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REVIEW

The current preference for the immuno-analytical ELISA method for quantitation of steroid hormones (endocrine disruptor compounds) in wastewater in South Africa

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Abstract The availability of national test centers to offer a routine service for analysis and quantitation of some selected steroid hormones [natural estrogens (17-β-estradiol, E2; estrone, E1; estriol, E3), synthetic estrogen (17-α-ethinylestradiol, EE2), androgen (testosterone), and progestogen (progesterone)] in wastewater matrix was investigated; corresponding internationally used chemical- and immuno-analytical test methods were reviewed. The enzyme-linked immunosorbent assay (ELISA) (immuno-analytical technique) was also assessed for its suitability as a routine test method to quantitate the levels of these hormones at a sewage/wastewater treatment plant (WTP) (Darvill, Pietermaritzburg, South Africa), over a 2-year period. The method performance and other relevant characteristics of the immuno-analytical ELISA method were compared to the conventional chemical-analytical methodology, like gas/liquid chromatography–mass spectrometry (GC/LC-MS), and GC-LC/tandem mass spectrometry (MSMS), for quantitation of the steroid hormones in wastewater and environmental waters. The national immuno-analytical ELISA technique was found to be sensitive (LOQ 5 ng/L, LOD 0.2–5 ng/L), accurate (mean recovery 96 %), precise (RSD 7–10 %), and cost-effective for screening and quantitation of these steroid hormones in wastewater and environmental water matrix. A survey of the most current international literature indicates a fairly equal use of the LC-MS/MS, GC-MS/MS (chemical-analytical), and ELISA (immuno-analytical) test methods for screening and quantitation of the target steroid hormones in both water and wastewater matrix. Internationally, the observed sensitivity, based on LOQ (ng/L), for the steroid estrogens E1, E2, EE2, is, in decreasing order: LC-MSMS (0.08–9.54) > GC-MS (1) > ELISA (5) (chemical-analytical > immuno-analytical). At the national level, the routine, unoptimized chemical-analytical LC-MSMS method was found to lack the required sensitivity for meeting environmental requirements for steroid hormone quantitation. Further optimization of the sensitivity of the chemical-analytical LC–tandem mass spectrometry methods, especially for wastewater screening, in South Africa is required. Risk assessment studies showed that it was not practical to propose standards or allowable limits for the steroid estrogens E1, E2, EE2, and E3; the use of predicted-no-effect concentration values of the steroid estrogens appears to be appropriate for use in their risk assessment in relation to aquatic organisms. For raw water sources, drinking water, raw and treated wastewater, the use of bioassays, with trigger values, is a useful screening tool option to decide whether further examination of specific endocrine activity may be warranted, or whether concentrations of such activity are of low priority, with respect to health concerns in the human population. The achievement of improved quantitation limits for immuno-analytical methods, like ELISA, used for compound quantitation, and standardization of the method for measuring E2 equivalents (EEQs) used for biological activity (endocrine: e.g., estrogenic) are some areas for future EDC research.

Keywords Endocrine disruptor compound · Steroid hormones · Immuno-analysis · Enzyme-linked immunosorbent assay · Gas/liquid chromatography–tandem mass spectrometry · Wastewater · Bioassay

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Introduction

Natural steroid hormones secreted by the adrenal cortex, testis, ovary, and placenta in human and other animals include progestogens, glucocorticoids, mineralocorticoids, androgens, and estrogens [1]. The steroid estrogens 17-β-estradiol (E2), estrone (E1), and estriol (E3), predominantly female hormones, are responsible for maintenance of health of the reproductive tissues, breast, skin, and brain. Progestogens (e.g., progesterone) can be viewed as a hormonal balancer, particularly of estrogens. Androgens (e.g., testosterone, tes) play an important role in tissue regeneration, especially of the skin, bones, and muscles. Synthetic steroids (e.g., 17-α-ethinylestradiol (EE2), mestranol) and progesterone (pro) are used as contraceptives. All humans and animals excrete natural steroid hormones through their bodies, via urine and excrement; these hormones can end up in the environment through sewage discharge or animal waste disposal [1]. The steroid hormones, which are chemically very stable, are excreted in the free form or as conjugates; the latter readily biotransform to the free conjugates [2,3].

The vast number of papers published in the literature confirms the detection of steroid hormones in raw sewage, treated sewage effluents, receiving rivers, and surface water [4]. Their eventual presence, even at very low nanogram per liter concentration, in the environment poses a significant potential problem of interference with normal function of the endocrine systems, and can thus affect reproduction and development in wildlife [5]. The steroid hormones of major concern in the aquatic environment, as a result of their endocrine-disrupting potential, are mainly the estrogens, namely 17-β-estradiol, estrone, and estriol, and contraceptives, namely 17-α-ethinylestradiol. Androgens and progestogens, which are also released from sewage treatment plants, may also pose a risk for aquatic fauna [6]. Some studies have described androgenic activities in environmental samples that have been attributed to human androgens, like testosterone [7].

One of the major sources of contamination of the aquatic environment is wastewater treatment plants (WTPs) because several steroid hormones and related compounds are not totally removed or degraded by biological treatments. As a result of incomplete elimination of steroid hormones from wastewater treatments plants, both steroid hormones and pharmaceutical products are found in ground and surface waters. These hormones have also been reported to be present at varying levels in treated wastewater (effluents) from sewage treatment works in countries around the world [8]. The observed international data to date is summarized in Table S1 (see Electronic Supplementary Material, ESM) [9].

Internationally, and also in South Africa, treated wastewater effluent has to comply with certain legal requirements before discharge into natural water courses. However, there is currently no legislation in South Africa as regards maximum allowable levels of these steroid hormones in water matrices. To date, the first report on the estrogen levels in treated sewage effluent in South Africa was by Swart and Pool [10], in the Kuils River water catchment area, using the immunoanalytical ELISA [11] technique. We recently reported [12] detection of the steroid estrogens estrone, and 17-β-estradiol, at our Darvill WTP, Pietermaritzburg, again using the immuno-analytical ELISA technique [9–11].

Several methods have been developed in the past few years to determine the concentration of steroid hormones at trace level in various matrices, e.g., immunoassay [10,13]. Within the last 15–20 years, the increasing use of liquid chromatography–mass spectrometry (LC-MS) has revolutionized environmental analysis, providing a new analytical tool that enables identification of highly polar organic pollutants without derivatization, down to nanogram per liter levels in all kinds of water bodies (raw sewage, treated sewage effluent, surface water, groundwater, and drinking water). The major innovation that enabled this involved the development of the appropriate ionization interfaces, like electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), to couple LC with MS. Further innovations have been made in rapid on-line extraction, microextraction, and on-line derivatization techniques in combination with gas chromatography–mass spectrometry (GC-MS), or GC–tandem mass spectrometry (MSMS), detection [14,15].

As a follow-up to our initial findings [12], we recently reported the detailed study outcome on the occurrence, fate, and environmental risk assessment of some steroid hormones (endocrine disruptor compounds) (EDCs) at the Darvill WTP, using the immuno-analytical ELISA method for steroid hormone quantitation [9]. We hypothesized that nationally, the immuno-analytical ELISA method is the commonly used test method for screening and quantitation of the selected steroid hormones; internationally, the conventional chemical-analytical gas/liquid chromatography–mass/tandem mass spectrometry (GC/LC-MS/MS) test methods are used. The aims of this study review were thus to establish, at international level, which are the commonly used analytical test methods for screening and quantitation of some selected steroid hormones (E1, E2, EE2, E3, tes, pro); to ascertain the availability of national test centers, and test methods used, for routine analysis of the selected steroid hormones in wastewater; an assessment of the immuno-analytical ELISA method, as a potentially preferred technique at national level, for accurate, precise, low-level quantitation of the selected steroid hormones in raw and treated sewage/wastewater, and in environmental waters, over other conventional, commonly used

chemical-analytical methods, like LC/GC–mass spectrometry (MS) or LC/GC–tandem mass spectrometry (MSMS).

Materials and methodology

National test centers, and analytical equipment suppliers, were consulted to provide test information services available, with test costs and method performance characteristics for screening and quantitation of the targeted steroid hormones, namely E1, E2, EE2, E3, pro, and tes, in wastewater and water matrix. The international literature was reviewed to cover the past decade, and prior, regarding reports and current analytical methods used for screening and quantitation of these selected steroid hormones, in wastewater matrix, with a focus on test method sensitivity.

All samples for the target steroid hormones were tested by the immuno-analytical ELISA test method at the University of the Western Cape (UWC). For the comparative, national study performance of the ELISA and LC-MSMS test methods, the same samples were analyzed by LC-MSMS, by outsourcing the duplicate samples to laboratory B in South Africa.

The activated sludge process at the Darvill wastewater treatment plant (WTP)

The Darvill WTP, part of Umgeni Water, the main sewage and wastewater treatment plant for Pietermaritzburg, serving over 300,000 people, treats raw municipal wastewater and treated industrial wastewater (75 million liters per day, ML/day). It operates through an activated sludge process [16]. At an operating temperature of 12–24 °C, the activated sludge process (hydraulic capacity 110 ML/day) has solids retention time of ±8 days and a mean hydraulic retention time of 7 h.

The immuno-analytical ELISA test method

Samples were collected as per the recommended procedure [9,10,12]. Details of the method validation, performance characteristics (ESM Table S2), and analytical test method procedure have been previously reported [9,10,12]. In summary, for the targeted six steroid hormones, the detection range was 2–4,000 ng/L, with an LOQ of 5 ng/L for all hormones; the LOD ranged from 0.2 to 5.0 ng/L. The mean recovery (±SD) was 96.18 % (±8.62) (% RSD 8.28±4.40). The inter-assay and intra-assay precision was 9.45 % (±4.45) and 7.38 % (±3.59), respectively.

Details of the sample collection, sample extraction, kits used, and test procedure for the immuno-analytical ELISA method are appended in ESM Text S3.

Results and discussion

The targeted steroid hormone levels at the Darvill WTP

The steroid hormone levels present in the raw wastewater (influent), treated wastewater, and river water at the Darvill WTP, obtained by the immuno-analytical ELISA method, are summarized in Table 1; a detailed discussion of the test results has been previously reported [9].

The total steroid hormone concentration in the raw wastewater (influent) was ±989 ng/L; pro was the most abundant (41 %, 408 ng/L), followed by tes (35 %, 343 ng/L) and E2 (12 %, 119 ng/L). For the treated wastewater (effluent levels), the total steroid hormone concentration was 66 ng/L; E1 was the most abundant (35 %, 23 ng/L), followed by E2 (30 %, 20 ng/L) and tes (17 %, 11 ng/L).

Risk assessment of the target steroid hormones

The aspect of health risk assessment has been briefly addressed [9]. It is the process or method of determining if an activity (man-made or natural) will negatively impact humans. Risk assessment can be used as a decision-making tool to support policies that protect public health and the environment, such as guideline development, which may include allowable limits.

Assessment of a new chemical entity should involve a complex toxicology package comprising acute, subacute, subchronic, and chronic studies in rodents and other mammals, together with carcinogenicity, repro- and genotoxicity studies [17]. Data are submitted to a regulatory agency (e.g., US Environmental Protection Agency, EPA) for evaluation and determination of risk assessments that include critical toxicity, the no-observed-adverse-effect level (NOAEL: “the greatest concentration or amount of a substance, found by experiment or observation, which causes no detectable adverse alteration of morphology, functional capacity, growth, development, or life span of the target organism under defined conditions of exposure”), and the lowest-observed-effect level (LOEL: “lowest concentration or amount of substance greatest concentration or amount of a substance, found by experiment or observation, that causes any alteration in morphology, functional capacity, growth, development, or life span of the target organism under defined conditions of exposure) in each test species [18].

Although it is an established fact that EDCs are a serious concern, standardized protocols on how to apply current human health risk assessment methodologies in order to assess the potential risks associated with EDCs, like the steroid hormones, are unavailable [19]. In addition, the current [19], and previous, World Health Organization (WHO) drinking water quality guidelines [20] do not specify any allowable limits for

Table 1 Steroid hormone levels in influent/effluent/river samples at the Darvill wastewater treatment plant determined by the immuno-analytical ELISA method

Sample site	Estrone (ng/L)	17-β-Estradiol (ng/L)	Estriol (ng/L)	17-α-Ethinylestradiol (ng/L)	Progesterone (ng/L)	Testosterone (ng/L)
Influent						
n[a]	11	11	8	8	8	8
Min	13	20	3	10	163	119
Max	351	199	9	95	904	635
Mean	84	119	5	30	408	343
Median	50	97	4	18	342	258
SD	97	83	2	29	220	226
% RSD[b]	115.12	70.11	47.48	97.16	54.03	66.00
Mean±2SD	0–278	0–285	1–9	0–88	0–628	0–795
Effluent						
n	11	11	8	8	8	8
Min	3	4	<1	1	0	0
Max	78	107	<1	8	25	26
Mean	23	20	<1	3	9	11
Median	13	7	<1	2	8	10
SD	25	31	<1	2	8	7
% RSD	108.70	155.00		66.67	88.89	63.64
Mean±2SD	0–73	48–96		0–7	0–25	0–25
% Removal efficiency[c] Mean±SD (% RSD)	72±12 (16.67)	78±12 (15.38)	100	90±3 (3.33)	98±2 (2.04)	96±1 (1.04)
"Upstream"[d]						
n	3	3	3	3	3	3
Min	2	1	<1	0	0	5
Max	10	28	<1	3	12	16
Mean	5	10	<1	1	7	10
Median	4	2	<1	1	9	10
SD	4	15		2	6	6
% RSD	78.06	148.14		114.56	89.21	53.30
Mean±2SD	0–14	0–40	<1	0–5	0–19	0–22
"Downstream"[d]						
n	12	12	10	10	9	9
Min	1	2	<1	1	0	3
Max	32	66	2	4	60	19
Mean	8	10		2	13	10
Median	6	5		1	9	8
SD	9	18		1	19	6
% RSD	107.74	171.56		65.17	140.16	57.36
Mean±2SD	0–26	0–46		0–4	0–51	0–22

[a] Total number of samples/day sampled

[b] Reproducibility standard deviation

[c] = [(Influent level − Effluent level)/Influent level]100

[d] River sites up- and downstream of the wastewater treatment plant (Darvill WTP)

the majority of EDCs, and the significant steroid hormones, like the estrogens E2, E1, and EE2.

It is general practice in health risk assessments to assume that toxic substances have some safe level (non-zero threshold/highest dose or concentration) to which no adverse health effects (NOAEL or NOEL) will occur over the lifetime of exposure to the substance [20]. This safe threshold is also referred to as the reference dose which is derived from an

acceptable daily intake (ADI). If a NOAEL is not available, a LOAEL (or LOEL) may be used. When a LOEL is used instead of the NOEL, an additional uncertainty factor (1–10) is normally used.

For each toxicity study, there is a NOEC (no observed effect concentration) and a LOEC (lowest observed effect concentration). The derived PNEC (predicted no effect concentration) is the lowest value: PNEC < NOEC < LOEC.

It is a known observation that both the natural steroid estrogens E1 and E2 and the synthetic EE2 have the potential to behave like EDCs in the environment. EDCs can cause reproductive disturbances in fish, including fertility, masculinization of females, and feminization of males.

In a recent report [21], the authors used long-term duration toxicity study (up to more than 180 days) NOEC values (ng/L) from 22 diverse fish species reproduction studies to derive the following PNECs for the steroid estrogens: 0.1–16 for EE2, 2.9–80 for E2, and 198 for E1 [21]. Their proposed PNECs (ng/L), for use in risk assessment in relation to aquatic organisms, are 6 for E1, 2 for E2, 60 for E3, and 0.1 for EE2 [21], for long-term exposures (more than 60 days) to steroid estrogens in surface water, consistent with that used by Williams et al. [22].

For environmental risk assessment the PNEC is the value that is typically used. If the PEC (predicted environmental concentration) or MEC (measured environmental concentration) divided by the PNEC is less than 1, then there is minimal risk. This is commonly reported as the PEC/PNEC or MEC/PNEC ratio, sometimes referred to as the risk quotient.

After careful review of the observed NOELs, it was recommended that aquatic risk assessments investigating the potential effects associated with shorter than full life-cycle exposures to steroid estrogens in surface water should rather use alternate, higher PNECs (ng/L) for the steroid estrogens: 20 for E1, 5 for E2, 200 for E3, and 0.5 for EE2 [21].

Four methods, or protocols, for risk assessment of the target six steroid hormones, at the Darvill WTP, were previously identified and applied [9]. One method involved use of an "average trigger value" of estrogenic activity using bioassays. In investigating the feasibility of a health risk assessment protocol for EDCs, South African researchers [23,24] proposed a framework for guidelines for drinking water based on a tiered approach. First level screening tests for reproductive endocrine-disrupting capability was recommended rather than testing for the presence of specific or individual chemical concentrations: screening using a battery of in vivo and in vitro tests quantitatively expressing the results of estrogen activity of a water sample containing a mixture of chemicals in terms of their relative potency was recommended. An approach similar to toxic equivalency factors can be used for hormones and their activity in water can be expressed in terms of equivalency factors. An activity value above which a more detailed assessment is recommended is the "average trigger value" (ATV) of estrogenic activity using bioassays. The derived ATV was 0.7 ng/L [23,24], based on the WHO value of estrogenic equivalency factor or quotients (EEQ). The fraction of the ATV (ratio EEQ/ATV, where EEQ = sample biological E2 equivalent concentration using bioassay) is then used for risk assessment, where a ratio greater than 1 would recommend a more detailed investigation to be carried out [23,24]. The most potent form of estrogenic activity is 17-β-estradiol and all other compounds are measured against this.

After application of these four risk assessment methods, risk assessment of the steroid hormone levels present in the Darvill WTP effluent, and the associated surface water (Umsunduzi River), indicated that EE2 and E2 pose the highest risk to human health and fish [9]. One of the study conclusions was the recommendation of a battery of tests: quantitative chemical assay, bioassay for estrogenic activity, and risk assessment methods, collectively, are preferred in order to make meaningful, accurate conclusions regarding potential adverse effects of these steroid hormones (EDCs) present in treated wastewater effluent or surface water, to the aquatic environment, human health, and wildlife systems [9].

Two recent related reports also addressed this aspect of human health risk assessment regarding two "groups" of EDC compounds in drinking water: pharmaceuticals [25] and steroid hormones [26]. The latter study [26] was similar to the South African study [23,24] in which trigger values were derived for the ERα (estrogen) [and AR (androgen), PR (progesterone), GR (glucocorticoid)] CALUX bioassays for agonistic hormonal activity in drinking water, which define a level above which human health risk cannot be waived a priori and additional examination of specific endocrine activity may be warranted. The derived trigger value, for estrogenic activity, in this study was 3.8 ng E2-equivalents (eq)/L [26], which enabled the calculation of the corresponding ratio, the benchmark quotient (BQ) (BQ = concentration of endocrine activity in water by bioassay/trigger value). Conservatively, at a BQ value of at least 1 in drinking water, a potential health concern cannot be waived if the water was to be consumed over a lifetime period.

In an earlier study [27], a much higher trigger value of 7 ng E2-eq/L was derived for estrogenic activity in water samples.

Potential effects on biological systems on exposure to observed levels of EE2

Some reported concentrations of EE2 in streams are in excess of 100 ng/L [21]. It must be noted that the observed mean level of 95 ng/L of EE2, at the Darvill WTP, was in the raw sewage/wastewater. Our study [9] showed a removal efficiency of 90 % for EE2 by the wastewater treatment process, with a value of 8 ng/L maximum, for EE2, found in the treated effluent, which is then discharged into the nearby Umsunduzi

River. These values exceed the LOEL (1 ng/L) [28] and PNEC (0.1 ng/L) for EE2 [21].

Regarding the fate of steroid estrogens in the aquatic environment, estrogens like EE2 bind rapidly to suspended solids owing to their relatively high octanol/water partition coefficients (K_{OW} 3.67–4.2). In natural water, they degrade under aerobic conditions. The half-life of 4–6 days for EE2 in water and sediments has been noted; our earlier study [9] showed that EE2 is much more resistant to biodegradation.

EDCs like EE2 have the potential to adversely affect the sensitive hormone pathways that regulate reproductive functions. In aquatic organisms, the adverse effects may be expressed in terms of reduced fertility and egg production in female fish, or reduced gonad size and feminization of male fish. Exposure to EE2 may also result in a variety of other effects that include induced production of vitellogenin in male fish, changes in sex ratio of progeny, and alterations in gene expression. It has been shown that EE2 toxicity varied over a wide range of concentrations, about 6 orders of magnitude (<0.1–4,100,000 ng/L) [29]. In essence, toxicity is a function of the type of aquatic organism, its life stage, length of exposure to the contaminant, and the end point used in the study. In general, fish are the most sensitive aquatic organisms to the effects of EE2. For the EE2 exposure concentrations of 100 ng/L, observed toxicity effect end points were embryo production (*Potamopyrgus antipodarum* – snail: invertebrates), sex ratio, population size (*Gammarus pulex* – amphipod: invertebrates), reproduction (*Hyalella azteca* – amphipod: invertebrates), egg masses, sex ratio, emergence, egg production (*Lymnaea stagnalis* – pond snail: invertebrates) [29]. The lowest observed effect concentration was produced by Metcalfe et al. [30], who reported a nominal concentration of 0.1 ng/L as a LOEC for the testis–ova induction (i.e., feminization) in Japanese medaka fish (*Oryzias latipes*) in a 100-day test.

In addition to the above potential adverse effects, a level of 95 ng/L of EE2 could, as a minimum, result in population level effects if it was maintained for prolonged periods (weeks to months); short-term exposure on the order of days would have minimal effects.

Standards ("allowable limits") for the steroid estrogens E1, E2, E3, and EE2?

For EE2, most field samples report levels less than detection limits. The measured concentrations of E2 in European surface waters ranged from less than detection limit to about 1 ng/L [21]. The recently reported PNECs (ng/L) are 20 for E1, 5 for E2, 200 for E3, and 0.5 for EE2, for short-term exposure, and 6 for E1, 2 for E2, 60 for E3, and 0.1 for EE2, for long-term exposure [21]. Based on the LOEC of 1.0 ng/L of EE2 for reproduction and egg production [29], the recommended guideline (average concentration) proposed, using a safety factor of 2, was not to exceed 0.5 ng/L of EE2 for the protection of freshwater aquatic life.

The earlier reported LOEL (ng/L) for E1, E2, and EE2 is 10, 10, and 1, respectively [28]. Using the "safety/uncertainty factor" of 10 (or 100) [29] gives a calculated guideline (allowable limit/standard) of 1, 1, and 0.1 ng/L for E1, E2, and EE2. However, some typical reported levels for these steroid estrogens in surface water, and sewage effluent, from the USA, Japan, Germany, Italy, and the Netherlands, are (ng/L) less than 0.1 to 27 for E1, 0.09–160 for E2, less than 0.1 to 73 for EE2, and 0.33–19.70 for E3 [31], which exceed the "allowable limits". It is therefore not practical to have any proposed "allowable limits" for the steroid estrogens.

Considering the current sensitivity of the developed immuno-analytical ELISA method (LOQ 5 ng/L), and interference by cross-reaction (0.1 % is usually considered negligible), measurement of EE2 concentrations as low as 0.1 ng/L (100 pg/L or 0.1 pg/mL) is required. The current immuno-analytical ELISA processes 1,000 mL of water sample, which is finally reconstituted in 1 mL of DMSO solvent, followed by a 1/10 dilution. This value equates to a required accuracy measurement, of an analytical test, of 0.1 pg/100 μL, or 0.001–0.10 pg/10 μL (10 % error). In a routine setting, such a measurement level of sensitivity, accuracy, and precision of most immuno-analytical test methods is not practical or achievable. It is therefore also not possible to have a standard, or "allowable limit", for the steroid estrogen EE2. Improvement of the LOQs of immuno-analytical methods, like ELISA, in the area of method development is thus one area for future research.

Comparison of the immuno-analytical ELISA test performance characteristics with conventional chemical-analytical methods for steroid hormone assay in wastewater and water matrix

As the target steroid hormones have been frequently investigated in environmental water, several analytical approaches, like the chemical-analytical GC-MS (Table 2, entries 1–32), LC coupled to various detectors (Table 2, entries 33–56), capillary electrophoresis (CE) methods (Table 2, entries 57–63), and the immuno-analytical immunoassay-radioimmunoassay (RIA) methods (Table 2, entries 64–69), have been developed in the past few years to determine their concentration at trace level in various matrices, like biological fluid (urine, serum), sediment, wastewater (raw and treated), environmental water (river, surface), and potable water (see refs. 78–104 in ESM Text S4). Various reviews of the analytical methodology employed have also been reported (see refs. 71, 105, and 106 in ESM Text S4).

Table 2 Relative sensitivity of some internationally reported conventional chemical- and immuno-analytical test methods for quantitation of selected steroid hormones in water and wastewater matrix

Entry	Method	Steroid estrogens/hormones[a] (EDCs)	Matrix	LOD	LOQ	Range	Ref.[b]	Year
	Chemical-analytical							
	GC							
1	GC-MS	E1, E2, EE2, E3	Influent	0.05–0.27 ng/L	NR	NR	[48]	2012
2	GC-MS	E1, E2, E3	Tap water, sewage	1.6–10 ng/L	NR	NR	[68]	2008
3	GC-MS	E1, E2, EE2, E3	Water	0.2–30.3 ng/L	NR	NR	[69]	2009
4	GC-MS	EE2	Water	0.01–0.06 ng/L	NR	NR	[70]	2007
5	GC-MS	E1, E2, EE2, E3	Water	0.03–100 ng/L	NR	NR	[71]	2009
6	GC-MS	Various EDCs	Water samples	12–32 ng/L	NR	NR	[72]	2004
7	GC-MS	E1, E2, EE2, E3	Water, effluent	0.02–0.48 ng/L	0.06–3.6 ng/L	500–500,000 ng/L	[42]	2012
8	GC-MS	Pro, tes	Surface water, sediment	0.1–0.5 ng/L	0.5–1.8 ng/L	1,000–5,000,000 ng/L	[4]	2013
9	GC-MS	E1, E2, EE2	Influent, sludge	100 ng/L	0.1–5 ng/L	1–100 ng/L	[73]	2005
10	GC-MS	E1, EE2, E3	Effluent	0.2 ng/L	NR	Up to 80 ng/L	[15]	2003
11	GC-MS	E1, EE2, E3	Effluent, surface water	0.05–0.1 ng/L	NR	NR	[15]	2003
12	GC-MS	E1, E2, EE2	River sediment	1.5–5 ng/g	NR	Up to 150 ng/g	[37]	2007
13	GC-MS	E2, EE2	Influent, effluent	0.3–2.4 ng/L	NR	NR	[35]	2000
14	GC-MS	E1, E2, EE2	Effluent	0.2 ng/L	NR	1.4–76 ng/L	[74]	2000
15	GC-MS	E2,	Effluent	58 ng/L	NR	NR	[74]	2000
16	GC-MS	E1, E2, EE2, E3	Influent, effluent	NR	1 ng/L	<1–13 ng/L	[74]	2000
17	GC-MS	E1, E2, EE2	Influent, effluent	0.5–1 ng/L	NR	1.7–220 ng/L	[74]	2000
18	GC-MS	EE2	Effluent	74 ng/L	NR	NR	[74]	2000
19	GC-HR-MS	E1, E2, EE2, tes	Influent, effluent	1.6–41 ng/L	NR	NR	[47]	2007
20	GC-MS (LVI)	E1, E2, EE2,	Water, effluent	0.031–0.12 ng/L	NR	0.1–20 μg/L	[33]	2008
21	GC-ESI/APCI-MS	E1, E2, EE2, E3	Water	0.1–75 ng/L	NR	NR	[71]	2009
22	GC-TOF-MS	E1, E2, EE2	River sediment	1.5–12 ng/g	NR	Up to 150 ng/g	[37]	2007
	GC-MSMS							
23	GC-MSMS	E1, E2, EE2	Effluent	0.1–2.4 ng/L	NR	NR	[74]	2000
24	GC-MSMS	E1, E2,	Effluent	1 ng/L	NR	NR	[74]	2000
25	GC-MSMS	E1, E2, EE2	Influent, effluent	0.1–1.8 ng/L	NR	<0.5 –140 ng/L	[74]	2000
26	GC-MSMS	E1, E2, EE2	Effluent	1 ng/L	NR	<1–55 ng/L	[74]	2000
27	GC-MSMS	E2	Effluent	0.2–4 ng/L	NR	<0.1–4.05 ng/L	[74]	2000
28	GC-MSMS	E1, E2, EE2	Sludge, sediment	NR	0.2–2 ng/g	0.1–200 ng/g	[75]	2002
29	GC-ESI-MSMS	E1, E2, EE2, E3	Water	1 to <100 ng/L	NR	NR	[71]	2009
30	GC-MSMS	Various EDCs	Water samples	0.05–2.4 ng/L	NR	NR	[76]	2006
31	GC-MSMS	E2, EE2	Influent, effluent	0.1–1.8 ng/L	NR	Up to 10 ng/L	[35]	2000
32	GC×GC-TOF-MS	E1, E2, EE2	River sediment	0.4–2.5 ng/g	NR	Up to 150 ng/g	[37]	2007

Table 2 (continued)

Entry	Method	Steroid estrogens/hormones[a] (EDCs)	Matrix	LOD	LOQ	Range	Ref.[b]	Year
	LC							
33	LC-fluorescence	EE2	Effluent	4 ng/L	NR	NR	[77]	1999
34	LC-fluorescence	E1, E2, EE2, E3, sulfonated/ glucuronated derivatives	Influent, effluent, river, sea	2.7–8.3 μg/L	NR	NR	[78]	2004
35	LCMS	E1, E2, EE2, E3, sulfonated/ glucuronated derivatives	Influent, effluent, river, sea	0.07–10.6 ng/L	NR	NR	[71]	2009
36	LC-MS	E1, E2, EE2, E3	Effluent	4.2–19.2 μg/L	0.9–4.3 ng/L	1–200 ng/L	[79]	2000
37	LC-DAD-MS	E1, E2, EE2, E3,	Influent, effluent	2–500 ng/L	NR	NR	[74]	2000
38	LC/DAD-MS	E1, E2, EE2, E3,	Influent, effluent	10–200 ng/L	NR	NR	[74]	2001
39	UPLC-Q-TOF-MS	E1, E2, EE2, E3, Pro	Potable, river	0.25–0.50 ng/L	0.83–1.67 ng/L	0–50 ng/L	[38]	2012
	LC-MSMS							
40	LC-MSMS	E1, E2, EE2	Influent, effluent	0.1–1.08 ng/L	NR	Up to 10 ng/L	[35]	2000
41	LC-MSMS	E1, E2, EE2, E3	Influent, effluent	NR	0.08–0.6 ng/L	NR	[43]	1999
42	LC-MSMS	E1, E2, EE2, E3	Influent, effluent	NR	0.5–1 ng/L	NR	[80]	2000
43	LC-MSMS	E1, E2, EE2	Influent, effluent	0.4–0.7 ng/L	NR	1–100 ng/L	[81]	2007
44	LC-MSMS	E1, E2, EE2, pro, tes	Surface water, ground water	IDL 0.008–0.18 μg/L MDL: 0.01–0.20 ng/L	NR See MDL	NR	[82]	2008
45	LC-MS/MS	E1, E2, EE2, E3, pro, tes	Influent, effluent	NR	1–1500 ng/L	NR	[83]	2009
46	LC-MSMS	Various EDCs	Water samples	0.08–33 ng/L	NR	NR	[72]	2004
47	LC	Various EDCs	Water samples	0.064–1.2 ng/L	NR	NR	[76]	2006
48	LC/ESI-MS/MS	Various EDCs	Water samples	3.5–44 ng/L	NR	NR	[84]	2001
49	LC-MSMS	E1, E2, EE2, E3, sulfonated/ glucuronated derivatives	Influent, effluent, river, sea	0.0025–34 ng/L	NR	NR	[71]	2009
50	LC-MSMS	E1, E2, EE2	Influent, effluent, sludge	NR	1 ng/L	NR	[85]	2001
51	LC-MSMS	E1, EE2, E2, pro, tes	Influent, effluent, river water	0.03–1.0 ng/L	NR	0.005–100 ng/L	[36]	2011
52	LC-MSMS	E1, E2	NR	NR	NR	NR	[86]	2000
53	LC-MSMS	E1, E2, EE2	Effluent	<0.5 ng/L	<1.5 ng/L	NR	[39]	2013
54	LC-MSMS	E1, E2, EE2, E3	Influent, effluent, (and sludge)	NR	0.44–9.54 ng/L	0.01–5 μg/L	[87]	2011
55	LC-MSMS	E1, E2, E3	Water, influent, effluent	0.73–43 ng/L	NR	NR	[88]	2013
56	LC-MSMS	E1, E2, EE2, E3	Influent, effluent	0.2–0.5 ng/L	NR	30–500 ng/L	[35]	2000
	Capillary electrophoresis/related methods							
57	CE-UV	E1, E2, E3	Only standard solutions were analyzed	NR	NR	NR	[89]	1993
58	CE-TOF/MS	E1, E2, E3 and corresponding glucuronide/sulfate conjugates	Urine	±0.40 μmol/L	NR	10–20 μmol/L	[90]	2011
59	CE-UV	E1, E2, E3, 2-hydroxyestrone, 2-methoxyestradiol, 17-α-estradiol	Only standard solutions were analyzed	NR	NR	NR	[91]	1997
60	MEKC-PDA	EE2, gestodene	Pharmaceutical products	0.6–1.3 mg/L	1.8–3.9 mg/L	2–64 mg/L	[92]	1999
61	MEKC-PDA	E1, E2, EE2, E3	River water	6.8–7.4 mg/L	NR	20–70 mg/L	[93]	2000

Table 2 (continued)

Entry	Method	Steroid estrogens/hormones[a] (EDCs)	Matrix	LOD	LOQ	Range	Ref.[b]	Year
62	pCEC-UV	Diethylstilbestrol, hexestrol, dienestrol	Fish muscle	1.6–4.2 μmol/L	NR	(5–100)×10^{-5} mol/L	[50]	2005
63	dCPE-MEKC	Diethylstilbestrol, hexestrol, dienestrol	Lake water Tap water	7,900–8,900 ng/L	26,300–29,700 ng/L	50–5,000 μg/L	[94]	2013
	Immuno-analytical							
64	Immunoassay	E2	Serum	0.49 ng/L[c]	0.49–1.09 ng/L[c]	1.8–250.04 ng/L[c]	[13]	2002
65	Immunoassay	EE2	Effluent, other	1 ng/L	NR	NR	[95]	1989
66	RIA	E2	Influent, Effluent	2 ng/L	NR	NR	[96]	1993
67	RIA	E2, EE2	Effluent	0.053–0.107 ng/L	NR	NR	[97]	1999
68	ELISA	E2	Water	2.5 ng/L	NR	NR	[98]	2007
	ELISA	E1, E2, EE2	Wastewater, biological	NR	50 ng/L	50–5,000 ng/L	[46]	2004
69	ELISA	E1, E2, EE2, E3, Pro, tes	Influent, effluent, potable water, raw water	0.2–5 ng/L	5 ng/L	2–4,000 ng/L	[10]	2007, 2010–2014
	Recent publications							
70	GC-MS	E1, E2, EE2	Stream	2–80 ng/L	NR	NR	[99]	2013
71	ELISA	E1, E2, EE2, tes	Influent, effluent	±0.97 ng/L	NR	NR	[100]	2013
72	MEKC-MS	E1, E2, EE2, E3	Mineral water, run-off, wastewater	6.22–110 μg/L	56.2–263 μg/L	100–1,000 μg/L	[101]	2014
73	GCMS ELISA	E1, E2, EE2, E3 E1, E2, EE2	Sediment	NR	5–20 ng/g 0.43–1.8 ng/g	NR	[102]	2014
74	LC-DAD/FD	E1, E2, EE2	Ultrapure water mineral, wastewater	0.3–1.81 μg/L	1.01–6.05 μg/L	1.0–61 μg/L	[103]	2014
75	LC-MSMS	Pharmaceuticals	Influent, effluent	0.07–5.2 ng/L	0.24–11.0 ng/L	NR	[104]	2014

E1 estrone, *E2* 17-β-estradiol, *EE2* 17-α-ethinylestradiol, *E3* estriol, *Pro* progesterone, *Tes* testosterone, *CE* capillary electrophoresis, *CZE* capillary zone electrophoresis, *DAD* diode array detection, *dCPE* dual cloud point extraction capillary electrophoresis, *FD* fluorescence detection, *LVI* large volume injection, *MEKC* micellar electrokinetic chromatography, *PDA* phototodiode array detection, *Ref.* reference, *RIA* radioimmunoassay, *TOF* time-of-flight, *NR* not reported (no data provided)

[a] In very few reports, other EDCs were also quantified but are omitted here

[b] References 68–106 are given in Electronic Supplementary Material Text S4

[c] Actual data provided in pmol/L units

Chemical-analytical GC methods

The chemical-analytical GC-MS method (Table 2, entries 1–22) is the most widely applied technique for the determination of steroid estrogens and progestogens in water, and is the most popular of all complex techniques for GC. GC-MSMS (Table 2, entry 23–32) is a hyphenated technique combining GC with tandem MS. Although various ionization methods are available, electron impact (EI) and chemical ionization (CI) are the most common for GC-MS analysis.

With the use of MS-MS instruments, the selectivity of the analysis is increased not only by a specific mass quantitation, but the specific mass can be related to a specific fragmentation of the product ions.

Chemical-analytical LC methods

As a result of limited sensitivity, fewer reports exist on methods for environmental analysis of the steroid hormones by LC using detectors other than MS (DAD, PDA, and UV) (Table 2, entries 33, 34, 37, 38). The use of spectrophotometric techniques, including diode array detectors (DAD), is common in HPLC systems, but high sensitivity determination in a very low concentration range (nanogram per liter), such as environmental samples, has not emerged. The adequate techniques of choice for analysis of the steroid hormones are LC-MS (Table 2, entries 35–39) and LC-MSMS (Table 2, entries 40–55). Before the advent of LC-MS, many of these polar compounds were difficult and sometimes impossible to measure.

In the last decade, LC-MS has experienced impressive progress, both in technology development and application. Interface designs have changed considerably and have become much more sophisticated and efficient. Today, the interfaces most widely used for LC-MS analysis of steroid hormones in the aquatic environment are ESI and APCI. LC-MS and LC-MSMS have been mostly applied in the selected ion monitoring (SIM) mode.

Chemical-analytical MS-based methods

High accuracy is a well-known attribute of MS because it is a very specific technique, owing to the mass spectrometric (or mass-selective) detector measuring the mass to charge ratio (m/z) of ions derived from fragmentation of the parent compound; use of stable isotope standards can be used to correct for recovery. The high overall specificity is achieved by one of the following methods: use of a chromatographic, electrophoretic, immunoextraction, or other resolving technique prior to MS detection; use of a high resolution form of MS, such as a dual-sector, time-of-flight (TOF), or ion cyclotron resonance instrument; use of one of several forms of tandem MS. In addition, many organic compounds can be analyzed, confirmed, and quantified simultaneously with LC-MS.

Capillary electrophoresis and other chemical-analytical techniques

For capillary zone electrophoresis (CZE), and related techniques, like micellar electrokinetic chromatography (MEKC) (Table 2, entries 57–63), the observed LOQs are, at best, in the milligram per liter down to microgram per liter range, even with an MS detector, as compared to the lower nanogram per liter range obtained by GC/LC-MS and ELISA. Such techniques (CZE, MEKC, etc.) appear to be extended to the analysis of other steroids, like diethylstilbestrol, hexestrol, and dienestrol. The apparently fewer number of reports regarding use of these techniques reflects their relatively limited application in this area, and this observation may be most likely due to their inadequate sensitivity.

The immuno-analytical ELISA method

ELISA is the abbreviation for enzyme-linked immunosorbent assay. It is a useful and powerful immuno-analytical method that can be used for estimating nanogram to picogram per milliliter levels of analytes in solution, such as in serum, urine, sperm, culture supernatant, water, and wastewater matrix (Table 2, entries 64, 65, 68, 69, 71, 73). The ELISA method has been widely used in life sciences research [11]. Compared to MS, the ELISA method requires specific primary, and secondary, antibodies. The sample preparation can be complicated with extended incubation times.

The immuno-analytical enzyme immunoassay (EIA) and ELISA methods are both widely used as diagnostic tools in medicine and as quality control measures in various industries; they are also used as analytical tools in biomedical research for the detection and quantification of specific antigens or antibodies in a given sample. These two procedures share similar basic principles and are derived from the immuno-analytical radioimmunoassay (RIA) method. RIA was first described by Berson and Yalow (1960), for which Yalow was awarded the Nobel Prize in 1977, to measure endogenous plasma insulin. RIA was then developed into a novel technique to detect and measure biological molecules present in very small quantities, paving the way for the analysis and detection of countless other biological molecules, including hormones, peptides, and proteins. Because of the safety concern regarding its use of radioactivity, RIA assays were modified by replacing the radioisotope with an enzyme, thus creating the modern-day immuno-analytical EIA and ELISA methods.

EIA/ELISA methods use the basic immunology concept of an antigen binding to its specific antibody, which allows detection of very small quantities of antigens such as proteins,

peptides, hormones, or antibody in a fluid sample. EIA and ELISA utilize enzyme-labeled antigens and antibodies to detect the biological molecules, the most commonly used enzymes being alkaline phosphatase and glucose oxidase. The antigen in fluid phase is immobilized, usually in 96-well microtiter plates. The antigen is allowed to bind to a specific antibody, which is itself subsequently detected by a secondary, enzyme-coupled antibody. A chromogenic substrate for the enzyme yields a visible color change or fluorescence, indicating the presence of antigen. Quantitative or qualitative measures can be assessed on the basis of such colorimetric reading. Fluorogenic substrates have higher sensitivity and can accurately measure levels of antigen concentrations in the sample.

The basic steps are the following: (1) Antigens are coated onto the ELISA plate. (2) The sample containing primary antibodies is added. (3) Non-antigen-binding antibodies are washed off the plate. (4) Secondary antibody-conjugated with an enzyme is added. (5) The excess secondary antibody is washed off the plate. (6) Chromogen (substrate for the enzyme) is added. (7) The enzyme reacts with the substrate producing color; intensity of the color correlates with the level of antigen.

The key step in the ELISA assay is the direct or indirect detection of antigen by adhering or immobilizing the antigen or antigen-specific capture antibody, respectively, directly onto the well surface. For sensitive and robust measurements, the antigen can be specifically selected out from a sample of mixed antigens via a "capture" antibody. The antigen is thus "sandwiched" between such capture antibody and a detection antibody. If the antigen to be measured is small in size or has only one epitope for antibody binding, a competitive method is used in which either the antigen is labeled and competes for the unlabelled antigen–antibody complex formation, or the antibody is labeled and competes for the bound antigen and antigen in the sample. Each of these modified techniques of ELISA can be used for a qualitative and quantitative purpose.

Various types of ELISA methods have been employed with modification to the basic steps: indirect ELISA, sandwich ELISA, competitive ELISA, and multiple-portable ELISA [11].

Sample preparation

The chemical-analytical test methods usually comprise the following preliminary steps: sampling, storage, sample preparation, extraction, followed by clean-up, analysis (off-line and on-line solid-phase extraction, SPE) and chromatographic separation with selective detection–mass spectrometry (MS). Other related chemical-analytical techniques are LC-MS [32], GC-MS [33,34], GC-MSMS [35], LC-MS-MS [36], orthogonal GC (GC × GC) [37], coupled with HSTOF-MS [37], and UPLC-QTOF-MS [38] (Table 2). The use of immunoassay has also been reported [10,13].

To improve stability of the target compounds, and also the precision and the sensitivity of the GC analysis, the sample extract is usually derivatized. Derivatization is carried out in the case of thermolabile, polar, and low volatile compounds, such as the steroid estrogens, to prevent or minimize thermal decomposition and to improve chromatographic separation and the sensitivity of the analysis. Unfortunately, there is sometimes a loss of the sample during the additional manipulation. Thus, the chemical-analytical LC-MS and LC-MSMS methods have some benefits over GC-MS analysis of the estrogen in environmental water. The immuno-analytical ELISA method does not require prior derivatization. LC is not limited by factors like non-volatility and high molecular weight, and enables determination of both conjugated and unconjugated estrogens without the need for derivatization. GC is applicable only to volatile steroid estrogens and not to non-volatile ones, such as the conjugated estrogens.

Screening

The chemical-analytical chromatographic techniques enable simultaneous screening of both the free steroid hormones and their conjugates. Biological methods, like the immuno-analytical immunoassay, are limited in the requirement for a separate kit for each steroid hormone. To aid in determining whether GC-MSMS or LC-MSMS methods should be done on a sample, preliminary screening of the groundwater samples for the presence of steroid hormones can be done using immuno-analytical ELISA methods or other chemical-analytical assays.

Specificity

Some researchers consider that using the chemical-analytical MS system in SIM mode, or MS-MS, is enough for confirmation. However, if there are interfering ions, or only one transition is monitored, the results cannot be considered valid. The use of MS-MS provides added specificity, which is necessary when analyzing samples of an increased matrix complexity. There are, however, significant differences between MS interfaces, e.g., APCI-derived spectra, via LC, are more limited than EI spectra, via GC-MS. The use of high accuracy mass spectrometers, such as TOF and hybrid quadrupole (Q-TOF), allows result confirmation.

Examination of the ELISA kits brochures used in this study for specificity data indicate that the estradiol ELISA kit measures the free estradiol, and has cross-reactivity for E3 (0.05 %), and E2 (0.2 %). The estrone ELISA kit measures free estrone, and has cross-reactivity for estrone-3-sulfate (4.9 %), E2 (2.2 %), estrone-3-glucoronide (1.2 %), and E2-3-glucoronide (0.14 %). The Estriol ELISA kit measures free

estriol; cross-reactivity, at 40 ng/mL estriol, for testosterone, E2, E1, and cortisol was not significant ("non detectable"). The EE2 ELISA kit measures free EE2, and showed less than 0.2 % cross-reactivity for E1, E2, 16-keto-E2, E2-17-glucuronide, E2-3-glucuronide, E2-3-sulfate-17-glucuronide, E3, 16-epi-E3, and E3-16-glucuronide.

It has been shown that the mean proportion between free and total steroid estrogens is 80–90 % in influent samples, and is lower in treated wastewater (57–93 %) [39]. As a result of higher variability for the latter, and because of the different treatment processes, it is therefore recommended that testing of influent samples requires a deconjugation step during analysis, especially when evaluating removal rates at the WTP. The implication of these figures is that, in the absence of a deconjugation step in sample preparation, steroid estrogen test results can be underestimated by ±10–20 % for influent samples, and by ±7–43 % for effluent samples.

When calculating the percentage composition (based on the total steroid hormone load) of pro and tes in the raw wastewater (influent) at the Darvill WTP [9], we found that the levels were 41 % and 35 % respectively, which is comparable to what has been reported in the literature. On the basis of the estimates done by Shore and Shemish [40], using human urine excretion data, E1 and E2 levels should account for about 50 % of the observed steroid estrogen levels in the raw wastewater (influent). However, the data for the Darvill WTP indicates a much lower figure of about 21 %. This demonstrates the importance of steroid conjugates, which can account for up to 50 % of the total steroid estrogen concentration in the raw wastewater (influent) [40,41], not detected by the current ELISA method used in this study. Thus, provided wastewater samples are appropriately treated to ensure conversion of conjugated to free steroid estrogen, the immunoanalytical ELISA method is able to selectively measure the steroid estrogen.

Sensitivity

A large number of the EDCs generally exhibit their "disruptive effects" at relatively low concentration (nanograms per liter), compared to other, similar toxic chemical compounds. It is therefore required that, in order to meet environmental requirements, such assay test methods employed must be fairly accurate at these rather low concentrations.

Regarding some of the reported analytical methods, listed in Table 2, like the chemical-analytical GC, LC, and the immuno-analytical immunoassay, the more significant LOQ data are not reported for many of the studies.

On the basis of LOQ, the literature indicates the superior sensitivity of the chemical-analytical GC-MS [42] and LC-MSMS methods [43] for quantifying steroid hormones in water matrix, as attested by the internationally reported limits of 0.06 ng/L and 0.08 ng/L, respectively (Table 2, entries 7, 41). The corresponding LOQs for the immuno-analytical immunoassay and ELISA methods are 0.49 ng/L (on serum sample) and 5 ng/L, respectively (Table 2, entries 64, 69).

Regarding the measures of LOQ and LOD, the former is generally a more accurate, and quantitative, measure, as it is generally validated with acceptable accuracy (e.g., 80–120 % recovery) and precision (at most 10 % RSD) (Umgeni Water standard operating procedure 20b) [44]. The latter measure of LOD is less meaningful, quantitatively. For chromatographic test methods, the signal to noise ratios of 10:1 and 3:1 can be used for the determination of the LOQ and LOD, respectively. The IUPAC method [45] uses the mean concentration and standard deviation (SD) from replicate analysis of a "blank" (ultrapure water) sample matrix, as per the following equations: mean+10 SD, for LOQ, and mean+3 SD, for LOD. This statistical approach, however, cannot be applied when a negative value is observed for the blank signal response.

Although sensitive chemical-analytical LC-MSMS methods using ESI and APCI are available, both of these ionization techniques can be susceptible to response of a loss due to ion suppression caused by matrix effects present in complex samples. Steroid hormone analysis by ESI-MS encounters three general problems: its response is often analyte-dependent, conditions in the overall system for highest sensitivity of each analyte may be different, and response can be very dependent on analyte purity (e.g., as an HPLC peak), which gets worse with high throughput (fast HPLC). These are due to increased matrix effects that suppress or enhance analyte signal; it was found that APCI can also have this problem.

Accuracy

Hirobe et al. [46] reported the development of ten kinds of the immuno-analytical ELISA methods and did a comparison between the immuno-analytical ELISA method with the chemical-analytical HPLC, GC-MS, and LC-MSMS methods, noting good correlations between the immuno-analytical ELISA method and the common chemical-analytical instrumental methods in all cases. The overestimation caused by matrices in environmental samples, considered to be an inherent problem with some immuno-analytical ELISA methods, can be eliminated when a proper cleanup method is adopted. They concluded that appropriate choice of antibody and proper sample pretreatment significantly reduced overestimation. For EDCs and the steroid estrogen ELISA methods, dichloromethane was selected as solvent for SPE, eluting as much target compound as possible, while minimizing elution of substances that might block the immunochemical reaction.

More recently, our comprehensive study findings [9,12] compare fairly well with similar, international studies and

Table 3 Test center for routine chemical- and immuno-analytical assay of the selected steroid estrogens/hormones in wastewater matrix in South Africa

Technique	Equipment//model	Estimated cost (rand)[a]	Laboratory/ equipment supplier	Steroid hormone	LOD (ng/L)[b]	LOQ (ng/L)[b]	Range (ng/L)[b]	Cost of assay (rand)	Established routine analytical service for		Ref.
									Water matrix	Wastewater matrix	
ELISA	Plate reader, vacuum manifold	100,000	Laboratory A: UWC[c]	E1	0.2	5	15–2,000	624	Yes	Yes	[9,10,54]
				E2	1	5	25–2,000	504	Yes	Yes	
				E3	1	5	3–1,000	624	Yes	Yes	
				EE2	5	5	5–1,250	1,164	Yes	Yes	
				Pro	3	5	3–4,000	1,164	Yes	Yes	
				Tes	2	5	2–1,600	624	Yes	Yes	
				(Total)				(4,704)			
LC-MSMS	Trap 1	4,000,000	Laboratory B	E1		10	10–200	1,350[d]	No	No	[55][e]
				E2		10	10–200		No	NO	
				EE2		10	10–200		No	No	
				E3		10	10–200		No	No	
LC-MS-TOF	TOF	5,345,000	Laboratory B	Tes[f]		±500		950	No	No	[55]
				Tre[f]		±500		950	No	No	
LC-MSMS	Trap 2	2,975,997	Laboratory C	E1					No	No	[55]
				E2	10 μg/L	100 μg/L		1,538[g]	Yes	Yes	
				EE2	10 μg/L	100 μg/L			Yes	Yes	
				E3	10 μg/L	100 μg/L			Yes	Yes	
				Pro	10 μg/L	10–50 μg/L			Yes	Yes	
				Tes					No	No	
LC-MSMS	Trap 3	4,598,000	Equipment supplier A[h]	E2	±5–10	±20–50					[h]
	Quad	4,388,000		EE2	±5–10	±20–50					
				E3	±5–10	±20–50					
				Pro	±5–10	±20–50					
				Tes	±5–10	±20–50					
LC-MSMS	AB Sciex: AB SCIEX 3200 Q Trap		Laboratory D: Rand Water[i]	E1	0.07	0.24	0–1	4,500[j]	No	No	[56]
				E2	0.10	0.32	0–1		No	No	
				EE2	0.07	0.24	0–1		No	No	
				E3					No	No	

Table 3 (continued)

Technique	Equipment//model	Estimated cost (rand)[a]	Laboratory/ equipment supplier	Steroid hormone	LOD (ng/L)[b]	LOQ (ng/L)[b]	Range (ng/L)[b]	Cost of assay (rand)	Established routine analytical service for		Ref.
									Water matrix	Wastewater matrix	
				Pro					No	No	
				Tes					No	No	
GC-MS (LVI)	Agilent: 7890A GC/5975 MSD	1,551,000	Equipment supplier B: Chemetrix[k]						No	No	[k]

*E1*estrone, *E2* 17-β-estradiol, *EE2* 17-α-ethinylestradiol, *E3* estriol, *Pro* progesterone, *PTV* programmable temperature vaporization, *Ref.* reference, *Tes* testosterone, *Tre* trenbolone, *TOF* Time-of-flight, *LVI* large volume injection

[a] Actual price will depend on configuration and associated HPLC unit/other accessories

[b] Units ng/L, unless otherwise stated

[c] University of the Western Cape; data applicable to water and wastewater

[d] For all four steroid hormones

[e] Data based on drinking water; method not yet validated/optimized for influent/effluent matrix

[f] Test not yet available: can be done but requires some prevalidation/method development work

[g] For all four steroid hormones; method not validated

[h] Data provided are estimates, for water; not yet validated

[i] Data based on environmental (river and potable) water matrix

[j] On special request only, includes 17-α-estradiol isomer; not validated for wastewater

[k] Agents for Agilent in South Africa (Mr N Solai); Service/test by GCMS not currently available in S Africa

are additional confirmation of fairly acceptable accuracy of the immuno-analytical ELISA technique.

Day-to-day precision

The significance of our recent comprehensive screening study of the steroid hormones in raw/treated wastewater/receiving river using the immuno-analytical ELISA method [9] needs to be critically reviewed in the light of other similar studies, using the conventional chemical-analytical instrumental methods, like LC-MSMS. Equally important factors to consider are the sampling period, composite vs. grab sampling, time of sampling, reproducibility imprecision of the immuno-analytical ELISA technique used for assay (as opposed to the commonly used chemical-analytical LC-MSMS/GC-MS methods), rainfall, storm water management, etc.

Although the study by Chang et al. [36] reported under 12 % RSD (reproducibility) for spiked samples over a 10-day period, using the chemical-analytical LC-ESI-MS/MS method, the actual variation for the test results on free steroid hormone levels (estrogen, progestogen, androgen) in real samples (influent/effluent) is not reported. Using flow proportional samplers, 24-h composite samples were collected every day, over a 3-week period. Examination of their data, over a 3-week period, gives a calculated mean RSD of 40 % for E1 (range 15–113 %), 73 % for E2 (50–106 %), 52 % for EE2 (14–91 %), 48 % (26–96 %) for tes, and 50 % (14–82 %) for pro for the influent (overall mean 53 %, range 14–113 %). The corresponding % RSD for the effluent samples was 60 % for E1 (range 12–120 %), 115 % for E2 (88–150 %), 152 % for EE2 (125–200 %), 72 % (20–200 %) for tes, and 25 % (13–23 %) for pro for the influent (overall mean 85 %, range 12–200 %).

Using the chemical-analytical GC-HRMS method to analyze municipal wastewater in Canada for steroid estrogenic organic contaminants, like E1, E2, and EE2, Fernandez et al. [47] reported 5–20 % RSD for most compounds, but higher RSD of 100 % for E2 and 66 % for E3; grab and composite samples were collected over 1–12 weekly intervals. The calculated RSD for steroid estrogens (E1, E2, E3, EE2) in sewage treatment plant effluents by Bellet et al. [48], using the chemical-analytical GC/MS, was 3–140 %; grab samples were collected, over 9 days, in the morning and composited. Nie et al. [49] sampled at 0200 to 0400 hours, for just 1 day during each season, because the hourly estrogenic fluctuation was insignificant, except in the morning [50].

In a study in France [39], 24-h flow proportional, composite samples, for up to 3 days, were collected to assess inter-day concentration variability of steroid estrogens and blockers in WTPs; sampling was not done in summer in order to obtain comparable results. The authors measured free and total steroid estrogen (by an enzymatic hydrolysis/deconjugation step during sample preparation) using the chemical-analytical LC-MSMS method. Despite these measures taken, the observed % RSD was 48–58 % for free/total steroid estrogen (E1, E2, α-E2, E3) in influent samples, and 56–166 % for free/total steroid estrogen in effluent samples. Again, whilst Bellet et al. [48] reported influent steroid estrogen values, the variation is not shown. Grab samples were collected in winter, over 1 h in the morning and were composited. The data gives a calculated RSD figure of 4–8 % for conjugated E1, E2, EE2, and E3 in influent, and 3–24 % for unconjugated steroid estrogen, in influent, using the chemical-analytical GC/MS method.

For their chemical-analytical LC-MS/MS method, the analytical uncertainty is evaluated at 20–30 % for high steroid estrogen concentration in influent/effluent, and can reach 50–100 % for concentrations between LOQ and 10 LOQ [39]. Some molecules (such as E1) can be produced during the wastewater treatment process by degradation of others (e.g., E2, EE2).

The grab samples collected, over 2 years, in our study [9] were during the morning period, between 0700 and 1200 hours, a peak time for domestic consumption (e.g., for bathing, laundering, etc.) and sewer discharge, which will be expected to contribute to larger variation in the wastewater volumes, and concentration of estrogens, entering the WTP. We similarly noted slightly higher, overall % RSD for the effluent samples (mean 80 %, range 0–109 %), compared to the influent samples (mean 75 %, range 54–115 %) (Table 1) using the immuno-analytical ELISA method. For E2, we noted 70 % RSD for the influent, and 155 % for the effluent. For the data reported by Chang et al. [36], the calculated mean RSD for E2 is 73 % for the influent, and 115 % for the influent, confirming the observation by Gabet-Giraud et al. [39]. We noted a maximum RSD of 115 % for E1 in the influent and a maximum of 155 % for E2 in the effluent. The results by Chang et al. [36] give a calculated maximum RSD of 73 % for E2 in the influent and 115 % for EE2 in the effluent.

The data in Table 1 indicate significant variation in the steroid hormone concentrations, obtained by the immuno-analytical ELISA method, received in the raw sewage (RSD 47–115 %). However, compared to conventional chemical-analytical instrumental methods, like LC-MS-MS and GC-MS [24,36,51], the observed RSD values vary over a wide range (±14–113 %, 48–166 %, and 5–100 %), for both influent and effluent samples. One factor that can contribute to the observed imprecision of the detection method used is the setting surrounding sample pretreatment. On the other hand, when looking at the data for EE2, the 97 % RSD value implies sporadic occurrences, and may be due to it not being released at such an elevated and constant rate from a population, compared to the other steroid hormones [52].

Despite the high variation (% RSD) noted for the steroid hormone influent/effluent levels, our results using the

immuno-analytical ELISA method confirm many previous study conclusions where other chemical-analytical instrumental methods were used, attesting to its accuracy.

Cost and technical requirements

It is evident that the chemical-analytical techniques, such as HPLC, GC-MS, LC-MS, and LC-MSMS, are generally employed for quantification of EDCs and steroid hormones in environmental samples. Although highly reliable, they have several potential drawbacks, including expensive instrumentation, large sample volume, extensive purification, utilization of large amounts 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 the steroid estrogens, and other steroid hormones, such as the immuno-analytical ELISA method.

In 2008, the US EPA recognized that the immuno-analytical ELISA method could be a beneficial technique for EPA regional offices, EPA ORD, state and local clients in that it is potentially much faster and more cost-effective (US$17–25 per sample) than the traditional chemical-analytical GC-MS (US$500–900 per sample) and LC-MS methods, and planned a verification study of ELISA kits, to accurately and reliably measure selected EDCs in environmental samples, in comparison to GC-MS methods. Two of their Environmental Technology Verification Reports, published in 2009, investigated the Abraxis Ecologenia EE2 ELISA and the Abraxis E2 Magnetic Particle ELISA kits [51,53].

Advantages and disadvantages of the immuno-analytical ELISA method

The immuno-analytical ELISA method does offer considerable advantages over conventional chemical-analytical analytical procedures because of its sensitivity, its speed, it requires small sample volumes, it has wide applicability, the ease of handling (no special skills necessary), the ease of pretreatment procedures, relatively cheap machines, which do readings, are also available in portable format for field studies, relatively fast measurement, high sample turnover, low quantification limit and acceptable costs, and can thus contribute to routine monitoring of environmental pollutants, as shown in this study. Some disadvantages include not being 100 % specific, it is vulnerable to cross-reactivity, requires independent confirmation (e.g., HPLC/GC-MS/MS), is not suitable for small sample loads, synthesis of antibody can be difficult, and only one substance/analyte can be analyzed at a time.

Current applicable international analytical techniques

A survey of the most current literature indicates fairly equal use of the chemical-analytical LC, GC-MS and the immuno-analytical ELISA test methods, for screening and quantitation of the target steroid hormones (E1, E2, EE2, E3, tes, pro), and related EDC compounds, like the pharmaceuticals in wastewater/water (Table 2). However, LOQ data and/or range is not reported for many test methods, where the reported LOD values imply detection at very low nanogram per liter levels. For any test method validation, the observed recoveries from spiked ultrapure water (raw, potable) matrix is, generally, superior to that obtained with relatively complex matrix, like raw sewage, or wastewater (influent). The observed method sensitivity, based on reported LOQ (ng/L), application to relevant wastewater, and/or water, matrix, and quantitation of at least E1, E2, and EE2 (Table 2), is, in decreasing order, LC-MMS (0.08–9.54) > GC-MS (1) (chemical-analytical) > ELISA (5) (immuno-analytical); based on observed LOD, the decreasing order is LC-MSMS (at least 0.0025) > GC-MS (at least 0.5) (chemical-analytical) > ELISA (at least 0.2) (immuno-analytical). The LOQ and LOD for the immuno-analytical ELISA method are 5 ng/L and 0.2–5 ng/L, respectively. On the basis of observed LOQ, the immuno-analytical ELISA method reported here is thus approximately 5–63 times less sensitive than the chemical-analytical GC/LC-MS/MS methods. Thus, it is evident that the hypothesis that the conventional chemical-analytical GC/LC-MSMS methods, internationally, are frequently used, is found to be valid.

Test centers and test methods for steroid hormone analysis at national level (South Africa)

In South Africa, test centers able to routinely analyze for the steroid hormones in both water and wastewater are rather limited. The common techniques available here (Table 3), as a routine service, are only the immuno-analytical ELISA method [10] (Table 3), by laboratory A, and the chemical-analytical LC-MSMS method, by laboratory C. Whilst the former immuno-analytical ELISA method has been fully validated for both water and wastewater matrix, the latter chemical-analytical LC-MSMS method has not.

The estimates of capital costs of analytical equipment for steroid hormone analysis in South Africa, obtained by suppliers, indicate that the chemical-analytical GC-MS and LC-MSMS methods are approximately 16-fold and 30–53-fold higher than that required for the immuno-analytical ELISA method. In a Third World country, like South Africa, such access to financial capital, by both the private and public sector testing laboratories can be a challenge. Comparison of the actual analysis costs indicates that the immuno-analytical ELISA method costs approximately two times as much as

the chemical-analytical LC-MSMS method to analyze a sample for the four steroid hormones: E2, EE2, E3, and pro. However, the immuno-analytical ELISA method cost analysis is comparable to that offered by laboratory D, for the chemical-analytical LC-MSMS method, but this latter service is only on an exceptional, "non-routine" basis.

Although very good LOQs (0.24–0.32 ng/L for E1, E2, EE2) were achieved by laboratory D (Rand Water) [54], in South Africa, on their developed chemical-analytical SPE-LC–tandem mass spectrometry method, using the AB Sciex 3200 Q Trap, this work was done only on river and potable water matrix. A recent technical application note [55], on the AB Sciex 4000 LC-MSMS instrument, shows method reporting limit (MRLs) of 0.1–2 ng/L for tes, E1, E2, EE2, and E3; this chemical-analytical method was, however, set up for drinking water matrix. Another reported use of the AB Sciex QTRAP 6500 and Triple TOF 5600 LC-MSMS instruments describes a chemical-analytical LC-MSMS test method for analyzing 80 EDCs and pharmaceutical and personal care products (PPCPs) in various water samples [56]; again, no data are reported for wastewater matrix.

For the unoptimized chemical-analytical LC-MSMS method (laboratory C), offered as a routine service, from the very little current information provided to date, and based on unvalidated data, an initial comparison of the LOQs show that the immuno-analytical ELISA method (LOQ 5 ng/L) is apparently more sensitive by a factor of 2,000–20,000 compared to LC–tandem mass spectrometry (LOQ 10–100 μg/L) (Table 3). For E1 and E2, 0.2 and 1 ng/L can be detected by the immuno-analytical ELISA method, while the corresponding reported limits of quantitation are generally, at best, ±10 μg/L for the chemical-analytical HPLC-MSMS technique. It must, however, be noted that the Q Trap, used by laboratory C, is an "entry level" model, compared to that used by laboratory B. For the non-routine service offered by laboratory D (chemical-analytical LC-MSMS method) [54], which was validated for water matrix only, LOQ comparison indicates that sensitivity of the LC-MSMS method (0.24 ng/L) is much better (lower), by a factor of approximately 21, than that of the immuno-analytical ELISA method (5 ng/L).

Comparison of the immuno-analytical ELISA and the chemical-analytical LC-MSMS method performance at national level

Our earlier comparative study of ELISA and LC-MSMS showed that the ELISA technique is more sensitive and more accurate [9] (Table 4). In that preliminary study, we sent eight samples: unspiked raw wastewater (influent), treated wastewater (effluent), samples taken up- and downstream of the wastewater treatment, and the corresponding spiked samples, at 20 ng/L of steroid estrogens E1 and E2, to two national test centers; one using ELISA (laboratory A) and the other, tandem mass spectrometry equipment (laboratory B). For ELIS A, the observed mean recovery±SD, based on duplicate sample testing, was 86±68 % (range 25–180 %) (16±8 % RSD) for E1 and 105±39 % (range 65–155 %) for E2 (22±19 % RSD). The observed recovery by the LC-MSMS method, by laboratory B, was 50±67 % (range −10 to 130 %) (50±67 % RSD) for E1 and 0 % for E2, for all spiked samples.

As some steroid estrogens display endocrine disruptor effects at concentrations below 1 ng/L, the LOQ obtained by this immuno-analytical ELISA method may not be fully adequate for meeting environmental quantification requirements. However, detection is possible at sub-nanogram per liter level, as the LOD is 0.2–5 ng/L.

Comparison of the developed immuno-analytical ELISA national method with similar international reports

Table 2 (entry 64) shows that although very good LOQ (0.49–1.09 ng/L) was reported for immunoassay, the matrix was blood serum. Other similar, reported immuno-analytical methods (entries 65–68, 71) do not provide the LOQ and/or LOD/range data. The report by Stewart et al. [57], with LOQ of 0.43–1.8 ng/g, is on sediment sample. The study by Hirobe et al. [46] reported an LOQ of 50 ng/L. The current immuno-analytical ELISA method reported (entry 69) here has been fully validated ESM Table S2 for all types of water matrix (wastewater influent/effluent, river, dam, industrial effluent). It is further applicable to six priority steroid estrogens (E1, E2, EE2, E3, tes, pro), with the following performance characteristics: range 2–4,000 ng/L, mean precision 7–9 % RSD, mean recovery 96 %, LOD 0.2–5 ng/L, and LOQ 5 ng/L. Its successful application has been adequately demonstrated in our national studies at the Darvill WTP [9,12].

Measures of biological activity vs. measures of individual chemical compounds

The mechanism of action of steroid hormones (EDCs) [58] is fairly well understood. The generally accepted paradigm for receptor-mediated responses involves a hormone binding to its receptor at the cell surface, in the cytoplasm or within the nucleus, followed by a complex series of events within the classical genomic pathway that lead to interaction of receptors with the DNA by binding to hormone response elements in the target gene promoter area. Off-target effects as well as cross-talk may occur as many transcription factors modulate transcription in a DNA-binding-independent fashion; because this mechanism is established, in vitro bioassays have been developed that can indicate whether compounds (e.g., EDCs) are binding to the receptor.

In addition to chemical-analytical and immuno-analytical methods which are intended to detect individual compounds,

Table 4 Preliminary comparative, national performance study on the immuno-analytical ELISA and chemical-analytical LC-MSMS test methods: precision and accuracy

Analytical method (laboratory)		Immuno-analytical ELISA (laboratory A)				Chemical-analytical LC-MSMS (laboratory B)			
Sample number	Sample site/description	E1(ng/L)	E1 (% recovery)	E2 (ng/L)	E2 (% recovery)	E1 (ng/L)	E1 (% recovery)	E2 (ng/L)	E2 (% recovery)
1	Darvill[a] influent	39		57		<10[b]		<10[b]; <10[c]	
2	Darvill influent duplicate	45		57		<10		<10	
	Mean	42		57					
	SD	4		0					
	% RSD	10		0					
3	Darvill influent spiked[d]	78	180	70	65	<10	0	<10	0
4	Darvill effluent	13		22		<10; 15[c]		<10; <10[c]	
5	Darvill effluent	18		14		<10		<10	
	Mean	16		18					
	SD	4		6					
	% RSD	25		33					
	Darvill effluent spiked[d]	34	90	41	115	31	80	<10	0
7	Duzi upstream[e] Darvill	3		3		<10; 14[c]		<10; <10[c]	
8	Duzi upstream Darvill spiked[d]	13	50	34	155	12	−10	<10	0
9	Duzi downstream[e] Darvill	5		5		<10; 15[c]		<10; <10[c]	
10	Duzi downstream Darvill spiked[d]	10	25	22	85	41	130	<10	0
11	Pilot plant permeate[f]	34		7		<10		<10	
12	Pilot plant permeate duplicate	29		11		<10		<10	
	Mean	32		9					
	SD	4		3					
	% RSD	13		33					
	Overall	Precision	Accuracy	Precision	Accuracy	Precision[g]	Accuracy	Precision[g]	Accuracy
		10	180	0	65		0		0
		25	90	33	115		80		0
		13	50	33	155		−10		0
			25		85		130		0
	Mean	16	86	22	105		50		0
	SD	8	68	19	39		67		

E1 estrone, *E2* 17-β-estradiol

[a] Darvill wastewater treatment plant

[b] LOQ

[c] 2 replicates

[d] At 20 ng/L for both E1 and E2

[e] Sample sites up- and downstream of the Darvill WTP

[f] Wastewater reclamation project in progress

[g] Not possible to calculate

biological methods, also known as bioassays, can measure total estrogenic and androgenic activity resulting from all the EDCs present in a water body, including unknowns. As the effects of chemical mixtures cannot always be elucidated from their concentrations, bioassays are an important component of examining the presence of, and integrating the effects of, complex mixtures of EDCs.

Bioassays are generally are significantly more sensitive than chemical-analytical and immuno-analytical assay methods. In addition they provide a combination of potency and dose; more importantly, they require no prior knowledge of the specific chemical nature of a sample. The option of testing a water sample for endocrine-disrupting activity using one or more of the available bioassays (ESM Table S5) is a

more feasible option where one obtains biological measures of exposure or biomarkers.

The in vitro bioassays (also called effect-directed bioassays or bioanalytical tools) are now recognized as sensitive monitoring tools to screen for contaminants on the basis of their biological action. As the specific chemical composition of a sample is often unknown, and mixture effects cannot be detected by chemical/immuno-analytical methods, in vitro bioassays are highly suitable tools to examine the presence of complex mixtures of low concentrations [59]. A number of in vitro (and in vivo) bioassays have been developed for estrogenicity, including mammalian cell- or yeast-based reporter gene assays, or proliferation assays of estrogen-responsive cells [60] (see ESM Table S5). The ERα CALUX reporter gene bioassay has good sensitivity and reproducibility [60] , and recently other CALUX in vitro bioassays for the detection of endocrine activity have been developed, which are able to detect androgenic, glucocorticoid, or progestogenic activity [60,61]. These recent CALUX bioassays consist of U2OS human osteosarcoma (bone cancer) cells, which are transfected with a luciferase gene under control of a specific endocrine receptor, such as the estrogen (ERα), androgen (AR), glucocorticoid (GR), and progesterone (PR) receptors [62].

Both chemical- and immuno- (compound-directed) analysis provides a method of absolute quantification of certain compounds in water samples, but the toxicological properties of these compounds are not always known. CALUX bioassays do not discriminate between different specific compounds but detect the total specific endocrine activity, and so the concentration of endocrine activity is expressed as an equivalent (eq) relative to a potent reference compound, e.g., 17-β-estradiol (E2) for ERα- (estrogen receptor), dihydrotestosterone (DHT) for AR- (androgen), dexamethasone (DEX) for GR- (glucocorticoid), and Org2058 for PR-(progesterone)-mediated activity [62].

By using in vitro bioassays, the combined biological activities of the mixture can be quantified and expressed as nanogram equivalents of a reference compound per liter. This enables unknown compounds to be detected by their activity and provides toxicological relevance (i.e., specific endocrine activity) of this mixture. Together with the sensitivity and robustness, these properties make the use of in vitro bioassays such as the CALUX bioassay battery very suitable as a screening tool for endocrine activity in water samples. Application of these CALUX bioassays demonstrated different specific hormonal activities in specific samples of waste and/or surface water [63]. By using in vitro bioassays, the combined biological activities of the mixture can be quantified, which enables unknown compounds to be detected.

Compounds causing endocrine effects are the subject to many processes after oral intake, including limited uptake and first pass metabolism (reducing absorption), binding to protein (reducing distribution), biotransformation reactions (metabolism) by, e.g., the liver, and excretion. As these absorption, distribution, metabolism, and excretion (ADME) processes are only marginally included by the CALUX bioassays, the quantitative translation of in vitro results from in vitro bioassays to the human health risks is seriously hampered. Therefore, relative endocrine potencies determined in in vitro bioassays cannot directly be used to predict risks or effects in humans.

The generation of data by means of these in vitro bioassays requires the establishment of limits of maximum tolerable (drinking) water concentrations for hormonal activity by which the cellular responses from water samples can be judged. As limit values for drinking water are aimed at the protection of human health, such limits should be sufficiently conservative to serve as a warning signal. On the other hand, such limits should not be too conservative, to avoid unnecessary and costly additional protection measures.

A trigger value of 7 ng E2-eq/L has been derived for estrogenic activity in water samples [27]. With the use of bioassays, South African researchers proposed an “average trigger value” of 0.7 ng E2/L [23], and a more recent report derived a value of 3.8 ng E2-eq/L [26], for agonist estrogenic hormonal activity in drinking water, using the ERαCALUX bioassay; the standard detection limit was 0.01 ng E2-eq/L water sample.

The bioassay-based screening study indicated that the ER-CALUX and E-Screen assays successfully detected estrogenicity in environmental water samples, even at very low levels, from 0.1 to 320 ng/L EEQ [23]; the MELN assay gave good qualitative data but the accuracy was more problematic, possibly because of matrix interferences, such as sewage samples.

The South African study [23] noted the following shortcomings of bioassays: running costs, tests are quite labor-intensive, lack of capacity in South Africa to perform these tests; another problem with bioassays is the presence of cytotoxic compounds. Other potential complicating factors include the presence of other EDCs in the water sample that can have agonistic (stimulation) or antagonistic (inhibition) effects.

In general, EEQs are calculated in different ways, depending on the laboratory doing the analyses. This issue requires standardization, and has been identified by the Water Research Commission (South Africa), University of Pretoria (South Africa), and the US EPA.

Conclusion

The international reports have shown sub-nanogram per liter LOQs achievable by the conventional chemical-analytical GC-MS and LC-MSMS test methods for steroid hormone analysis, on environmental water, potable water, and

wastewater. A snapshot of some technical application notes on the use of available LC-MSMS equipment confirms the excellent LOQs achieved on water matrix for steroid hormones, and other EDCs, PPCPs, and emerging chemical contaminants (ECCs).

The current study indicates fairly equal use of the chemical-analytical LC, GC, and the immuno-analytical ELISA test methods, for screening and quantitation of the target steroid hormones (E1, E2, EE2, E3, tes, pro), at international level; relative sensitivity of test methods decreases in the order LC-MSMS > GC-MS > ELISA (chemical-analytical > immuno-analytical).

At national level, the immuno-analytical ELISA method, fully validated for both water and wastewater matrix, is being used for routine, preliminary steroid hormone profiling and for quantitative measurement in South Africa.

For raw water sources, drinking water, and wastewaters, the use of bioassays, with trigger values, is a useful screening tool option to decide whether further examination of specific endocrine activity may be warranted, or whether concentrations of such activity are of low priority, with respect to health concerns in the human population.

Future research must focus on, inter alia, the achievement of improved quantitation limits for immuno-analytical methods, like ELISA, for quantitation of individual compounds, and standardization of the method for measuring E2 equivalents (EEQs), for the purpose of establishing biological (endocrine: e.g., estrogenic) activity for EDCs.

We are currently investigating the development and validation of a chemical-analytical GC-MS method, using large volume injection [33], for quantitation of some priority steroid estrogens (E1, E2, and EE2) in wastewater/water matrix. Further detailed results will appear in a follow-up publication.

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