_{inj} can be observed for E2 in Fig. 1 (T_{CIS} fixed at 60 °C). As it can be observed, the highest injection volume and injection speed provided the best responses (peak area/peak width ratio) and were, therefore, fixed at 45 μL and 6 μL/s, respectively. The rest of target analytes showed a similar behaviour.

As a resume, while cryo-focusing temperature is maintained at 60 °C, 45 μL of the n-hexane extract are injected at 6 μL/s of speed. The solvent is vented at 50 mL/min and 7.7 psi pressure for 5 min. Afterwards, the vent valve is closed and the analytes are introduced to the column for 1.5 min. Finally, the vent valve is re-opened and the inlet is purged at 100 mL/min in order to avoid any carryover effect before the next sample injection.

3.3. Figures of merit of the developed method

Calibration curves were built in the SIM mode in 0.15–10 ng range. Correlation coefficients obtained after correction with E2-d_3 were up to 0.996 for all the analytes and the precision (n = 5) was below 9% in all the cases.

The optimized analysis method was applied to different environmental samples (estuarine water, wastewater, fish bile and fish homogenate) and some of the most important results are summarized below.

Estuarine water collected at the estuary of Bilbao (Spain) was spiked at ~10 ng/L and good recovery values (74–129%, n = 3) were obtained (see Table 4). Target analytes were not detected in the non spiked samples and precision was in the 2–8% range.

In the case of wastewater samples, in a first approach, the same method as that applied to estuarine samples was used but the

Table 3
Design matrix and responses, defined as the peak area/peak width ratio ($\times 10^5$), obtained for the central composite design.

T_{CIS} (°C)	V_{inj} (μL)	v_{inj} (μL/s)	E1	E2	E2-d_3	MeEE2	EE2	E3
40	33	4	14.35	8.49	6.00	6.45	11.54	8.25
80	33	4	15.02	8.88	6.08	7.10	13.26	9.39
60	20	4	6.64	4.13	2.95	2.45	4.97	5.03
60	45	4	17.97	11.20	8.04	9.16	16.67	12.64
60	33	2	13.67	8.76	5.71	6.15	12.15	8.65
60	33	6	17.19	10.80	7.19	7.80	15.26	11.47
48	25	3	12.65	6.36	4.46	3.73	7.14	6.20
72	25	3	11.09	6.72	4.97	5.20	9.54	7.52
48	40	3	18.20	11.25	7.71	8.77	17.76	12.02
72	40	3	16.14	10.07	7.26	7.87	15.01	10.79
48	25	5	14.26	9.28	6.29	6.72	14.07	9.68
72	25	5	13.49	8.11	5.87	5.97	11.32	9.44
48	40	5	18.81	12.48	8.39	9.88	17.43	12.40
72	40	5	19.87	12.89	8.87	9.54	18.36	12.79
60	33	4	16.10	10.29	7.07	8.23	14.61	10.88
60	33	4	16.58	10.99	7.66	7.99	15.31	11.15
60	33	4	15.64	10.13	6.89	7.52	14.37	10.71

Table 4
Recoveries and standard deviation for spiked estuarine water from Zorrotza (Nerbioi-Ibaizabal estuary, Spain) and spiked effluent and influent water from the WWTP in Bakio (Basque Country, Spain) ($n = 3$).

	Recovery (%)				
	Zorrotza (estuarine water) Oasis HLB	Bakio (effluent) Oasis HLB	Bakio (effluent) Oasis HLB + Florisil	Bakio (influent) Oasis HLB + Florisil	Standard additions Bakio (effluent) Oasis HLB
E1	129 ± 10	97 ± 3	99 ± 8	114 ± 6	77 ± 3
E2	105 ± 5	106 ± 6	83 ± 2	74 ± 2	94 ± 8
MeEE2	94 ± 2	181 ± 17	90 ± 4	85 ± 2	105 ± 7
EE2	102 ± 2	230 ± 9	99 ± 4	92 ± 2	76 ± 4
E3	98 ± 8	76 ± 5	85 ± 3	77 ± 1	100 ± 2

(–) Not analyzed.

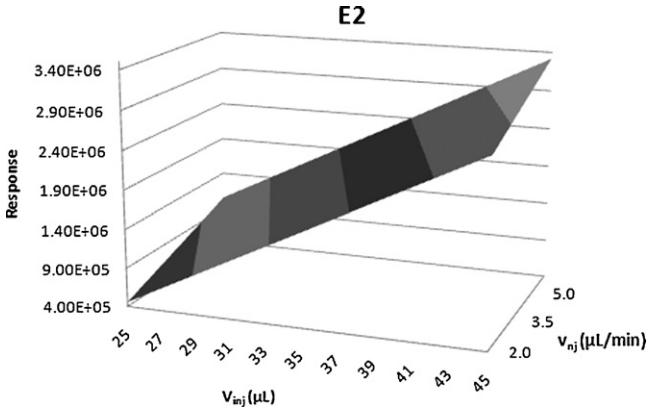

Fig. 1. Response surface obtained for E2 using only the significant ($p < 0.05$) parameters. Cryofocusing temperature 60 °C.

recoveries obtained for certain analytes (MeEE2 and EE2) exceeded by far the 100%, even after correction with the non-spiked sample (181–230%), as included in Table 4. In order to improve the previous values two alternatives were studied: the use of standard additions and a further clean-up of the ethyl acetate extract using 1-g Florisil® cartridges, as previously reported in the literature [42]. In the case of standard additions, 4 additions were performed to aliquots of previously spiked sample, before SPE preconcentration step. Additions of $x/2$ ng/L (where x was the spiked concentration) were performed.

The results obtained are summarized in Table 4. Both alternatives provided good results, although, in our opinion, the second approach is simpler since the use of standard additions requires a previous knowledge of the concentration of the analytes and at least 3–4 additions should be performed onto each sample, increasing the number of samples to be processed. Precision was in the 1–8% and 2–9% ranges for the method using a Florisil® clean-up of the extracts and for the standard addition method, respectively.

Table 5
Comparison between limits of detection (LODs) and limits of quantification (LOQs) obtained in this work for water, fish bile and fish homogenate after LVI-PTV-GC–MS and others found in the bibliography.

Water (LODs, ng/L, this work)

Compounds	Estuarine	Effluent	Influent
E1	0.07	0.06	2.57
E2	0.05	0.02	0.06
MeEE2	0.15	0.02	0.04
EE2	0.04	0.02	0.05
E3	0.17	0.05	6.07

Water (LODs, ng/L)

Compounds	LVI-PTV-GC–MS [43]	GC–MS [47]	GC–MS [38]	GC-–NCI-–MS [48]	GC–MS [27]	LC–MS [49]
E1	0.041	0.03	0.95	0.2	1.7	1
E2	0.046	0.03	0.35	0.3	3.4	2
MeEE2	–	–	1.66	–	–	–
EE2	0.031	0.07	1.00	–	0.8	2
E3	–	0.09	0.44	–	–	–

Bile (LODs and LOQs)

Compounds	LODs (ng/g) this work	LOQs (ng/g) this work	LODs (ng/g) [46]	LODs (ng/mL) [18]	LOQ (ng/g) [50]	LOQ (ng/g) [51]	LODs (ng/g) [52]
E1	0.23	0.76	–	0.7	40	20	0.95
E2	0.05	0.16	5	0.4	40	30	3.04
MeEE2	0.14	0.45	–	–	–	–	–
EE2	0.04	0.13	–	0.4	100	–	5.67

Fish homogenate (LODs)

Compounds	This work (ng)	LODs (ng) [45]
E1	0.1	–
E2	0.2	1.5
MeEE2	0.6	–
EE2	0.4	–
E3	7.5	–

Table 6
Concentrations (ng/L) and standard deviation (s) of the analytes in the influent and effluent of the WWTP of Bakio and Gernika (Biscay, Spain), (n = 3).

	TOC (mg/L)	E1	E2	MeEE2	EE2	E3
Bakio						
Influent	286	152 ± 5	56 ± 3	43 ± 2	0.8 ± 0.2	682 ± 127
Effluent	203	<LOD	<LOD	<LOD	<LOD	<LOD
Gernika						
Influent	145	121 ± 20	130 ± 16	11 ± 1	2.2 ± 0.4	101 ± 13
Effluent	78	28 ± 2	18 ± 1	3.7 ± 0.1	2.1 ± 0.6	12 ± 1

In the case of fish bile and fish homogenate, recovery values obtained are similar to those previously reported [34,46].

LODs, defined as three times the signal to noise ratio of the blanks, are included in Table 5. In the case of water samples, LODs were in the 0.02–6.07 ng/L range, similar to those obtained in the bibliography using LVI-PTV-GC–MS [43] or GC–MS after pre-column trimethylsilyl derivatization [47] and better than those obtained after splitless injection followed by GC–MS, both in the electron impact ionization mode [38] or negative chemical ionization mode [48], and better than the results obtained by LC–MS/MS analysis [49].

In the case of fish bile, LODs are two orders of magnitude better than those obtained in our research group using splitless injection onto a GC–MS [46], better than those obtained using GC–MS/MS [18] and much better than those obtained by means of GC–MS [50–52].

Finally, in the case of fish homogenate, LODs improved ten times compared to our previous results using splitless injection [45].

The optimized method was applied to influent and effluent samples from Gernika and Bakio (Basque Country, Spain) WWTPs. Results obtained are included in Table 6. Concentrations in the 0.8–682 ng/L (n = 3) range were observed for influents while in the 2.1–28.2 ng/L range for the effluents. Problems in the precision of EE2 (RSD ~25%) were observed for the determination of this analyte in the influent from the WWTP in Bakio. These values are similar to those found in a trickling filter/solid contact treatment plant in Canada (15–150 ng/L) [16], in a sewage treatment plant in Germany (not quantified 470 ng/L) [53,54] and effluents from Ulm WWTP in Germany (not quantified 13 ng/L) [55].

4. Conclusions

LVI-PTV-GC–MS has been successfully optimized for the determination of estrogens (E1, E2, MeEE2, EE2 and E3) in environmental samples after derivatization with BSTFA + 1% TMCS in an ultrasonic cup booster. Problems due to the use of plastic material during derivatization step and pyridine use during LVI-PTV injection arose, but they were solved by using glass vials during derivatization and pyridine exchange to n-hexane before LVI-PTV-GC–MS analysis.

LODs obtained with the developed method are in low ng/L level, better than those obtained with splitless inlets in GC–MS and better than those for tandem mass spectrometry coupled either to GC or LC.

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