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Research Article

Development and Validation of a Gas Chromatography-Mass Spectrometry Test Method for Screening and Quantitation of Steroid Estrogens (Endocrine Disruptor Compounds) in Water and Wastewater Using Large Volume Injection

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Keywords Steroid estrogen; Endocrine disrupting compound; Wastewater; Liquid-liquid extraction; Large volume injection; Gas chromatography-mass spectrometry; Selected ion monitoring

Abstract

An improved, Liquid-liquid Extraction (LLE) procedure, without derivatisation, using large volume injection, followed by separation with Gas Chromatography (GC), and mass spectrometric detection, in Selected Ion Monitoring (SIM), has been fully validated and applied in the quantitation of three priority female steroid estrogens (natural estrogens: 17-β-estradiol (E2), estrone (E1) and the synthetic estrogen:17-α-ethinylestradiol (EE2)), in water and in raw influent wastewater matrix. The method has been validated, over the range 10-100 µg/L, showing, for all target analytes, good linearity (mean r2 = 0.997), recovery (mean = ± 99%), and precision (mean RSD = ± 5.5%) in both water and wastewater matrix. The Method Detection Level (MDL) was: 5 ng/L for E1, 2 ng/L for E2 and 5 ng/L for EE2. The LOQ was 10 ng/L for E1, E2 and EE2. The signal/ Noise (S/ N) ratio method gave an LOD and LOQ of 1 ng/L (S/ N = 17-61) for all three estrogens. The method was successfully applied to the determination of the target estrogens in raw wastewater, treated wastewater and river water. The estrogen levels, in all tested matrices, obtained by the GC-MS method compared fairly well with the previously used Enzyme-Linked Immunosorbent Assay (ELISA). The method showed to be a viable option to ELISA and Liquid Chromatography- tandem Mass Spectrometry (LC-MS/MS).

Introduction

The exposure to natural and synthetic chemicals, which can interfere with the reproductive system and its development, is a controversial topic in environmental science due to the potential risks to wildlife and humans. These chemicals, shown to having a significant effect on reproductive systems in wildlife [1] and humans, are termed Endocrine Disrupting Compounds (EDCs) [2-4]. The endogenous estrogens: estrone (E1), 17-*β*-estradiol (E2) (Figure 1), estriol (E3), and progestogens, are natural compounds in humans and animals. The exogenous estrogens (exoestrogens) include: phytoestrogens, synthetic estrogens (e.g., 17-*α*-ethinylestradiol (EE2)) (Figure 1). Female steroids are widely used as contraceptives and also as medicaments, and in hormone-replacement therapy [5]. There is considerable increase in the consumption of estrogens in human medicine, primarily contraception: EE2 is one of two most common components contained in combined oral contraception [5].

One major problem of steroid therapy is the pollution of the global environment; the presence of estrogenic compounds in aquatic systems is well described [6]. As the steroid estrogens are not usually entirely metabolized, as human waste-borne, they enter the aquatic environment mainly by effluents from wastewater (sewage) treatment plants (STPs) [2]. The natural steroid estrogen hormone E2, its metabolites (E1 and E3) and conjugates (glucuronides and sulfates) are mainly excreted in the urine of mammals [7]. The synthetic steroid hormone EE2 is also excreted in urine of women on such medication [7]. The EE2 conjugates can be degraded in STPs, releasing the active parent compound [8].

Steroid hormones, like many EDCs, have potent activity at ng/L, and lower (pg/L), concentration in target tissue. Thus, effective analysis in relevant liquid and environmental samples is necessary. For the trace level determination of steroids with similar structures, being present in complex matrices, sensitive, accurate and precise analytical methods are therefore required. To this end, various analytical methodology, like ELISA [9], hyphenated GC [10] and LC [11], MS, tandem

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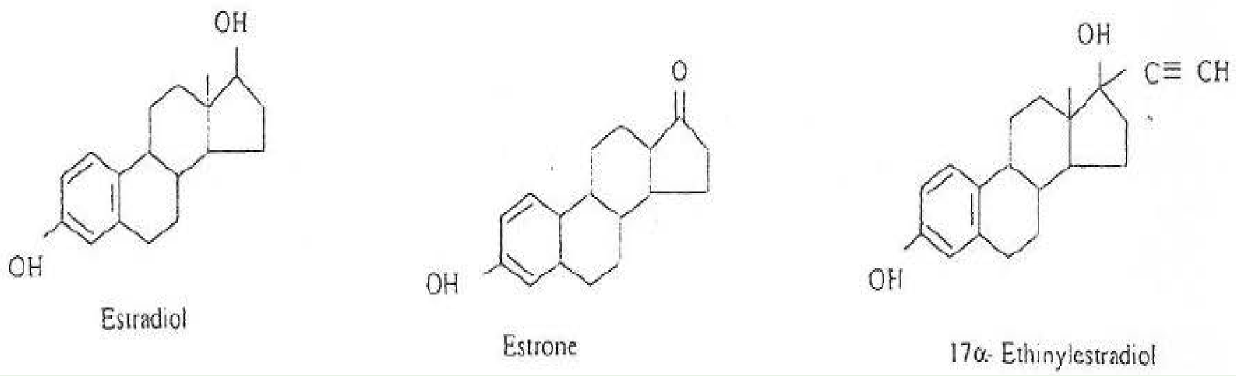

Figure 1: Chemical structure of the steroid estrogens, showing E2, E1, EE2.

mass spectrometry (MS/MS) [12], electrophoresis [13] have been developed, and related reviews [14] have been published.

For analysis of estrogens at the picogram per liter level, without derivatisation, (as in our present study), the sensitivity achieved by the use of a GC-MS instrument can be a challenge. More sensitive results can be obtained with GC-MS/MS but a derivatisation step is still needed. Although the use of MS-based chromatographic (GC/LC) detection methods have the main distinct advantage of simultaneous multi-analyte screening and specificity, they have several potential drawbacks, including expensive instrumentation, large sample volume, extensive purification, utilization of large amount of solvents, and the need for technical expertise in operation. The analysis of a large number of samples may be both cost and time-prohibitive.

The Darvill Waste Water Works, in Pietermaritzburg, of Umgeni Water, is the main sewage treatment plant for the Pietermaritzburg area, serving over 300 000 people. Treated sewage is then discharged directly into the adjacent Umsunduzi (*Duzi*) River. This river joins the Umgeni River, which is the main water supply to the Inanda dam. The latter supplies raw water to Durban Heights and Wiggins potable water treatment works, managed by Umgeni Water, to supply potable water to the greater Durban area. Currently the dam supplies about 300 m^3/d. As the Inanda impoundment (~ 240 x 10^6 m^3) has a retention time in the order of 2 years, estrogenic contaminants are not currently a concern. However, direct recycling options are being considered at Umgeni Water; a recent pilot project investigated the use of Membrane Bioreactors (MBR), followed by advanced treatment, to treat waste water to a standard where it is drinkable. The necessity of monitoring of the water quality was therefore a critical requirement to assess performance of the MBR; testing for some selected steroid estrogens was done largely by the immuno-analytical ELISA method, by outsourcing samples to the University of the Western Cape (UWC) [15,16]. This nationally available routine service, however, has cost implications, sample transport and stability implications.

Hu et al., [17] reported an optimized SPE-based method, using the GC-MS method, for quantitation of the steroid estrogens E1, E2 and EE2 in water matrix only; MDLs of 0.031-0.046 ng/L were obtained for E1, E2, and EE2 in environmental waters [17]. Due to the availability of a number of GC-MSD units in our state-of-the-art ISO/IEC 17025-accredited routine, water testing laboratory at Umgeni Water (Head Office, Pietermaritzburg), and the latter report [17,18], we considered setting up a simple GC-MS based in-house chemical-analytical test method as an option to the immuno-analytical ELISA method for steroid estrogen analysis in water, and in raw influent wastewater matrix. The aim of this study was thus to evaluate, develop and validate a GC-MS analytical method for analysis of E1, E2 and EE2 in water and in raw influent wastewater matrix. We now report on an improved procedure and the full, preliminary method validation data and method performance.

Materials and Methodology

Materials

Estrone (E1) (b.p. 445.2°C/760 Pa), 17-*β*-estradiol (E2) (b.p. 445.92°C/760 Pa) and 17-*α*-Ethinylestradiol (EE2) (b.p. 457.2°C/760 Pa) (≥ 98% purity), sodium sulfate (anhydrous), Dichloromethane (DCM), Dimethyl Sulfoxide (DMSO) (pesticide grade), and ethanol (HPLC grade) were purchased from Capital Lab Supplies (South Africa), local agents for Sigma-Aldrich. Ultrapure water (mean conductivity 0.055 μs/cm and mean TOC 1.67 μg/L) was obtained from a Siemens unit. Nitrogen gas was obtained from Airflex (Pietermaritzburg, South Africa). The low pressure drop GC, single taper split inlet liner, with glass wool, deactivated (4,0 mm ID, 6.3 mm OD x 78,5 mm length) (Agilent # 5183-4711) was obtained from Chemetrix (Midrand), agents for Agilent. The Supelco # 2-0510-05 GC inlet liner was obtained from Capital Lab Supplies (South Africa). A 20 mg/L composite primary stock standard of the three steroid estrogens were prepared by weighing 5 mg each of estrone (E1), 17-*β*-estradiol (E2) and 17-*α*-ethinylestradiol (EE2) into a 250 ml volumetric flask, and dilution to the mark with DMSO. A composite, intermediate secondary stock solution of 200 μg/L in DCM was prepared. Five composite, working standard solutions of 10 μg/L, 15 μg/L, 20 μg/L, 50 μg/L and 100 μg/L were prepared from the intermediate secondary stock solution in DCM (50 mL). Storage and stability information has been previously reported [9,15,16]. As per the recommended procedure [9,15,16], wastewater influent (site WDV 1) and effluent (site WDV 20) grab samples were collected during August 2014, from DWWW and a river sample, taken from the Duzi River, upstream of the DWWW effluent discharge point/zone was also obtained, as a "control" sample. A "blank" sample was not taken. The total number of sampling runs was 2. Samples were immediately transported, on ice packs, to the testing laboratory; where this was not possible, collected samples were stored overnight at 4°C-8°C [9,15,16].

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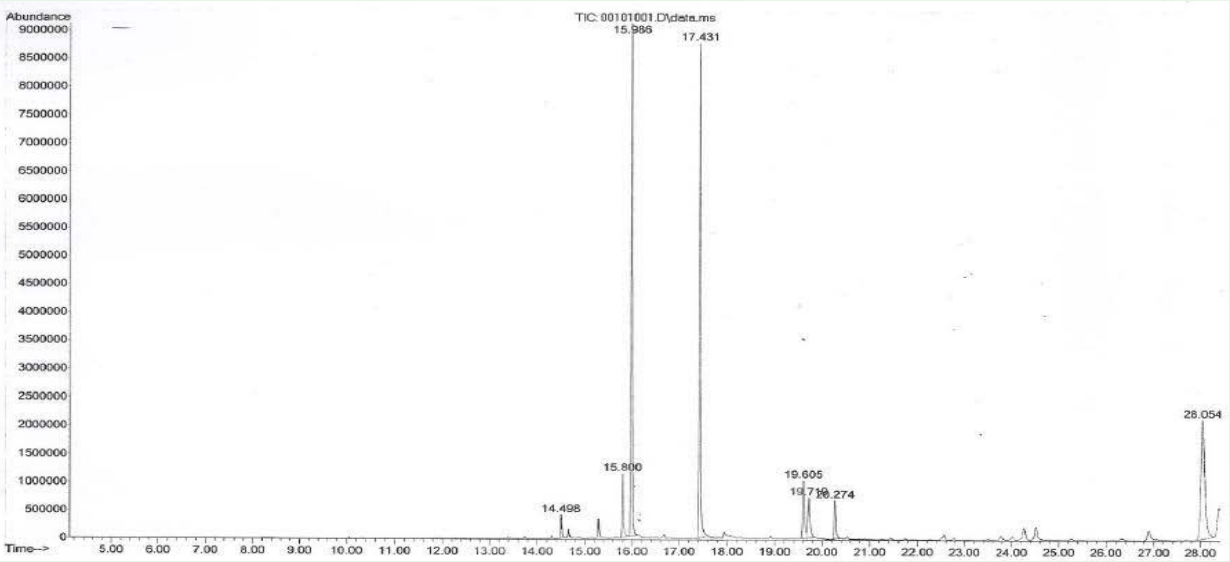

Figure 2: SIM chromatogram of a DCM 10 μg/L standard (10 μg/L) of steroid estrogens: E1 at 19.605 min, E2 at 19.719 min, EE2 at 20.274 min.

Methodology

A 1 L water sample was extracted with DCM (60 mL x 3) in a 2 L separating funnel. The combined DCM extracts were dried over anhydrous sodium sulphate and evaporated to 2 mL, under reduced pressure, using a BUCHI R210 rotatory evaporator. After being transferred into a calibrated GC auto sampler vial, the 2 mL extract was concentrated to a final volume of 1 mL by gentle blowing with a fine stream of a regulated supply of nitrogen gas, from a cylinder, via a syringe needle, at room temperature. The final reduced volume of 1 mL DCM extract was then analyzed by GC-MS. Analysis were carried out with an Agilent 7890A gas chromatograph equipped with a PTV inlet, for solvent and Multi-Mode Injection (MMI), in the fast injection mode (0 s injection interval), Agilent 7693A auto-sampler, coupled to an Agilent Technologies 5975C inert, Triple- Axis MSD. The GC column was an Agilent Technologies HP-5MS (30 m x 0.25 μm x 0.250 mm); helium gas flow was at 1mL/min. The temperature of the GC-MS interface line was maintained at 230°C; quad temperature was 150°C. The inlet mode was set to "PTV solvent vent"; pressure was 14 psi, with total flow 54.738 mL/min and 3 mL/min septum purge flow. The purge flow to the split vent was 50mL/min at 6.5 min. The inlet temperature program was: 40°C/4.2 min, then 300°C/min to 350°C, for 5 min. Using a 10 μL syringe, the "Multiple" injection type was used with 10 Injection Repititions of 5 μL volume. The GC oven temperature program was: 40°C initial, for 6.1 min, then 30 °C/ min to 230 °C, for 1 min, and 10 °C/ min to 290°C, for 9 min. The Electron Impact (EI) ionization mode (emission current = 34.6 μA), with EI source temperature of 230°C was used, with 70 eV electron energy, tuned with Perfluoro-Tri-Butlyamine (PFTBA). Full scan mass spectra were obtained over the scan range 40-500 amu. SIM mode was used for quantitation with 4 minute solvent delay. Monitored fragment ions used were m/z: 270 (100%), 185, 146 for E1, 272 (100%), 213, 160 for E2 and 213 (100%), 160, 296 for EE2; a dwell time of 100 ms was used for all ions. The base peak was used for calibration and quantitation. The retention time windows were: 19.500-19.650 min for E1, 19.690-19.800 min. for E2, and 20.200-20.400 min. for EE2. Qualitative identification of the target estrogens E1, E2, and EE2 was established by analysis of liquid standards in DCM, made from the commercial material, in full scan MS mode, based on retention time analysis, and by comparison of the observed mass spectra, with that of the NIST Library, on the GC-MSD instrument Software, and the NIST mass spectra. In full scan mode, *m/z* ion abundances obtained by us, on the Agilent MSD, were: 270 (100%), 185 (82%), 146 (86%) for E1, 272 (100%), 213 (66%), 160 (61%) for E2, and 213 (100%), 160 (100%), 296 (98%), for EE2, respectively. Mass spectral verification on real water samples was done by comparison of relative abundance values of the quantification and qualification ions to the same values obtained from the standard samples. The initially reported [19] GC-MS parameters were utilized as a starting point. Preliminary work involved the effect of GC inlet liner. The "straight through" purge-trap liner (Supelco: # 2-0510-05), and the split liner (Agilent # 5183-4711), not previously investigated, were investigated for optimum sensitivity, precision and accuracy. The oven temperature programme (first ramp) was changed from 20°C/min to 30°C/min. in order to improve resolution of E1 and E2. The steroid estrogen levels in all samples were determined from the calibration graph. The calibration standard solutions data were fitted to a straight line of the form: $y = mx + c$ (y = signal response; m = gradient; x = unknown concentration; c = y-intercept).

Recoveries were calculated using the observed values on the "blank" samples, the spiked samples and the expected (theoretical concentration): $\% R = (C_s - C_{us}/C_t)\ 100\%$, where C_s = estrogen level on spiked sample, C_{us} = estrogen level on "blank" sample, C_t = theoretical estrogen (spike) level.

Results

Figure 2 and 3 shows the TIC chromatogram, and the extracted ion chromatogram, of a 10 μg/L DCM standard. The average retention times were: (± SD) (%RSD): 19.590 (± 0.109) min. (0.55) for E1, 19.787 (± 0.106) min (0.53%) for E2, and 20.307 (± 0.107) min. (0.52%) for EE2. Figure S1 shows the SIM chromatogram of an extracted ultrapure water sample, spiked at 1 ng/L.

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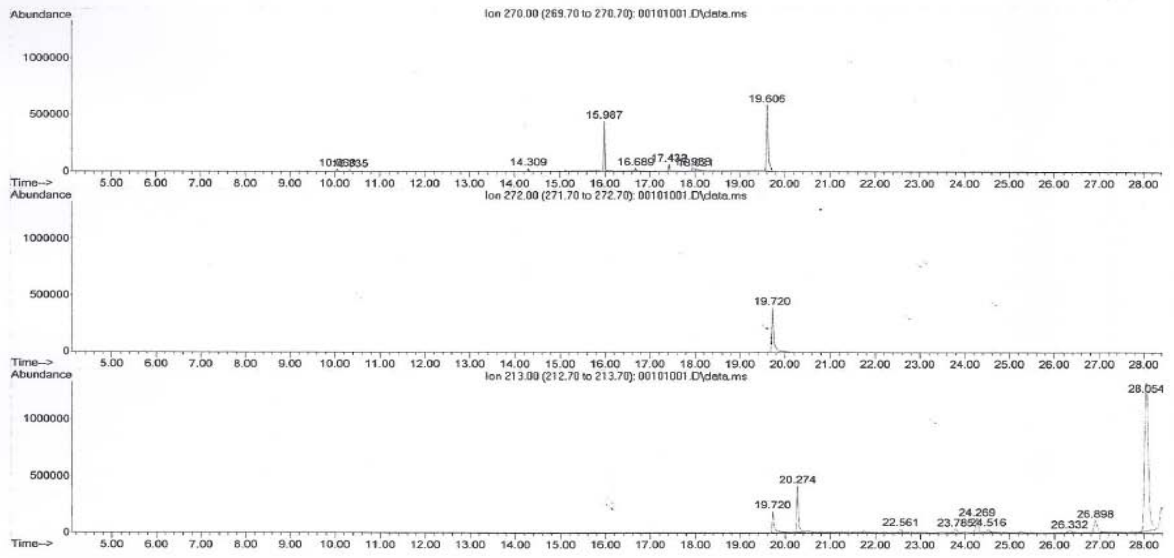

Figure 3: SIM Extracted ion chromatogram of a DCM standard (10 μg/L) of steroid estrogens: E1 at 19.606 min, E2 at 19.720 min, EE2 at 20.274 min.

Evaluation of ultrapure water indicated average values of well below the LOQ and LOD for all three estrogens. Regression analysis showed good linearity. The correlation coefficient r^2, determined over 13 days, averaged 0.996 (range = 0.994-0.997) for E1, 0.996 (range = 0.9959-0.9963) for E2, and 0.998 (range = 0.996-0.999) for EE2, indicating acceptable linearity. The linear regression equations were, on average: $y = 582414x - 1438852$, for E1, $y = 125710x - 193517$ for E2, and $y = 1034883x - 2526525$ for EE2. Due to the 1/1000 concentration effect during the liquid-liquid extraction procedure, the estrogen levels on all matrix samples were obtained directly from the calibration graph, in ng/L units. For spiked ultrapure water, wastewater influent and effluent, at 10, 50 and 100 ng/L, the observed concentrations were plotted as a function of average area response (n = 3). The mean (± SD) (% RSD) regression parameters was: $r^2 = 1.000$ (range = 0.999-1.000) , m = 411 806 (± 19 327) (4.69%), c = 4 882 108 (± 5 722 891) (117.22%) for E1; r^2 = 0.949 (range = 0.850-1.000), m = 107 207 (± 33 192) (30.96%), c = 2 719 356 (± 2 340 937) (86.08%) for E2; r^2 = 1.000 (range= 0.999-1.000), m = 1 533 767 (± 77 110) (5.03%), c = 2 881 454 (± 2 650 580) (91.99%) for EE2. Thus, good correlation was observed for all three analytes in all three matrices. The average m (gradient) values decrease in the order: EE2 > E1 > E2, indicating maximum sensitivity (area response) for EE2, followed by E1 and E2, in all three matrices. The % RSD for the m values, for the three matrices, is approximately 5% for both E1 and EE2, indicating fairly constant sensitivity. Instrument precision (repeatability) was determined by assay of 10 replicates of composite standards, in DCM, at 25 and 100 μg/L. Using peak areas, RSD % was 4.56 and 2.98% (mean = 3.77) for E1, 5.47 and 1.52% (mean = 3.50) for E2, 5.11 and

Table 1: Observed estrogen levels, recovery, precision and bias of analytes in ultrapure water matrix.

Matrix	Estrogen	Concentration added (ng/L)	Concentration found Mean ± SD (ng/L)#	RSD (%)	Recovery (Accuracy) Mean ± SD (%)	RSD (%)	Bias (%)
Ultrapure water	E1		< 10				
(unspiked)	E2		< 10				
	EE2		< 10				
Ultrapure water	E1	10	10.08 ± 0.17	1.67	100.83 ± 1.69	1.67	+ 0.83
(spiked)		50	43.26 ± 4.32	9.98	86.51 ± 8.63	9.98	- 13.49
		100	92.77 ± 2.72	2.93	92.77 ± 2.72	2.93	- 7.23
	Mean				93.37 ± 7.18	7.69	-6.63
	E2	10	10.08 ± 0.19	1.93	100.80 ± 1.95	1.93	+0.80
		50	46.04 ±1.59	3.46	92.09 ± 3.19	3.46	-7.91
		100	92.18 ± 8.90	9.65	92.18 ± 8.90	9.65	-7.82
	Mean				95.02 ± 5.00	5.26	-4.98
	EE2	10	9.98 ± 0.03	3.32	98.63 ± 2.29	2.33	-1.27
		50	45.19 ± 1.93	4.28	90.37 ± 3.86	4.28	-9.63
		100	90.94 ± 9.10	10.00	90.94 ± 9.10	10.00	-9.06
	Mean				93.31 ± 4.61	4.94	-6.65

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Table 2: Observed estrogen levels, recovery, precision and bias of analytes in wastewater matrices.

Matrix	Estrogen	Concentration added (ng/L)	Concentration found Mean ± SD (ng/L)#	RSD (%)	Recovery (Accuracy) Mean ± SD (%)	RSD (%)	Bias (%)
Darvill influent	E1		22.43 ± 1.29	5.73			
(unspiked)	E2		29.33 ± 0.56	1.91			
	EE2		8.42 ± 0.91	10.81			
Darvill effluent	E1		17.99 ± 0.68	3.80			
(unspiked)	E2		19.97 ± 0.26	1.31			
	EE2		7.25 ± 0.44	6.03			
	Mean				95.02 ± 5.00	5.26	-4.98
Darvill effluent/treated wastewater	E1	10	10.09 ± 1.70	16.84	100.87 ± 16.99	16.84	0.87
(spiked)		50	41.07 ± 0.93	2.27	82.15 ± 1.86	2.27	-17.85
		100	94.39 ± 1.84	1.95	94.39 ± 1.84	1.95	-5.61
	Mean				92.47 ± 9.51	10.28	-7.53
	E2	10	9.76 ± 0.85	8.75	97.63 ± 8.54	8.75	-2.27
		50	49.71±1.96	3.95	99.41 ± 3.93	3.95	-0.59
		100	92.76 ± 1.73	1.87	92.76 ± 1.73	1.87	-7.24
	Mean				96.60 ± 3.44	3.56	-3.37
	EE2	10	11.53 ± 1.34	11.66	115.27 ± 13.44	11.66	15.27
		50	47.72 ± 0.19	0.40	95.43 ± 0.38	0.40	-4.57
		100	101.43 ± 1.25	1.24	101.43 ± 1.25	1.24	1.43
	Mean				104.04 ± 10.17	9.78	4.04
Darvill Influent/raw wastewater	E1	10	14.82 ± 2.34	15.79	148.20 ± 23.40	15.79	48.20
(spiked)		50	50.21 ± 1.54	3.07	100.42 ± 3.08	3.07	0.42
		100	99.56 ± 0.60	0.61	99.56 ± 0.60	0.61	-0.44
	Mean				116.06 ± 27.84	23.99	-0.01
	E2	10	14.69 ± 2.54	17.32	146.87 ± 25.43	17.32	46.87
		50	50.13 ± 1.25	2.49	100.25 ± 2.92	2.85	0.25
		100	102.65 ± 2.92	2.85	102.65 ± 2.92	2.85	2.65
	Mean				116.59 ± 26.25	22.52	16.59
	EE2	10	7.13 ± 2.05	28.67	71.33 ± 20.45	28.67	-28.67
		50	48.53 ± 1.18	2.42	97.07 ± 2.35	2.42	-2.93
		100	106.23 ± 2.56	2.41	106.23 ± 2.56	2.41	6.23
	Mean				91.54 ± 18.09	19.77	-8.46

#After blank correction.

1.75% (mean = 3.43) for EE2. For a composite standard of E1, E2, and EE2, at 10, 50 and 100 μg/L, in ultrapure water, 10 replicate injections (within-batch) gave an RSD of: 4.60%, 4.90 and 2.40%, for E1, 5.40 %, 4.30 and 1.50%, for E2, and 5.10, 5.40 and 1.70%, for EE2. The mean precision (± SD), at the 10-100 ng/L level, for all three estrogens is 4.91 % (± 3.89) RSD. The mean precision (± SD), at the 10-100 ng/L level, for all three estrogens, is 6.10% (± 6.27) % RSD. Individual precision (RSD) (± SD) increased in the following order: 3.23% (± 2.51) for E2, 4.58% (± 3.84) for EE2 and 10.50% (±8.48) for E1. Standards of concentration 10, 5 and 2 ng/L in ultrapure water were analyzed in replicates of 3. The LOQ was found to be 10 ng/L, for E1, 10 ng/L for E2, and 10 ng/L for EE2 (Table S1). The LOD was found to be 5 ng/L, for E1, 5 ng/L for E2, and 10 ng/L for EE2, with RSD = 13.93% (accuracy = 89.80), For comparison purposes, use of the S/N ratio method for chromatographic methods, gave a LOD and a LOQ of 1 ng/L for E1 (mean S/N = 61, RSD = 9.58%), E2 (mean S/N = 17, RSD = 9.58%), and EE2 (mean S/N = 27, RSD = 23.85%), based on the lowest standard used (1 ng/L in ultrapure water) (Figure S1).

For unspiked ultrapure water, observed levels for all three estrogens (E1, E2, and EE2) were below the method LOQ (Table 1). The overall mean recovery, (± SD) (mean %RSD), for spiked ultrapure water matrix, over the range 10-100 ng/L, for all three estrogens, was 93.90% (± 0.97) (5.87) (Table 1).

Recovery results for wastewater matrix is summarized in Table 2:

The overall mean recovery (± SD), for the Darvill influent, over the range 10-100 ng/L, for all three estrogens, was 108.06% (± 14.31)

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(22.09). For the Darvill effluent, the overall mean recovery (± SD), over the range 10-100 ng/L, for all three estrogens, was 97.70% (± 7.71) (7.87). The overall mean recovery (± SD), for each estrogen, in raw and treated waste water is: 104.27% (± 22.65) for E1, 106.60% (± 20.01) for E2 and 97.79% (± 14.81) for EE2. The overall mean recovery (± SD), for both raw and treated wastewater matrix, over the range 10-100 ng/L, for all three estrogens, was 102.88% (± 7.33). Replicate analysis (n = 3, within-batch) results of the raw sewage and treated wastewater is summarized in Table 2. Figure S2 shows the chromatogram of an extracted, unspiked wastewater (influent) sample. Comparative results (n = 1 for GC-MS; n = 3 for ELISA) for a river sample, taken from the Duzi River, "upstream" of the normal, routine Darvill effluent discharge site (WDV 020), in the vicinity of Darvill Wastewater Works, is also listed in Table 1.

Discussion

Some of the recently international, reported analytical methods, used for trace analysis of the target steroid estrogens (E1, E2, EE2) in water and wastewater, are GC-MS, with BSTFA derivatisation [18], LC-DAD/FD [19], LC-MSMS [20], ELISA [21] and electrophoresis [22], with LODs and LOQs in the higher μg/L range [19,21]; many reports do not provide important LOQ data [19,21]. Various sensitive GC-MS methods have been reported [10,23] with LOQs of 0.06-30 ng/L. However, these methods required prior derivatisation [10,23]. Various methods, like passive sampling, Solid-Phase Extraction (SPE), Solid Phase Micro-Extraction (SPME), Single Drop Micro-Extraction (SDME), dispersive liquid-liquid micro-extraction, Vortex-assisted Liquid-liquid Micro Extraction (VALLME), immunosorbent extraction, molecularly imprinted SPE, continuous LLE, and LLE, have been reported [14]. To reach low (ng/L) detection levels, high extraction (sample) volumes are required. The SPE method by Hu et al. [17] used 1 L of water sample. For sample preparation, we used liquid-liquid extraction of a 1 L sample, for ease of setup and the need to achieve optimum sensitivity. Steroid hormones possess polar and non-ionic characteristics that enable use of reversed-phase purification and separation strategies. The application of sample preparation with high pre-concentration is necessary to achieve LODs at the ng/L level in liquid, and solid, samples. Most analytical methods include a SPE step alongside a more time-consuming purification step (e.g., gel-permeation chromatography). The concentration of steroid hormones in real samples is extremely low, usually pg/L to ng/L; the great variety of steroid hormones enhances the difficulty of their detection. A selective separation method and sensitive detection is therefore required. Currently, the most important methods used for steroid determination are LC-MS, LC-UV/FD, GC-MS, and immunoassays Considering the low LODs, and LOQs (ng/L or ng/g), GC-MS/MS and especially LC-MS/MS are the methods of choice.

With traditional liquid injection techniques in capillary gas chromatography, most inlets and columns can only handle 1- 2 μL at a time. Attempts to increase the injection volume can lead to broadened and distorted analyte peaks, large and long solvent peak tails, and saturated or damaged detectors. The purpose of increasing the injection volume is normally to improve detection limits in trace analysis. The development of the programmable temperature vaporizer technique [24], which offers the capability of Large-Volume Injection (LVI) [25], can improve sensitivity of the GC-MS analysis for steroid estrogens. In the current study, the Agilent Multi Mode Inlet (MMI) was used, having the following operational modes: Hot split/splitless, Cold split/splitless, Solvent vent and Direct modes. As in the other evaporation techniques, many factors can affect performance and efficiency of the LVI: solvent type, injection volume, the inlet temperature, the vent flow of evaporation gas, and the analyte boiling point. In addition, the inlet pressure during evaporation and the inlet liner have an impact on the rate of solvent removal and analyte recovery.

We utilized the previously reported parameters [17], DCM solvent, initial inlet temperature of 40°C, and 50 μL sample injection volume. Using the programme: initial: 40°C for 6.1 min, then 20 °C/ min to 230 °C for 1 min, and finally 10 °C/ min to 290 °C for 9 min), it was initially noted that signal responses (GC peaks) for E1 and E2 peaks were co-eluting, at ± 19.7 min. The following GC oven program was subsequently adopted: initial: 40°C for 6.1 min, then 30 °C/ min to 230 °C for 1 min, and finally 10 °C/ min to 290 °C for 9 min, which improved the resolution of E1 and E2.

Our chemical tests on water are ISO/IEC 17025-accredited. Our internal Standard Operating Procedure for Method Validation, based on the ISO/IEC 17025 guide for testing laboratories, and the national South African National Accreditation Standards (SANAS) reference document TR 26-02, was followed; typical validation parameters, like linear range, recovery, precision, sensitivity (LOD, LOQ), etc., were evaluated.

Four different inlet liners (multibaffle (5183-2037), fritted glass (5183-2041), single baffle (5183-2038 with glass wool, and 5-83-2036)), were previously evaluated [19]; the multibaffle liner was found to be suitable [19]. We found that the Agilent # 5183-4711 liner gave optimum accuracy and precision compared to the "pure-and-trap" liner. Using area responses of the selected quantification ions (*m/z* 270 for E1, 272 for E2, and 213 for EE2) from composite standards in DCM, and the observed concentrations automatically calculated from the DCM calibration standards, it was noted that the split liner (Agilent # 5183-4711) gave the best, overall accuracy, and precision.

Of note is the observation that while the LOD is reported for most of the methods, there appears to be no corresponding Method Detection Level (MDL) or LOQ data for most of the international reports. A recent review [26] on this subject also reports only the LOD of the various methods, determined at a Signal: Noise ratio of 3: 1.

The Method Detection Level (MDL) [27] and Level of Quantitation (LOQ) (Minimum Quantitation Level (MQL)) has been defined [28]. The IUPAC method [28] uses the mean concentration and Standard Deviation (SD) from replicate analysis of a "blank" (ultrapure water) sample matrix, as per following equations: mean + 10 SD, for LOQ, and mean + 3 SD, for LOD, respectively. This statistical approach, however, cannot be applied when a negative value is observed for the blank signal response. Based on the former definitions [27], and our validation results (Table 1), our MDL is: 5 ng/L for E1, 5 ng/L for E2 and 2 ng/L for EE2. Standards of concentration 10, 5 and 2 ng/L in ultrapure water were analyzed in replicates of 3 to determine the LOQ and LOD, based on the lowest standard which met the criteria of observed recovery (limit = 80-120%) and concentration precision (RSD ≤ 10%), our internal laboratory limits. The observed data comply with our general acceptable internal limits for recovery and precision. Our laboratory water quality tests methods (assays) generally utilize this technique for LOD and LOQ determination. The serial dilution technique, although it results in higher LOD and LOQ,

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would tend to be more accurate, being based on compliance to actual recovery, and precision, limits; selection of the "noise" region in a chromatogram, using the S/N method, is subjective, due to choice by the analyst. Composite standards of < 1 ng/L concentration in ultrapure water were not investigated.

Our previous study [29] showed that, at international level, there is fairly equal use of chemical-analytical LC, GC and immuno-analytical (ELISA) test methods for screening and quantitation of the steroid estrogens (E1, E2, EE2) in water matrices; relative sensitivity (LOQ - ng/L) of the test methods decrease in the following order:

LC-MS/MS (0.08-9.54) > GC-MS (1) > Immunoassay (e.g., ELISA) (5) (chemical-analytical > immuno-analytical) [29]. Although the sensitive LC-MS/MS methods Electrospray Ionisation (ESI) and APCI are available, both modes of ionization are susceptible to loss of signal response due to ion suppression caused by matrix effects in complex samples.

Improved recovery was noted in this study (overall ± 99%), for the three matrices: ultrapure water, influent and effluent, compared to the lower recoveries obtained in the earlier work [19], (> 50%, who also investigated the liquid-liquid extraction, with large volume injection), presumably due to the Agilent inlet liner used in our study. For the SPE technique, 79-98%, at 10 ng/L spike concentration, was obtained. However, recovery data for raw wastewater is not reported [17]. The mean precision for recovery (% RSD) of E1, E2 and EE2, for the three different matrices: ultrapure water, raw and treated wastewater, increases in the order: ultrapure water (5.34%) < effluent (9.00%) < influent (22.69%). This order can be expected based on the expected increase in percentage solids in moving from ultrapure water (minimal solids/dissolved solids) to influent matrix (maximum solids/dissolved solids).

Due to the unavailability of SPE equipment, our preliminary investigation focused on the liquid-liquid extraction technique only.

In ultrapure water, one would not expect any concentration of steroid hormones - the observed results confirm this, indicating levels of below the method LOQ for all three hormones: E1, E2 and EE2. In the raw wastewater (influent), the observed steroid levels decrease in the order: E2 > E1 > EE2. There is a noticeable decrease in the steroid levels in the treated wastewater (influent), due to some removal by the wastewater treatment process at the wastewater plant. The observed removal decreases in the following order: E2 (32%) > EE2 (14%) > E1 (2%); our previous work, using ELISA, showed an observed mean removal efficiency as follows: EE2 (90%) > E2 (78%) > E1 (72%). The observed relative levels in the effluent again decrease in the order: E2 > E1 > EE2. As expected, the observed levels in the "control" sample site (river), were lower than that of both the influent and effluent sample points; the observed relative levels in this river sample again decrease in the order: E2 > E1 > EE2.

Table 3 summarizes the comparison of the estrogen levels obtained in this study, using GC-MS, with that obtained in our previous study, using ELISA [16].

These results show fairly good correlation for the effluent levels, which is a relatively cleaner matrix, compared to the raw sewage influent. The comparison also shows that the mean steroid estrogen levels, obtained by GC-MS, are approximately four times lower than that obtained by ELISA. Overestimation caused by matrices in environmental samples, like the raw sewage influent, has been considered to be an inherent problem with some ELISAs [30]. It must also be noted that our earlier study [16] was over a 2-year period, where many more sampling runs (n = 8-11) were carried out.

The method performance characteristics of some previously reported GC-MS-based test methods, over the past 4 years, for the analysis of steroid estrogens, and other EDCs, are summarized in Table 4; detailed method comparisons has been reported, inter alia, in many previous review studies (Manickum, 2015) [29].

The priority estrogens analyzed are: E1, E2, and EE2, in most of the reported studies. The tested matrices include: environmental, tap, surface water, wastewater, river water, activated sludge, sewage sludge, and liquid-solid sludge. The edible/food matrices tested, include the following: human breast milk, milk, dairy products, egg products, meat and fish. The extraction methods that have been used include the following: liquid-liquid, SPE, ULE, Soxhlet, mechanical extraction, micro-extraction, MSPE, dispersive SPE and CLLE. It is clear that the majority of GC-MS-based methods utilize some mode of derivatisation (Entry: 1 to 8). Beside our current work (Entry 10), there has been one other study (Entry 9) [17], where derivatisation is not used. Regarding the sensitivity, although the LOD is reported in some references, the LOQ, which is more useful, (as it is generally validated at an acceptable level of precision and recovery), is not reported in most of the references (Entry 1, 2, 6, 7, 8). The lowest reported LOQ is 0.7-6.5 ng/L (Entry 3) [31], where derivatisation was used. In those cases where there is no prior derivatisation, (Entry 9) [17], the LOQ is not reported. The reported, observed precision ranges from 2-10% for the reported test methods. We observed fairly similar precision of 3.2-10.5% RSD or our test method. The observed

Table 3: Comparison of the steroid estrogen levels determined by the ELISA and GC-MS methods.

Sample	**Raw wastewater/Influent: concentration (ng/L)**						**Treated wastewater/Effluent: concentration (ng/L)**						**River sample: concentration (ng/L)**	
Test	ELISA[a]			GC-MS[a]			ELISA[a]			GC-MS[a]			ELISA	GC-MS
Estrogen	Mean ± SD (% RSD)	Range	Rel.[a] %	Mean ± SD (% RSD)	Range	Rel. %	Mean ± SD (% RSD)	Range	Rel. %	Mean ± SD (% RSD)	Range	Rel. %	Mean	Conc.
E1	84 ± 97 (115)[b]	13-351	36	22.4 ± 1.3 (6)[b]	21.6-23.9	37	23 ± 25 (108)	3-78	50	18.0 ± 0.7 (4)	17.7-18.8	40	5 (2-10)[c]	0.4
E2	119 ± 83 (70)	20-199	51	29.3 ± 0.6 (2)	29.0-30.0	49	20 ± 31 (155)	4-107	44	20.0 ± 0.3	19.7-20.2	45	10 (1-82)	18.3
EE2	30 ± 29 (97)	10-95	13	8.4 ± 0.9 (11)	7.5-9.4	14	3 ± 2 (67)	1-8	7	7.3 ± 0.4 (6)	6.9-7.2	15	1 (0-3)	3.0

[a] n = 11 sampling runs for ELISA; n = 2 sampling run/3 replicates for GC-MS; Rel = relative (composition of the estrogens)

[b] For ELISA day-to-day; for GC-MS within-day

[c] range.

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Table 4: Performance characteristics of some referenced GC-MS methods for analysis of priority steroid estrogens and other EDCs.

Entry	Article type, Corresponding/ First Author (year)	Matrix	Analytical technique/ test methods	Extraction methods reported	GC Derivati sation	Priority estrogens tested	Other EDCs tested	GC-MS Method performance/validation data					Comments
								Range	LOD	LOQ	RSD (%)	Recovery (%)	
1	Research, Prieto A, et al, (2011) [31]	river water, wastewater	GC-MS-LVI	Micro-extraction	BSTFA-1%TMCS	E1, E2, EE2, E3	NT, BPA, TT, EQ, cis-ADT, 4-OP, Me-EE2, DES, 4t-OP, 4n-NP, 4-NP, CHL	LOD-1000 ng/L		nr[a]	4-22 within-day 6-23 inter-day	75-109 81-103 57-66	
2	Review, Tomsikova H,et al, (2012) [23]	water, river water, activated sludge, wastewater, sewage sludge, liquid-solid sludge	GC-MS, LC-MS, LC-MSMS, LC-DAD/FD/ UV/PDA	SPE, ULE, liquid-liquid, Soxhlet and mechanical extraction	PFBBr, BSTFA-TMCS, BSTFA, BSTFA-TMS, MSTFA, PFPA, acetic anhydride-BSTFA	female steroid hormones, estrogens, progestogens		nr	0.01-500 ng/L	nr	nr	nr	All GC methods used Derivat isation
3	Research, Avbersek M, et al, (2013) [32]	tap, surface, waste influent/ effluent	GC-MS	SPE	MSTFA	E1, E2, EE2, E3		2-610 ng/L	0.2-2 ng/L	0.7-6.5 ng/L	9-Feb inter-day	nr	
4	Review, Socas-Rodriguez B, et al, (2013) [33]	human breast milk, milk, dairy products	GC-MS, GC-MSMS, LC-MS, LC-MSMS, LC, UHPLC-MSMS		BSTFA-TMCS,	E1, E2, EE2, E3,	DES, DS, HEX, α-ZAL	nr	1.0-1.2 ng/kg 0.01-1.3 µg/kg	nr	nr	91-104 85-111	
5	Review, Adamusova H, et al, (2014) [34]	edible; milk, egg products meat, fish, packaging products, milk products	LC-MSMS, LC-MS, LC-UV, LC-fluorescence, GC-MS, GC-MSMS	Extraction, SPE, LLE, MSPE, dispersive SPE, micro-extraction-SPE, SPME, SBSE	BSTFA-TMCS, MSTFA, PFBBr, TMIS, DTE, BSTFA	Selected EDCSs, including: E1, E2, EE2, α-E2	Others HEX, DES, DIE, BPA, BPF, BPZ,OP, NP						
6	Research, Rocha MJ, et al, (2015) [35]	river water	GC-MS	SPE	BSTFA-TMCS	E1, E2, EE2	4-OP, 4-t-OP, nonyl= phenol, polyethoxy=lates, BPA, phyto= estrogens, sitosterol	10-375 ng/L	nr	2.8-4.4 ng/L	nr	nr	Very little/ no method validation data
7	Research, Belhaj D, et al, (2015) [36]	wastewater, activated sludge	GC-MS	SPE	BSTFA-pyridine	E1, E2, EE2, E3		nr	nr	nr	nr	nr	Very little/ no method validation data
8	Research, Huang Z, et al, (2015) [37]	environmental water	GC-MS	Dispersive SPE	MSTFA-pyridine	E1, EE2, E3	Diethyl= stilbestrol	90-45000 ng/L	6-230 ng/L	nr	2.2-13	71-119	
9	Research, Hu R, et al, (2007) [17]	water, wastewater,	GC-MS-LVI	SPE, liquid-liquid (LL), CLLE		E1, E2, EE2		0.1-20 µg/L	0.031-0.046 ng/L	nr	2.21-9.52 with in-day 4.55-7.78 between batch	54-98.4	No derivat isation
10	Research This study: Manickum T, et al, -2015	water, wastewater,	GC-MS-LVI	liquid-liquid (LL),		E1, E2, EE2		10-100 µg/L	5-10 ng/50-110[b] pg/L	10 ng/L 16-595[c]pg/L	3.23-10.50	99	No derivat isation

[a]not reported

[b]calculated, at signal: noise = 3: 1

[c]calculated, at signal: noise = 10: 1

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recovery, for the derivatised methods, range from 57-119%. We observed ± 99% mean recovery for our un-derivatised method, for ultrapure water and influent-effluent matrix; much lower recovery (> 50%) was obtained by the previous work (Entry 9) [17]. Some advantages of our method over those reported include the following: no need to derivatise the extracted steroid estrogens; validated LOQ (10 ng/L; 0.02-0.60 ng/L at signal: noise = 10: 1), similar, and better LOQ compared to the lowest one reported, which used derivatisation (0.7-6.5 ng/L, Entry 3); improved recovery.

It is evident that techniques such as HPLC, GC-MS, LC-MS and LC-MS/MS are generally employed for quantification of EDC's and priority steroid estrogens in environmental samples. Although highly reliable, they have several potential drawbacks, including expensive instrumentation, large sample volume, extensive purification, utilization of large amount of solvents, and the need for technical expertise in operation. The analysis of a large number of samples may be both cost and time-prohibitive. There is thus a strong need for rapid, simple, and cost-effective methods for quantitative analysis of steroid estrogen hormones. In our country, South Africa, test centers able to routinely analyze for the steroid estrogens in both water and wastewater is rather limited. The common analytical techniques available here, as a routine service, are only the immuno-analytical ELISA method [9,15,16], which has been fully validated for both water and wastewater matrix, with an LOQ of 5 ng/L for E1, E2 and EE2. However, the cost implications, together with outsourcing implications (sample packaging, stability, transport to Western Cape) [9,15,16] are added factors. Our simple, validated chemical-analytical GC-MS method, without the need for prior derivatisation, appears to be a viable option to both ELISA, and LC-tandem mass spectrometry for screening and quantitation of E1, E2, and EE2.

Conclusion

Whilst both ELISA and LC-MS/MS techniques have their advantages and disadvantages, in general, the use of MS, as a detector, has the main distinct advantage of simultaneous, multi-analyte screening, which is not possible with ELISA. However capital costs implications associated with tandem mass spectrometry can be a financial hurdle for many institutions, especially Third World countries, like S Africa. The international reports have shown pg/L LOQs achievable by the chemical-analytical GC-MS/MS and LC-MS/MS test methods, with and without derivatisation, for the targeted steroid estrogens analysis (E1, E2, EE2), in environmental water, potable water and wastewater matrix.

We have now developed and fully validated a simple, easy to set up, liquid-liquid extraction, chemical-analytical GC-MS method for screening and quantification of E1, E2, and EE2, using large volume injection, without the need for prior derivatisation. Preliminary method performance indicates that the test method is fairly sensitive, accurate and precise for monitoring and quantitation of these three priority female steroid estrogens in both water and wastewater matrix.

South Africa is the world's 30th driest country in the world. Re-use options to supplement dwindling natural water resources is a definite need. The use of reclaimed wastewater for potable re-use will no doubt necessitate the requirement for much more stringent water quality testing due to the vast range of Emerging Chemical Contaminants (ECCs), EDCs, Personal Care Products (PCPs), etc., from wastewater influent and their subsequent potential presence in the treated effluent. Large volume injection, with GC-MS is a viable alternative to the traditional analytical methods for steroid estrogen analysis in water matrix.

Future work will involve optimization of the current liquid-liquid extraction method, by investigation of: other sample extraction methods, like the widely used SPE, use of labeled internal standards for accurate recovery, prior derivatisation of extracted analytes, other GC inlet liners, improved GC resolution of E1 and E2, relatively polar GC separating columns, lower (< 1 ng/L) standard concentrations for sensitivity optimization, reduction of analysis/run time, higher GC inlet and MS interface temperatures, suitable quality control material/quality assurance procedures, and application to other relatively important steroid hormones, like estriol (E3), progesterone and testosterone.

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