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Simultaneous Determination of Four Estrogens in Compost Based on Ultrasonic Solvent Extraction, Solid-Phase Extraction Clean-Up and Analysis by UHPLC-MS/MS

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Abstract

A rapid, cost effective and reliable analytical method was developed and validated for the simultaneous determination of four estrogens (17 β-estradiol, 17 α-ethinylestradiol, estrone, and estriol) in compost samples from the biodegradation of biological infectious hazardous wastes. Ultrasonic solvent extraction, using methanol as extraction solvent, coupled with SPE clean-up, using cartridges HLB 60 mg - 6 ml Supelco® and acetonitrile for reconstitution of eluents, was used for the simultaneous extraction of the four estrogens. Mean recoveries in the range of 98% - 107% were obtained. All compounds were separated in a single gradient run by UHPLC Kinetex™ 2.6 µm XB-C18 100 Å LC (50 × 4.6 mm) column. Analytes were detected via multiple reaction monitoring (MRM) using an AB SCIEX API-5000TM triple quadrupole (Applied Biosystems/MDS SCIEX) with electrospray ionization in negative mode. Isocratic mobile phase of Water:ACN (50:50) resulted to be the optimum. Limits of detection and quantification were on the order of 0.66 ng·g^{-1} and 2 ng·g^{-1} for all the estrogens. These limits were lower than most of the values reported in the literature for similar matrices. Suitable level of linearity, good repeatability and reproducibility with coefficients of variation is lower than 11.7%, 6.8% and 8.3%, respectively.

Keywords

Compost, Estrogens, Solid-Phase Extraction Clean-Up, UHPLC-MS/MS, Ultrasonic Solvent Extraction

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1. Introduction

Hospitals are commonly intensive generators of biological infectious hazardous wastes (BIHW); moreover, hospital biological wastes constitute a very complex matrix, loaded with microorganisms, pharmaceutical products, toxic chemicals and hormones [1]. Placenta is considered one of the most frequent BIHW from hospitals and is mainly constituted of biodegradable organic matter [2]. During pregnancy, the placenta produces large amounts of estriol and estrone, which are also the primary estrogens produced by adipose tissue in men and postmenopausal women [3]. Hormones of anthropogenic origin have been identified as the major contributors to endocrine disrupting activity in the aquatic environment [4]. Thermal aerobic treatment has been recently used in Mexico to degrade placentas as an economic and sustainable alternative to transform biodegradable waste into nutrient-rich compost that can be used as a fertilizer or soil conditioner [2]. Absence of estrogens in compost must be ensured before its disposal into the soil to avoid potential pollution of soil and water, and human health risk. Indeed, natural estrogens (17 β-estradiol, estrone, and estriol) and the synthetic estrogen ethinylestradiol, all of the endocrine disrupting compounds (EDCs), have frequently been detected in surface waters, sediments, groundwater, and even drinking water at various levels. They are ubiquitous in the environment despite low solubility and high affinity to organic matter [5].

Analysis of hormones in complex matrices is particularly challenging because of their low concentrations, the complex nature of the samples, complicated sample clean-up procedures based on classical approaches, and the difficulty to separate these compounds from interference [6] [7]. Among the reported methods, pressurized liquid extraction (PLE) has been applied in the determination of estrogenic compounds [8] and steroid hormones [9] in soil. Other techniques such as microwave-assisted extraction [10], ultrasonic assisted extraction [11] and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) [12] have been recently used in soil analysis of hormonal compounds. The determination of these compounds in very complex matrices requires a powerful tool such as mass spectrometry (MS) coupled to gas chromatography (GC) or liquid chromatography (LC) to provide high quality results [13]-[17]. In the last few years, high performance liquid chromatography (HPLC) coupled with tandem spectrometry (MS/MS) or combination with gas chromatography–mass spectrometry (GC–MS) have become popular techniques for the determination of estrogens in solid matrices due to its specificity and versatility [4] [18]-[22]. However, most of them have just been developed and validated for a specific sample matrix and specific compounds, and still important bottlenecks such as clean-up concerns, intensive labor, high cost and time consuming have to be solved.

Thus, in this study, an analytical method based on ultrasonic solvent extraction (USE) coupled with solid-phase extraction (SPE) clean-up and analysis by UHPLC-MS/MS was developed and validated for the simultaneous extraction, detection and quantification of four estrogens (17 β-estradiol, estrone, 17 α-ethinylestradiol and estriol) in compost from biological infectious hazardous wastes. These estrogens were selected because they are present in placenta and have been found in surface waters, and endocrine disrupting effect has been reported. **Table 1** summarizes the characteristics of each estrogen. The analytical method developed and validated in this study, based on the criteria and procedures of the ICH HARMONISED TRIPARTITE GUIDELINE solves such bottlenecks due to proper sample preparation, adequate solid-phase extraction clean-up, less labor needed, acceptable accuracy, shorter analysis time and lower costs. The analytical method is reliable with better recoveries and limits of detection and quantification than most of the methods and techniques reported for the determination of estrogens in solid matrices such as soil, amended soil, sludge, digested sludge, sediments and composted biosolids. This is remarkably relevant because the analysis of estrogens in complex matrices is particularly challenging because of the low concentrations, the complex nature of the samples, complicated sample clean-up procedures, and the difficulty to separate these compounds from interference.

2. Materials and Methods

2.1. Reagents and Standards

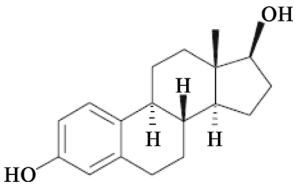
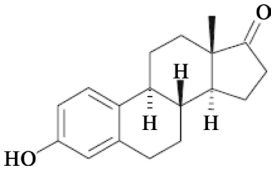
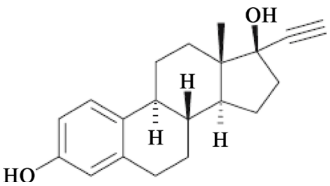
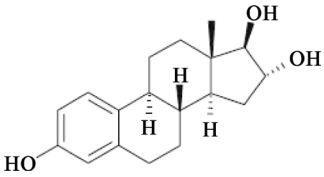
Analytical grade acetonitrile and methanol were purchased from J.T. Baker (Center Valley, PA, USA); analytical grade hexane, ethyl acetate, methyl tert-butyl ether (MTBE) and formic acid from Fisher Scientific (Monterrey, Mexico) and ammonium hydroxide from Fermont (Monterrey, Mexico). The estriol (99%), 17 α-ethinylestradiol (99%) and estrone (99%) standards were purchased from Sigma-Aldrich (Toluca, Mexico); and the 17 β-estradiol (99%) standard was from Fluka Analytical (Steinheim, Germany). Stock solutions for each estrogen standard at concentration level of 100 mg·L^{-1} were prepared in methanol and stored at 4˚C. These stock solutions were used

to prepare working standard solutions at different concentrations.

2.2. Sampling of Compost

Thermal aerobic treatment was applied in this study to transform biological infectious hazardous wastes into nutrient-rich compost that can be used as a fertilizer or soil conditioner (**Figure 1**). A pilot system was installed

Table 1. Characteristics of the estrogens used in this study.

Compound	Structure	Molecular mass ($g \cdot mol^{-1}$)	pKa	Log Koc ($1 \cdot kg^{-1}$)	Solubility ($mg \cdot L^{-1}$)
17 β-Estradiol (E2)		272.38	10.7	3.10 - 4.01	13.0
Estrone (E1)		270.37	10.3 - 10.8	2.45 - 3.34	6.0 - 13.0
17 α-Ethinylestradiol (EE2)		296.40	10.3 ± 0.2	2.91 - 3.04	4.8
Estriol (E3)		288.38	10.4	2.13 - 2.62	32

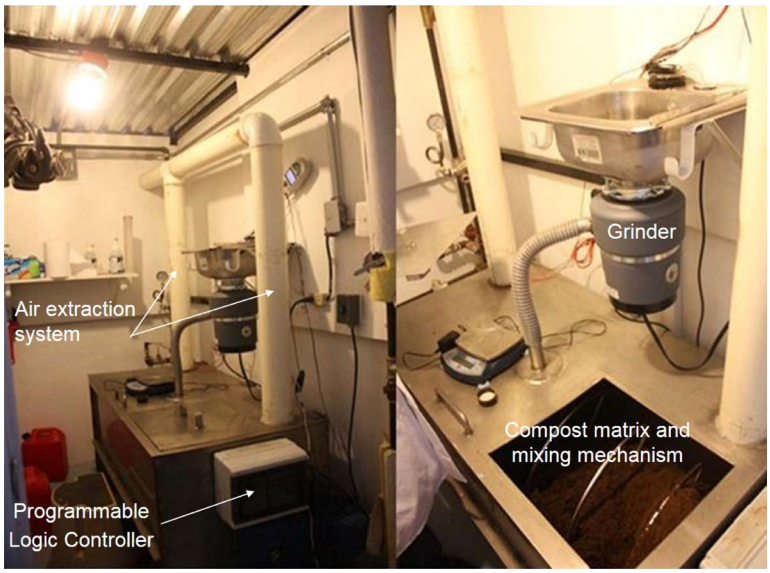


Figure 1. Pilot of a thermal aerobic treatment system installed at the San José hospital. Monterrey, Mexico.

and operated at the San José Hospital in Monterrey, Mexico. Placentas were minced with a food grinder and incorporated into the reactor of the system that uses sawdust as a matrix. The degradation process was conducted under the following conditions: matrix moisture content, 50% - 60%; mixing frequency, 6 times per day; and temperature, 50˚C - 60˚C. Compost samples were collected from the biological reactor and spiked with the target estrogens as described below.

2.3. Extraction Method

Samples of compost (0.5 g) were put into centrifuge tubes and spiked with standard mixtures of estrogens with a known concentration; then, the samples were homogenized using high speed vortex (1 min) and kept at 4˚C for 12 h to ensure pharmaceuticals sorption. Control samples of nonspiked compost were also prepared.

Volumes of 5 mL of methanol were added to the spiked compost samples; then, the mixtures were vortex for 1 min, ultrasonicated for 15 min and centrifuged at 4000 rpm and 4˚C for 5 min. The supernatants were then decanted and evaporated with a gentle stream of nitrogen until dryness at 40˚C and reconstituted with 1 mL of methanol (10%). Next, solid phase extraction (SPE) with cartridges HLB 60 mg-6 ml Supelco® was applied for clean-up. First, cartridges were conditioned with 3 mL of methanol and 5 mL of ultrapure water; then, the extracts (1 mL) were filtrated and filtrates discarded. Cartridges were dried for 1 min under vacuum conditions (<2 kPa) and then washed with 6 mL methanol (10%). Dryness was applied again to the cartridges for 1 min under vacuum conditions (>2 kPa) and elution with 6 ml of methanol (10%) was conducted. Next, eluent was concentrated with a gentle stream of nitrogen until dryness at 40˚C and reconstituted with 500 µL of acetonitrile (ACN). Then, filtration with 0.2 µm PTFE Whatman filters was conducted and the extracts were transferred to 1 mL vials for UHPLC-MS/MS analysis.

The extraction efficiency, reported as the mean recovery, was determined based on the analysis of extracts of ten replicates of compost samples spiked with the standard mixtures with known concentrations of estrogens, low (6.0 ng·g^{-1}), medium (60.0 ng·g^{-1}) and high (compost: 180 ng·g^{-1}). The coefficient of variation of the mean recovery must be lower than 15%.

2.4. Ultrahigh Pressure Liquid Chromatography and Mass Spectrometry Method

Estrogens contained in the reconstituted extracts were separated and analyzed by UHPLC-MS/MS. The Acquity Ultra High Pressure LC-system (Waters) consisting of a degasser, binary gradient pump, auto sampler (5˚C) and a column oven (40˚C) was used for separations. All the compounds were separated in a single gradient run using an UHPLC Kinetex™ 2.6 µm XB-C18100 Å LC (50 × 4.6 mm) column. The chromatographic conditions were flow rate 1 mL·min^{-1} and injection volume of 10 µL. Analytes were detected via multiple reaction monitoring (MRM) using an AB SCIEX API-5000TM triple quadrupole (Applied Biosystems/MDS SCIEX) with electrospray ionization (ESI) in negative mode. The mobile phase was isocratic and consisted of Water:ACN (50:50). The MS parameters were cad gas and curtain pressures 8 psig and 10 psig, respectively; the GS1/nebulizer and GS2/turbo GAS flow rates were set in 20 L·min^{-1} each. The ion spray voltage (IS) and the source temperature were fixed as −4500 V and 400˚C, respectively.

2.5. Method Validation

Validation was conducted based on the guidelines of the International Conference on Harmonization for Validation of Analytical Procedures [23]. These guidelines are commonly used for product purity tests in pharmaceutical industry rather than for the environment monitoring; however, some tests are similar in validation [24]. In this study, validation of the method was carried attending the following criteria: linearity, limits of detection (LOD) and quantification (LOQ), precision as intraday precision (repeatability) and inter-day precision (reproducibility).

2.5.1. Linearity

Linearity of the calibration curves for each analyte was verified by the coefficient of determination ($R2 > 0.98$) and the coefficient of variation between response factors (CV < 15%). To generate the calibration curves, standard mixtures of estrogens were prepared to yield solutions of five different concentrations (10 ng·mL^{-1}, 50 ng·mL^{-1}, 100 ng·mL^{-1}, 500 ng·mL^{-1} and 1000 ng·mL^{-1}). 0.5 g (dry based) compost samples were spiked with

100 µL of those solutions yielding concentrations for each estrogen of 2.0 ng·g^{-1}, 10.0 ng·g^{-1}, 20.0 ng·g^{-1}, 100.0 ng·g^{-1} and 200.0 ng·g^{-1}; then, the estrogens were extracted as described in the section 2.3. The extracts were analyzed by UHPLC-MS/MS as described in the section 2.4. Calibration curves for the four estrogens were prepared plotting the analytical signal (peak area) versus nominal (known) concentration.

2.5.2. Limits of Detection (LOD) and Quantification (LOQ)

The limits of detection and quantification were determined by analyzing extracts (by quintuplicate) of compost samples spiked with the standard mixtures of known concentration of estrogens. The LOD was defined as the minimum concentration that provides a signal to noise ratio greater than three for the four estrogens. The LOQ was set as the minimum quantifiable concentration whose average value is not deviated beyond ± 20% (CV < 20%) of the nominal (known) concentration for the four estrogens.

2.5.3. Precision

Precision was considered at two levels, repeatability and reproducibility. The repeatability (intraday precision) was obtained by quantifying the concentration of control samples at three different concentration levels, namely low 6.0 ng·g^{-1}, medium 60.0 ng·g^{-1} and high 180.0 ng·g^{-1} in five replicates of the spiked compost extracts during the same day. The reproducibility (inter-day precision) was determined by measuring over three different days, with two different analysts and with a daily-prepared standard curve, the concentration of the control samples at three different concentration levels, namely low 6.0 ng·g^{-1}, medium 60.0 ng·g^{-1} and high 180.0 ng·g^{-1} in five replicates of the spiked compost extracts. The intraday and inter-day precisions were evaluated in terms of the coefficient of variation that must be lower than 15%.

3. Results and Discussion

3.1. Optimization of the Extraction Method

Based on the literature reviewed [6]-[22], a great number of tests with different quantities of samples, extraction solvents (methanol, ether-ethyl acetate, hexane-ethyl acetate, hexane-MTBE, methanol-formic acid, methanol-ammonium hydroxide, ACN-formic acid, and ACN-ammonium hydroxide), extraction steps (vortex, ultrasonication, centrifugation, evaporation, and reconstitution), extraction times and the use of SPE for clean-up (Cartridges HLB Supelco® 60 mg-6 ml) were evaluated in order to develop an optimum extraction procedure. An ANOVA statistical analysis (data not shown) was applied to the results obtained from the tests. From this analysis, the ultrasonic solvent extraction coupled with SPE for clean-up, described in Section 2.3, resulted to be the optimum procedure for the simultaneous extraction of the four estrogens. **Table 2** presents the recovered concentrations and the mean recoveries of the four estrogens obtained with the ultrasonic solvent extraction method coupled with SPE for clean-up. Mean recoveries were calculated as the percentage of the extracted pharmaceutical compared to the spiked level. As observed, the extraction method exhibited a low variability response, with the CV ranging between 3.0% for estriol at 60 ng·g^{-1} to 6.2% for estrone at 60 ng·g^{-1}. As seen, the mean recoveries (98% - 107%) were better than those values reported by other researchers for similar matrices such as amended soil, sewage sludge, treated wastewater sludge, composted biosolids, sediments, manure and agricultural soils [7]-[15] [17]-[20] [25]-[27]. Proper sample preparation and adequate clean up were to the factors responsible of having high recoveries. This is in accordance with that reported in the literature [28] [29] in the sense that sample preparation and adequate clean up steps are of great importance to minimize the matrix interference during the final quantification step.

3.2. Optimization of the Ultrahigh Pressure Liquid Chromatography and Mass Spectrometry Method

Twelve mobile phase configurations, MRM conditions in positive and negative mode and estrogens' molecules fragmentation were evaluated for the finest performance of the method. At least three ions/transitions were selected for each target analyte. But, for quantification purposes, the most probable ion product (greater area) was selected as the quantification ion/transition and the other ions/transitions served as qualifier ions to confirm the detection of the suspected compound. From several experiments conducted with compost extracts, an isocratic mobile phase consisting in Water:ACN (50:50) resulted to be the optimum with electrospray ionization in negative mode. **Table 3** shows the mobile phase configurations, the ions/transitions selected for each estrogen and

Table 2. Recovered concentrations and mean recoveries obtained with the ultrasonic solvent extraction method coupled with solid-phased extraction clean-up.

Compound	Spiked Conc. ($ng \cdot g^{-1}$)	Recovered Conc. ($ng \cdot g^{-1}$)[a]	Standard Deviation ($ng \cdot g^{-1}$)	Coefficient of variation (%)	Mean Recovery (%)[a]
17 *β*-estradiol	6.0	6.4	0.36	5.59	107.33
	60.0	62.2	3.18	5.10	103.70
	180.0	182.0	7.81	4.29	101.13
Estrone	6.0	6.3	0.30	4.84	104.90
	60.0	62.1	3.82	6.15	103.57
	180.0	177.8	9.61	5.40	98.80
17 *α*-ethinylestradiol	6.0	6.4	0.26	4.03	106.17
	60.0	64.3	3.06	4.76	107.23
	180.0	193.5	9.34	4.81	107.49
Estriol	6.0	6.1	0.21	3.45	100.97
	60.0	61.2	1.85	3.03	101.93
	180.0	181.0	9.14	5.04	100.58

a. Mean of ten replications.

Table 3. Mobile phase configurations evaluated, ions/transitions selected, and multiple reaction monitoring (MRM) conditions for the analytes detection.

Mobile phase	Mobile phase
Water: ACN (50:50)	MeOH: Formic acid (50:50)
Ammonium acetate: ACN (50:50)	MeOH: Ammonium hydroxide (50:50)
ACN: MeOH (50:50)	ACN: Formic acid (50:50)
ACN	ACN: Ammonium hydroxide (50:50)

Compound	RT[a] (min)	Precursor Ion ($m \cdot z^{-1}$)	Product Ion ($m \cdot z^{-1}$)	DP[c] (V)	EP[d] (V)	CC[e] (V)	CCEP[f] (V)
17 *β*-estradiol	1.06	271.0	239.2, 145.3, 144.4, 143.1[b]	−150	−14	−70	−10
Estrone	1.41	269.2	158.9, 145.1[b], 142.5	−150	−7	−40	−15
17 *α*-ethinylestradiol	1.27	295.1	213.2, 198.9, 170.6, 159.0[b], 144.7, 142.4	−160	−7.7	−60	−20
Estriol	0.56	287.1	255.0, 210.8, 171.1[b], 158.8, 144.7	−150	−9	−60	−15

a. RT: Retention time; b. Ion transition selected for quantification purposes; c. DP: Declustering Potential; d. EP: Entrance Potential; e. CC: Collision Cell; f. CCEP: Collision Cell Exit Potential.

the optimized ESI and MS/MS parameters.

Figure 2 shows a chromatogram of a compost extract with the final retention times of the four estrogens for the MRM conditions shown in **Table 3**. As seen, the total analysis time was much shorter than in conventional HPLC techniques. Furthermore, the separation of the four estrogens was successfully achieved in only 1.5 min due to the better resolution and more narrow peaks in UHPLC. Shorter retention times will contribute in reducing the analysis time, elongation of the column life, reduction of elution times, elution good enough for all the target compounds and finally, reduction of the cost for routine analyses.

3.3. Validation of the Method

As mentioned in Section 2.5, validation was conducted based on the guidelines of the International Conference on Harmonization for Validation of Analytical Procedures [23]. These guidelines are commonly used for product purity tests in pharmaceutical industry rather than for the environment monitoring; however, some tests are similar in validation [24].

3.3.1. Linearity

The analytical procedure proposed in this study resulted with a suitable level of linearity. The coefficient of

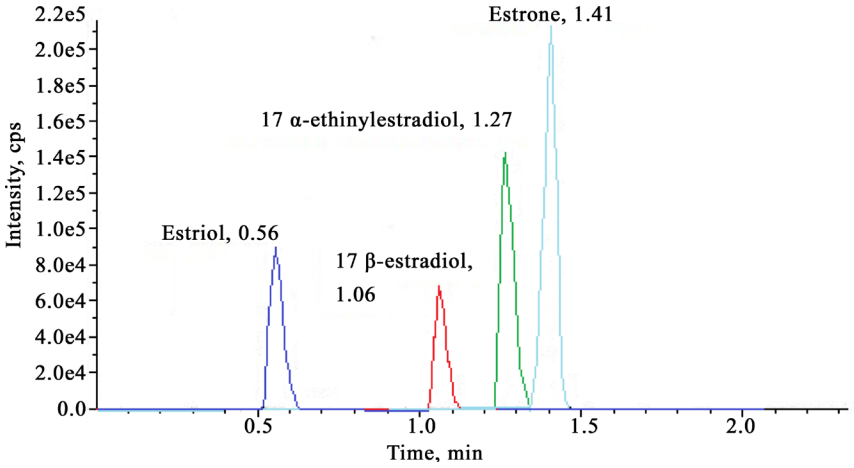


Figure 2. Chromatogram of a compost extract with the final retention times of the four estrogens for the MRM conditions shown in **Table 3**.

Table 4. Regression equations, coefficients of determination, recovered concentrations and mean recoveries of the five concentrations used to generate the calibration curves.

Compound	Regression equation	R^2	SC[a] ($ng \cdot g^{-1}$)	RC[b] ($ng \cdot g^{-1}$)	MR[c] (%)	CV (%)
17 *β*-estradiol	Y = 0.9774x + 1.5228	0.9977	2.0	1.9	95.0	7.08
			10.0	9.7	97.0	1.72
			20.0	20.6	103.0	4.29
			100.0	106.1	106.1	6.65
			200.0	193.7	96.85	3.81
Estrone	Y = 0.9936x + 0.4184	0.9998	2.0	2.0	100.0	9.37
			10.0	10.3	103.0	6.08
			20.0	19.8	99.0	6.34
			100.0	101.8	101.8	6.09
			200.0	198.2	99.1	3.31
17 *α*-ethinylestradiol	Y = 0.9831x + 1.0892	0.9987	2.0	2.0	100.0	11.65
			10.0	10.0	100.0	9.37
			20.0	20.0	100.0	7.65
			100.0	104.7	104.7	5.80
			200.0	195.2	97.6	2.95
Estriol	Y = 1.0056x − 0.3771	0.9998	2.0	2.1	105.0	9.58
			10.0	9.6	96.0	4.26
			20.0	20.5	102.5	8.39
			100.0	98.2	98.2	2.95
			200.0	201.7	100.9	2.10

a. Spiked concentration. b. Recovered concentration. Mean of five replicates. c. Mean recovery. Mean of five replicates.

determination for the four estrogens was greater than 0.98, in the range of 2 $ng \cdot g^{-1}$ to 200 $ng \cdot g^{-1}$, fulfilling the validation criteria [23]. **Table 4** presents the coefficient of determination and the regression equation for each estrogen. The regression equations were obtained by least-squares analysis. **Table 4** also shows the recovered concentrations and the mean recoveries of the five different concentrations (five replicates each) used to generate the calibration curves for the four target estrogens. The coefficient of variation in all cases was found to be lower than 11.7%; thus, the analytical procedure had a suitable level of linearity in terms of this parameter due

to the CV was lower than 15%. The four estrogens were detected and quantified even at the lowest concentration tested (2 ng·g^{-1}). Therefore, the method proposed in this study is reliable to analyze the target compounds in the compost samples.

3.3.2. Limits of Detection (LOD) and Quantification (LOQ)

The limits of detection and quantitation determined in this study for the four estrogens are shown in **Table 5**. As seen, both limits are commensurable due to the coefficient of variation is lower than 11%, fulfilling the acceptance criteria. A concentration of 0.66 ng·g^{-1} was set as a target LOD in order to go beyond the values reported in the literature. The value was set as the limit of detection for the simultaneous analysis of the four estrogens. This value (minimum concentration) gave a signal to noise ratio much greater than three for the four estrogens; therefore, the limit of detection might be lower than the set in this study for all the estrogens. The LOD defined in this research is lower than most of those values reported by the literature (0.002 ng·g^{-1} - 175 ng·g^{-1}) for GC-MS/MS and HPLC-MS/MS techniques for estrogens in matrices such as broiler manure, compost manure, amended soil, soil, sediments, composted biosolids, activated sludge and digested sludge [4] [7] [8] [10]-[12] [17]-[21] [26]. Thus, the analytical method proposed in this study is suitable for the determination of the four estrogens in the compost at low concentration level.

On the other hand, the LOQ was in the order of those values reported in the literature (0.02 ng·g^{-1} - 375 ng·g^{-1}) for GC-MS/MS and HPLC-MS/MS techniques for estrogens in matrices such as soil, amended soil, sludge, digested sludge, sediments and composted biosolids [8] [12] [17]-[19] [25] [26]. Therefore, at environmentally relevant concentrations, the limit of quantification determined in this research indicates that the analytical method developed is reliable for the analyses of complex matrices such as compost.

3.3.3. Precision

The quality of data obtained from the analyses of estrogens in compost is the major issue of concern. Therefore, good precision, as repeatability and reproducibility, is required to demonstrate the performance efficacy of the method. **Table 6** summarizes the results of the repeatability determination for the target estrogens. The repeatability (intraday precision) was obtained by quantifying the concentration of control samples at three different concentration levels, namely low 6.0 ng·g^{-1}, medium 60.0 ng·g^{-1} and high 180.0 ng·g^{-1} in five replicates of the spiked compost extracts during the same day. As seen, the coefficient of variation in all cases was below 6.8% and the mean recoveries in the range of 98% and 109%, denoting an acceptable repeatability of the method.

Table 7 presents the results of the reproducibility of the method for all the target estrogens. As explained in Section 2.5.3, the reproducibility was determined by measuring over three different days, with two different analysts and with a daily-prepared standard curve, the concentration of the control samples at three different concentration levels, namely low 6.0 ng·g^{-1}, medium 60.0 ng·g^{-1} and high 180.0 ng·g^{-1} in five replicates of the spiked compost extracts. The coefficient of variation in all analyses was below 8.3% and the mean recoveries between 99% and 111%, denoting a satisfactory reproducibility. The results of reproducibility were as good as those of repeatability for the four target estrogens at the three concentration levels.

Evaluation of the matrix effect was out of the scope of this study. However, proper sample preparation and adequate clean up procedure were essential to minimize the matrix interference during the final quantification step. Because of the better resolution and more narrow peaks in the UHPLC, analytes will co-elute less with interferences during ionization, therefore, the matrix effects could be lower, or even eliminated [30]. When the effect of this parameter is suspected or the confirmation of the parameter effect is required, validation of such

Table 5. Limits of detection and quantification of the four estrogens used in this study.

Compound	17 β-estradiol	Estrone	17 α-ethinylestradiol	Estriol
LOD[a] (ng·g^{-1})	0.66	0.66	0.66	0.66
(signal-to-noise ratio)[b]	14.4	21.8	30.0	25.8
CV (%)	10.53	9.95	10.00	6.37
LOQ[c] (ng·g^{-1})	2.22	2.11	2.10	2.39
Std. Dev.	0.06	0.07	0.05	0.06
CV (%)	2.73	3.19	2.64	2.41

a. Spiked concentration: 0.66 ng·g^{-1}. b. Mean of five replicates. c. Mean of five replicates. Spiked concentration: 2.0 ng·g^{-1}.

Table 6. Repeatability of the analytical method for the target estrogens.

Spiked Conc. ($ng \cdot g^{-1}$)		17 β-estradiol	Estrone	17 α-ethinylestradiol	Estriol
6.0	RC ($ng \cdot g^{-1}$)[a]	6.40	6.11	6.11	6.02
	MR (%)	106.7	101.8	101.8	100.3
	Std Dev	0.36	0.34	0.19	0.24
	CV (%)	5.57	5.57	3.05	4.01
	AD (%)[b]	6.73	1.87	1.80	0.4
60.0	RC ($ng \cdot g^{-1}$)[a]	62.16	62.28	64.00	60.88
	MR (%)	103.6	103.8	106.7	101.5
	Std Dev	2.68	2.53	3.47	1.65
	CV (%)	4.31	4.06	5.42	2.72
	AD (%)	3.60	3.80	6.67	1.47
180.0	RC ($ng \cdot g^{-1}$)[a]	182.80	176.48	195.16	179.04
	MR (%)	101.6	98.0	108.4	99.5
	Std Dev	7.35	8.12	13.22	8.14
	CV (%)	4.02	4.60	6.77	4.55
	AD (%)	1.56	1.96	8.42	0.53

a. RC = Recovered concentration. Mean of five replicates. b. AD = Absolute deviation

Table 7. Reproducibility of the analytical method for the target estrogens.

Spiked Conc. ($ng \cdot g^{-1}$)		17 β-estradiol	Estrone	17 α-ethinylestradiol	Estriol
6	RC ($ng \cdot g^{-1}$)[a]	6.48	6.48	6.63	6.09
	MR (%)	108.0	108.0	110.5	101.5
	Std Dev	0.36	0.27	0.33	0.18
	CV (%)	5.60	4.12	5.00	2.90
	AD (%)[b]	4.49	3.16	2.11	1.53
60	RC ($ng \cdot g^{-1}$)[a]	62.28	62.00	64.68	61.44
	MR (%)	103.8	103.3	107.8	102.4
	Std Dev	3.67	5.11	2.65	2.06
	CV (%)	5.89	8.24	4.09	3.35
	AD (%)	4.26	5.42	7.80	2.40
180	RC ($ng \cdot g^{-1}$)[a]	181.28	179.2	191.80	183.04
	MR (%)	100.7	99.6	106.6	101.7
	Std Dev	8.26	11.10	5.47	10.13
	CV (%)	4.56	6.19	2.85	5.54
	AD (%)	3.75	4.29	6.56	1.69

a. RC = Recovered concentration. Mean of five replicates. b. AD = Absolute deviation.

parameter must be conducted. Undoubtedly, evaluation of the matrix effect will contribute in having more reliability of the method and it is recommended to be conducted for matrices different to the used in this study.

It is important to remark that conventional analytical methods for the determination of estrogens in complex solid matrices commonly use internal standards or surrogates to get accuracy; nevertheless, they are time consuming and increase the cost of the analysis. Furthermore, most of them have just been developed and validated for a specific sample matrix and specific compounds, and still important bottlenecks such as clean-up concerns, intensive labor, high cost and time consuming have to be solved.

The analytical method developed and validated in this study, based on the criteria and procedures of the ICH HARMONISED TRIPARTITE GUIDELINE solves such bottlenecks due to proper sample preparation, adequate SPE clean-up, less labor needed, acceptable accuracy, shorter analysis time and lower costs. The analytical method is reliable with better recoveries and limits of detection and quantification than most of the methods and techniques reported for the determination of estrogens in solid matrices. This is remarkably relevant because the analysis of hormones in complex matrices is particularly challenging because of the low concentrations, the complex nature of the samples, complicated sample clean-up procedures, and the difficulty to separate these compounds from interference. The analytical method proposed in this study could be applied for estrogens with similar structure and negative ionization. This is an important issue to take into consideration for the future application

of the protocol developed in this study.

4. Conclusion

An analytical method based on ultrasonic solvent extraction coupled with solid-phase extraction clean-up and analysis by UHPLC-MS/MS was developed and validated for the simultaneous extraction, detection and quantification of four estrogens in compost from biological infectious hazardous wastes. The method fulfilled the validation criteria of the International Conference on Harmonization for Validation of Analytical Procedures. Suitable level of linearity, acceptable limits of detection and quantification, good repeatability and reproducibility were obtained. Proper sample preparation, ultrasonic extraction with methanol as extraction solvent and adequate clean-up by using cartridges HLB 60 mg-6 ml Supelco® and acetonitrile for reconstitution of eluents were essential to have high recoveries of the four estrogens. Suitable separation of compounds was achieved in only 1.5 min by using an isocratic mobile phase of water and ACN (50:50) with electrospray ionization in negative mode. The limit of detection of the method was lower than most of those values reported by the literature for conventional GC-MS/MS and HPLC-MS/MS techniques and similar matrices. The LOQ was on the range of those values reported in the literature for similar matrices such as soil, amended soil, sludge, digested sludge, sediments and composted biosolids. Thus, the analytical method is recommended to determine estrogens with negative ionization and similar structure to those used in this study. Its application will contribute in having suitable compounds recovery, acceptable levels of detection and quantification, shorter analysis time and lower costs when working with complex matrices such as compost.

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