Advances in Sample Preparation 12 (2024) 100132

Contents lists available at ScienceDirect

Advances in Sample Preparation

journal homepage: www.elsevier.com/locate/sampre

Determination of steroid hormones in sea urchins by microwave-assisted extraction and ultrahigh-performance liquid chromatography tandem mass spectrometry

Irene Rodríguez-de Cos [a], Raibel Núñez-González [b], Rayco Guedes-Alonso [c,*], Sarah Piaugeard [d], María Esther Torres-Padrón [c], José Juan Castro-Hernández [b], Zoraida Sosa-Ferrera [c], José Juan Santana-Rodríguez [c]

[a] Faculty of Marine Sciences, University of Las Palmas de Gran Canaria, Spain
[b] Instituto Universitario EcoAqua, University of Las Palmas de Gran Canaria, 35017 Tafira, Spain
[c] Institute for Environmental Studies and Natural Resources, University of Las Palmas de Gran Canaria, Spain
[d] Faculté d'écologie-éthologie, Université de Tours, France

ARTICLE INFO

Keywords:
Steroid hormones
Sea urchins
Bioindicator
Microwave-assisted extraction
UHPLC
Mass spectrometry

ABSTRACT

Marine pollution poses significant threats to ecosystems by contaminating habitats and degrading marine life. This involves the need to develop efficient methodologies to evaluate the compounds that affect marine organisms, such as steroid hormones. The study of the presence of these compounds in marine organisms like sea urchins is very interesting given their role as bioindicators because they feed on algae and are constantly in contact with sediments. Given the low concentrations of steroid hormones in marine environments, it is necessary to develop extraction procedures that allow these pollutants to be extracted and preconcentrated before chemical analyses. Of all the extraction methods, microwave-assisted extraction (MAE) has been used for its many advantages compared to traditional extraction techniques, such as easy sample handling or scarce organic solvents use, and for providing very selective extractions. This study presents the novel MAE optimisation for the extraction of 15 hormones, including five oestrogens, three androgens, four progestogens and three glucocorticoids from sea urchin tissues. The extracted hormones were subsequently determined by high-performance liquid chromatography tandem mass spectrometry. To the best of the authors' knowledge, this approach has not been previously developed. To perform extraction optimisation, different variables were studied following factorial experimental designs. The optimised extraction method showed very appropriate analytical parameters, with limits of detection between 0.21 and 20.4 $ng \cdot g^{-1}$ for the four families of studied steroid hormones, and recovery extractions over 60 % for most target compounds. After optimisation, the analytical methodology was applied to samples of three different sea urchins species (*Arbacia lixula, Paracentrotus lividus, Sphaerechinus granularis*) caught in different locations around the Gran Canaria island (Canary Islands, Spain). The results showed the great applicability of the optimised methodology and two target hormones, boldenone and prednisolone, which were quantified in different samples and locations. This indicates the potential of sea urchins as bioindicators of the health of marine ecosystems and of anthropogenic contamination.

1. Introduction

Currently, coastal areas are significantly impacted by various sources of pollution, including industrial and agricultural activities, hospital wastewater and effluents from wastewater treatment plants (WWTPs) [1]. Emerging pollutants are not entirely removed by WWTPs and are, thus, released to aquatic environments [2]. Of these pollutants, steroid hormones are a particular concern and are classified as emerging contaminants (ECs) [3]. They are known for being potentially harmful given their role as endocrine-disrupting chemicals (EDCs) [4]. These compounds may interfere with the signalling pathways of hormones [5] by mimicking or blocking them and altering the normal functions of different organisms [6]. Steroid hormones are the most potent endocrine disrupters, even at nanogram per litre ($ng \cdot L^{-1}$) levels [7], which means

* Corresponding author.
E-mail address: rayco.guedes@ulpgc.es (R. Guedes-Alonso).

https://doi.org/10.1016/j.sampre.2024.100132
Received 30 June 2024; Received in revised form 14 September 2024; Accepted 20 September 2024
Available online 21 September 2024

that they could be potentially toxic for marine organisms. Every year, 30,000 kg of natural steroids (oestrone, oestradiol and oestriol), and approximately 700 kg of synthetic oestrogen 17α-ethinyl oestradiol, are discharged from the world's human population [8]. In this context, it is crucial to develop methods for the identification, detection and quantification of molecules with endocrine activities and their metabolites in organisms. Understanding how these compounds affect marine organisms is essential for preventing deleterious effects on marine ecosystems.

Accordingly, sea urchins are considered bioindicators of marine pollution for their ability to accumulate more pollutants than other organisms, such as algae, molluscs or crustaceans, because the average life span of echinoderms is longer [9]. Besides being considered representative animals of the marine ecosystem, the reasons why they are chosen for environmental quality indicator studies are their wide distribution, abundance, benthic behaviour, rapid response and high sensitivity to contaminants [10]. Sea urchins feed on algae and marine phanerogams, and are also able to make good use of other food sources under rough conditions [11]. It is also known that echinoderms easily come into contact with all pollutants in marine waters because compounds tend to accumulate in sediments, which are close to the place from where animals extract their food [12]. On this matter, studies have shown that exposure to EDCs affects various parameters in sea urchins, including regenerative growth, histological patterns, egg diameter and gonad maturation [10].

To determine EDCs in biological solid samples, it is necessary to use extraction techniques that allow compounds of interest to pass from the sample to a solvent. In the last few years, extraction methods based on liquid partitioning with ultrasonic extraction (USE), pressurised liquid extraction (PLE) or supercritical fluid extraction (SFE) have been used for these types of pollutants [13]. Of them, microwave-assisted extraction (MAE) stands out as a extraction technique for providing not only selective and rapid extractions, but also low energy use, small volumes of solvents, low toxicity of the used solvents and generally less waste than other extraction techniques. This means that, compared to conventional procedures, it can be considered to be a greener extraction technique [14]. For these reasons, MAE has been successfully applied to extract several families of ECs from environmental samples [15]. In addition, MAE has been successfully combined with ultrahigh-performance liquid chromatography (UHPLC), a powerful analytical technique to analyse steroid hormones in environmental samples. Combining these techniques increases selectivity and sensitivity by reducing the limits of detection and quantification (LOD and LOQ) to the ng·L^{-1} – μg·L^{-1} range [16,17].

This study aimed to optimise an analytical method based on MAE combined with UHPLC and triple quadrupole mass spectrometry (UHPLC–MS/MS) to determine a group of 15 steroid hormones in sea urchin tissues, specifically shell, spines and lanterns. It focused on four families of steroid hormones: five oestrogens, three androgens, four progestogens and three glucocorticoids. As the literature about the harmful effects of xeno-steroids in invertebrates is limited, the studied hormones were selected after considering previous studies with fish that indicate compounds like 17β-oestradiol (E2), oestrone (E1) and synthetic hormone 17α-ethynylestradiol (EE2), which provoke oestrogenic effects on male fish [18]. The other target hormones were chosen following previous experiments on the extraction of hormones in biological marine samples on the Canary Islands (Central-east Atlantic), Spain [19], for their role as EDCs. The target compounds and their corresponding physico-chemical parameters are presented in Table 1. The variables that affect MAE (type of solvent, solvent volume, extraction time, extraction temperature, sample weight) were optimised by developing experimental designs that permit the influence of the variables in the extraction process to be evaluated. The method was validated at different spiking levels to evaluate both the extraction efficiency and the accuracy and sensitivity of the optimised methodology. This was applied to three sea urchins species (*Arbacia lixula, Paracentrotus lividus* and *Sphaerechinus granularis)* sampled from five different locations around the Gran Canaria Island. The obtained information could be relevant to study how echinoderms act as bioindicators of marine pollution in coastal areas.

2. Materials and methods

2.1. Materials, solvents and reagents

HPLC-grade methanol, acetonitrile and acetone were used as extractants. The LC/MS-grade methanol, LC/MS-grade water and ammonia for the mobile phase pH adjustment were obtained from Panreac Química (Barcelona, Spain). Ultrapure water was provided by a Milli-Q system (Millipore, Bedford, MA, USA). The 15 hormones with > 99 % purity were purchased from Sigma-Aldrich (Madrid, Spain). Each compound was dissolved in methanol to obtain 1000 mg·L^{-1} stock solutions and stored in glass-stoppered bottles at −20 °C. A hormone mixture solution at 10 mg·L^{-1} in methanol was prepared from stock solutions and stored in a glass bottle at −20 °C as a working solution.

2.2. Sampling

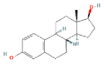
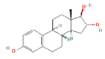
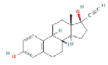
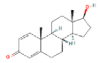
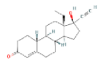
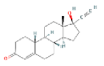
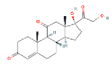
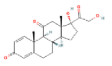
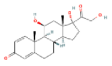
The study samples of the three sea urchins species (*Arbacia lixula, Paracentrotus lividus and Sphaerechinus granularis*) were collected from five different coastal locations around the Gran Canaria island, as shown in Fig. 1 and Table S1 during two different seasons: September 2020 and March 2021. The coastal locations were: Bañaderos (northern coast, point A) is a more inhabited area with one submarine outfall and one discharge point 600 m and 1 km away from the sampling area, respectively; San Cristóbal (northeast coast, point B) is a high-density inhabited area of the island's capital city with two wastewater discharge points at 600 m and 1.6 km away, and one submarine outfall at almost 3 km from the sampling area; Arguineguín (point C), at the southwest of the island, is characterised for being a tourist zone with four wastewater discharge sources <700 m away from the sampling area and a submarine outfall 1.3 km away; Tasartico (west coast, point D) is not influenced by anthropogenic activity and has no sewage discharge in its proximity; La Aldea (west coast, point E) is a sparsely inhabited area with one discharge point 900 m away from the sampling area.

The three studied sea urchin species were found on rocky substrates and sand bottoms. *Paracentrotus lividus* and *Arbacia lixula* can be found together in the intertidal zone, inside crevices and between rocks, and on the shallow infralittoral bottoms where calcareous algae appear. *P. lividus* is herbivorous and feeds on algae around the crevices where it lives [20]. *A. lixula* is phytophagous and feeds basically on calcareous algae. *Sphaerechinus granularis* is usually found in the shallow sublittoral and manifests cryptic behaviour by camouflaging with algae and shells [21].

2.3. Instrumentation

A Multiwave Microwave Sample Preparation System, equipped with a 6 EVAP rotor and 6 MF100 vessels (Anton Paar, Graz, Austria), was used to extract the target analytes. To perform the separation and quantification of the steroid hormones under study, an UHPLC–MS/MS system was used. This system consisted of a quaternary pump acting as a solvent manager, a column oven, an autosampler that holds up to 96 samples and a triple quadrupole detector with an electrospray interface (ESI) (Waters Chromatography, Barcelona, Spain). Components were managed with the MassLynx mass spectrometry software (Waters™). Chromatographic separation was done inside an Kinetex EVO C_{18} LC column (50 mm x 2.1 mm, 1.7 μm particle size) from Phenomenex (Barcelona, Spain). The injected sample volume was 10 μL. Analyte separation was carried out using water with 0.1 % (v/v) of ammonia and methanol at a flow rate of 0.4 mL min^{-1} in the gradient mode. All the separation and detection conditions for steroid hormones were optimised according to Guedes-Alonso et al. [19]. To homogenise the sea

Table 1
Physico-chemical parameters of the steroid hormones.

Family	*Compound*	*Solubility in water[a,b] (mg·L^{-1})*	*Vapor pressure[a,b] (mmHg)*	*Log Kow[a]*	*Formula[a]*	*Structure[a]*
Estrogens	Estrone (E1)	3.00	$2.49 \cdot 10^{-10}$	3.13	$C_{18}H_{22}O_2$	
	17β -estradiol (E2)	3.90	$6.39 \cdot 10^{-9}$	4.01	$C_{18}H_{24}O_2$	
	Estriol (E3)	27.3	$9.93 \cdot 10^{-12}$	2.45	$C_{18}H_{24}O_3$	
	17α-ethynylestradiol (EE)	11.3	$1.95 \cdot 10^{-9}$	3.67	$C_{20}H_{24}O_2$	
	Diethylstilbestrol (DES)	12.0	$1.40 \cdot 10^{-8}$	5.07	$C_{18}H_{20}O_2$	
Androgens	Testosterone (TES)	23.4	$1.71 \cdot 10^{-8}$	3.32	$C_{19}H_{28}O_2$	
	Nandrolone (NAN)	24.0	$3.50 \cdot 10^{-8}$	2.62	$C_{18}H_{26}O_2$	
	Boldenone (BOL)	20.0	$2.00 \cdot 10^{-8}$	3.05	$C_{19}H_{26}O_2$	
Progestogens	Progesterone (PRO)	8.81	$3.59 \cdot 10^{-4}$	3.87	$C_{21}H_{30}O_2$	
	Megestrol acetate (MGA)	6.50	$2.10 \cdot 10^{-10}$	3.20	$C_{24}H_{32}O_4$	
	Norgestrel (NOR)	2.05	$3.90 \cdot 10^{-10}$	3.48	$C_{21}H_{28}O_2$	
	Norethisterone (NORET)	7.04	$3.14 \cdot 10^{-7}$	2.97	$C_{20}H_{26}O_2$	
Glucocorticoids	Cortisone (COR)	140	$3.00 \cdot 10^{-15}$	1.47	$C_{21}H_{28}O_5$	
	Prednisone (PRD)	77.5	$3.82 \cdot 10^{-13}$	1.46	$C_{21}H_{26}O_5$	
	Prednisolone (PRDNL)	223	$1.18 \cdot 10^{-13}$	1.62	$C_{21}H_{28}O_5$	

[a]Obtained from Pubchem database: https://pubchem.ncbi.nlm.nih.gov/.
[b]Obtained from Chemspider database: http://www.chemspider.com/.

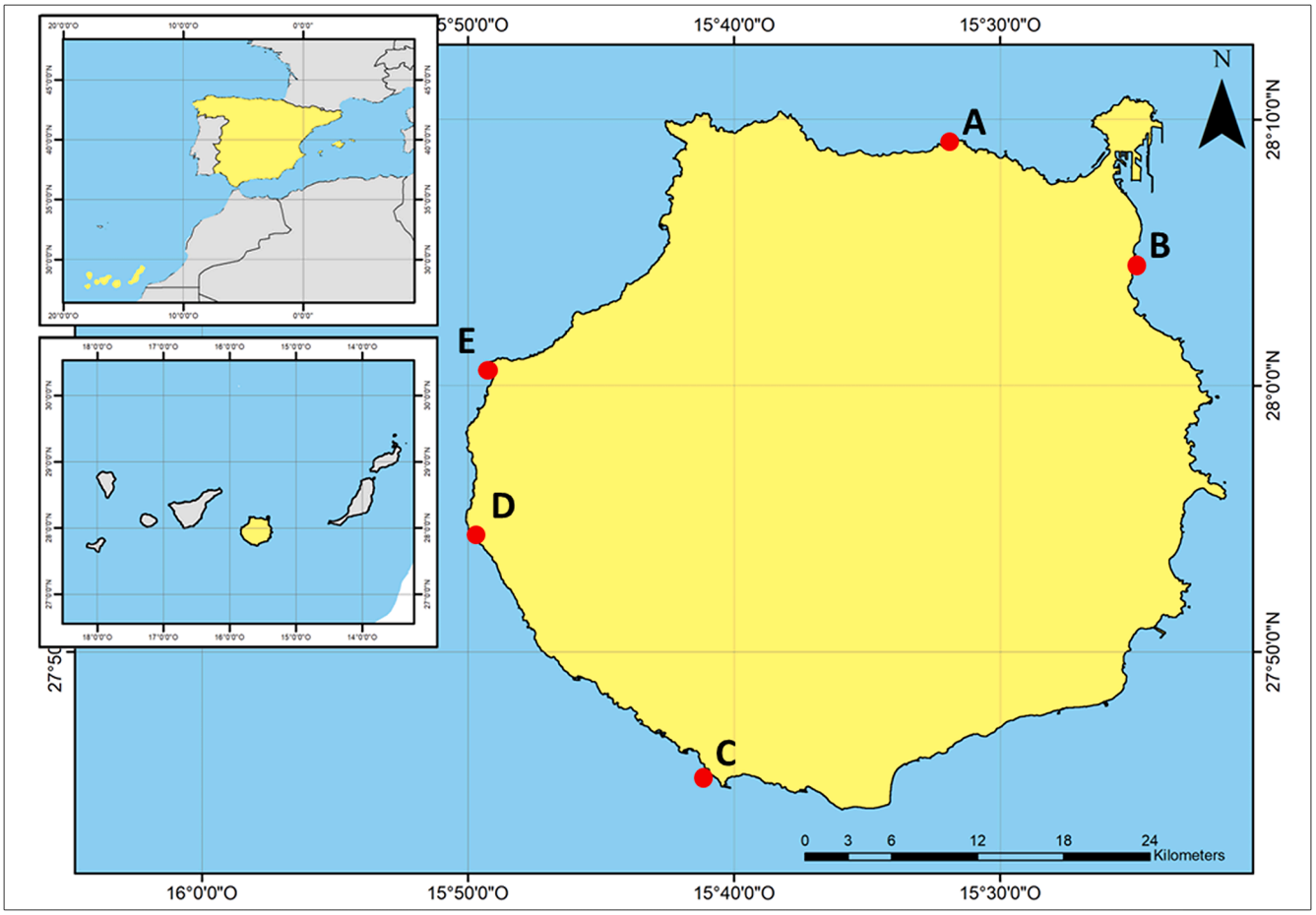


Fig. 1. Map of the Gran Canaria island with sampling points. A: Bañaderos, B: San Cristóbal, C: Arguineguín, D: Tasartico, E: La Aldea. Created with the ArcGIS software. Map extracted from CartoBase ANE 2006–2020 CC-BY 4.0 ign.es.

urchin samples, they were ground using a laboratory vibratory mill, Model MM301, from RETSCH (Asturias, Spain).

2.4. Pretreatment and extraction procedure

In order to carry out extraction optimisation, *Arbacia lixula* samples were used. The samples of lanterns, spines and shells were separated from gonads and lyophilised at -55 °C and then ground to dust. The working solution of 10 $mg{\cdot}L^{-1}$ was used to spike samples for the method's optimisation. Experiments were performed after evaporating the methanol of the standard solution. In this study, the spiking level was 250 $ng{\cdot}g^{-1}$ to ensure that the analytical signal was enough to observe any differences between experiments.

To perform the extractions of the target analytes, 200 mg of the lyophilised sea urchin tissue from shells, spines and lanterns were placed inside each microwave polytetrafluoroethylene (PTFE) vessel with a triplicate, and 10 mL of methanol were added as the extractant. Next vessels were closed and placed inside the microwave rotor in a symmetrical arrangement.

Once the rotor was placed inside the microwave, extraction was done under the optimised conditions (Table S2). Once the extraction process had finished, the solution was centrifuged for 10 min at 1507 RCF to obtain a supernatant without solids, and was then evaporated in a gentle N_2 stream to concentrate hormone residues in 1 mL of extract.

2.5. Greenness evaluation of the sample preparation method

The sustainability of the proposed sample preparation method was evaluated using the AGREEprep tool, as described by Wojnowski et al. A score reflecting greenness, which ranges from 0 (least compliant) to 1 (most compliant), was determined based on 10 green sample preparation principles, with each principle weighted according to its significance [22]. These scores were generated with specific software [23] and the guidelines provided by Pena-Pereira et al. [24].

3. Results and discussion

3.1. MAE optimisation

3.1.1. 2^3 and 3^2 factorial experimental designs

In order to perform MAE optimisation, a series of parameters that affect extraction was studied (i.e. solvent volume, extraction time and extraction temperature) using 100 mg of sample. To evaluate them, a 2^3 experimental design was developed that studied these three variables at two different levels using various solvents. This factorial design permitted the interaction among all these variables and the significance of each one during the extraction process to be evaluated. To avoid carry-over effects, runs were randomised (Table S3) and experiments were conducted with the three different types of solvents. The studied variables were solvent volume using 5 and 10 mL, extraction times of 5 and 15 min, and extraction temperatures of 60 and 90 °C, with three different solvents: methanol, acetone and acetonitrile. Fig. 2 shows the Pareto charts obtained from a compound of each family of steroid hormones extracted with methanol, and used as being representative for all the analytes.

Solvent volume was the variable with the strongest influence during the extraction process (Fig. 2, variable C), while time and solvent volume were the most significant combination among variables (variables

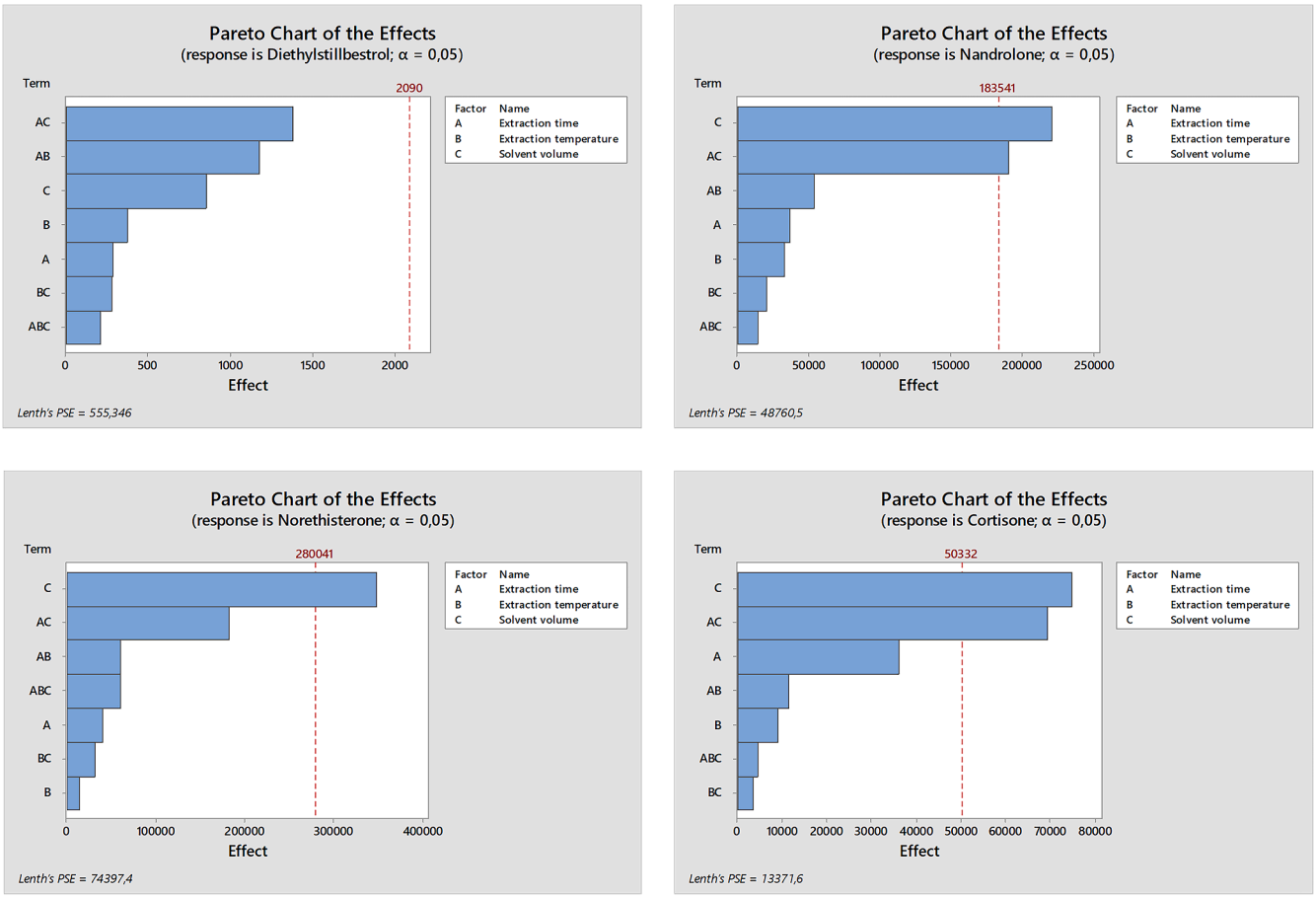


Fig. 2. Pareto Charts for the representative studied analytes of each steroid hormone family obtained during the 2^3 experimental design.

A and C in Fig. 2, respectively). This trend was observed for all the studied analytes. The solvent volume and extraction time (AC) was significant and positive for most analytes, which denotes that the combination of higher solvent volumes and longer extraction times synergistically improved extraction yields. After performing an ANOVA analysis of the results, the solvent volume presented a significant effect for norethisterone and the p-values were close to the threshold for other compounds, such as testosterone, megestrol acetate or levonorgestrel (Table S4). Moreover during this experimental design, methanol was the solvent that provided the bigger peak areas and, subsequently, higher recovery rates. Lower recovery yields were obtained with acetone and acetonitrile similarly to the studies by Guedes-Alonso et al. for the extraction of target steroid hormones in biological samples [19]. However, the extraction temperature was not a significant variable (Fig. 2) after performing the ANOVA analysis (p-values were higher than 0.05 for all the compounds). Hence it was considered to be irrelevant. Taking this into account, solvent volume and extraction time were chosen for the 3^2 factorial design, and methanol was used as the solvent.

To confirm the information obtained with the Pareto charts and the ANOVA analysis, and to evaluate the chosen variables for the second experimental design, partial correlations were calculated for each variable (Table S5). Any correlations near zero implied the slightest influence of the studied variable on the method, while those close to −1 or 1 indicated a stronger influence of the variable on the extraction process. For most target compounds, correlations near zero were obtained for the extraction temperature. Thus when considering the null influence of this variable, a temperature of 60 °C was chosen as the optimal value to avoid excessive extractant solvent evaporations during the extraction process. In contrast, solvent volume showed highly positive correlations, which indicated that extraction yields were higher when extraction volumes were increased. Medium correlations were observed for extraction times, which denoted that longer extraction times provided slightly better recoveries than short ones. Consequently for the second factorial design, bigger volumes and longer times were considered.

During the second factorial experimental design, the chosen variables were studied at three different levels (5, 12.5, and 20 min) for the extraction time, with 5, 10, and 15 mL for the solvent volume (Table S6). Fig. 3 depicts for all families of the target steroid hormones that the trend was similar, and the highest recovery rates were obtained with 10 mL of extraction solvent after 20 min of microwave extraction. In all cases, a sharp decrease was observed in the analytical signal obtained after extractions for times shorter than 12 min. However, longer extraction times were not evaluated to avoid not only the degradation of the studied compounds during the extraction process, but also extractant solvent evaporation because one of the advantages of microwave extraction is short extraction times.

3.1.2. *Sample weight*

In order to choose the optimal amount of sea urchin tissue to be used in the method, four extractions using 50, 100, 200, and 300 mg of sample were evaluated under the best achieved conditions. Samples were spiked to obtain the same final concentration of the target analytes, and bigger peak areas for all the steroid hormone families were achieved using 200 mg. Fig. 4 shows the normalised peak areas considering the peak area obtained in the extraction with 100 mg as the basis for calculation. Not only did the extractions with 200 mg provide a bigger peak area, but they also significantly differed from the rest for most of the studied compounds.

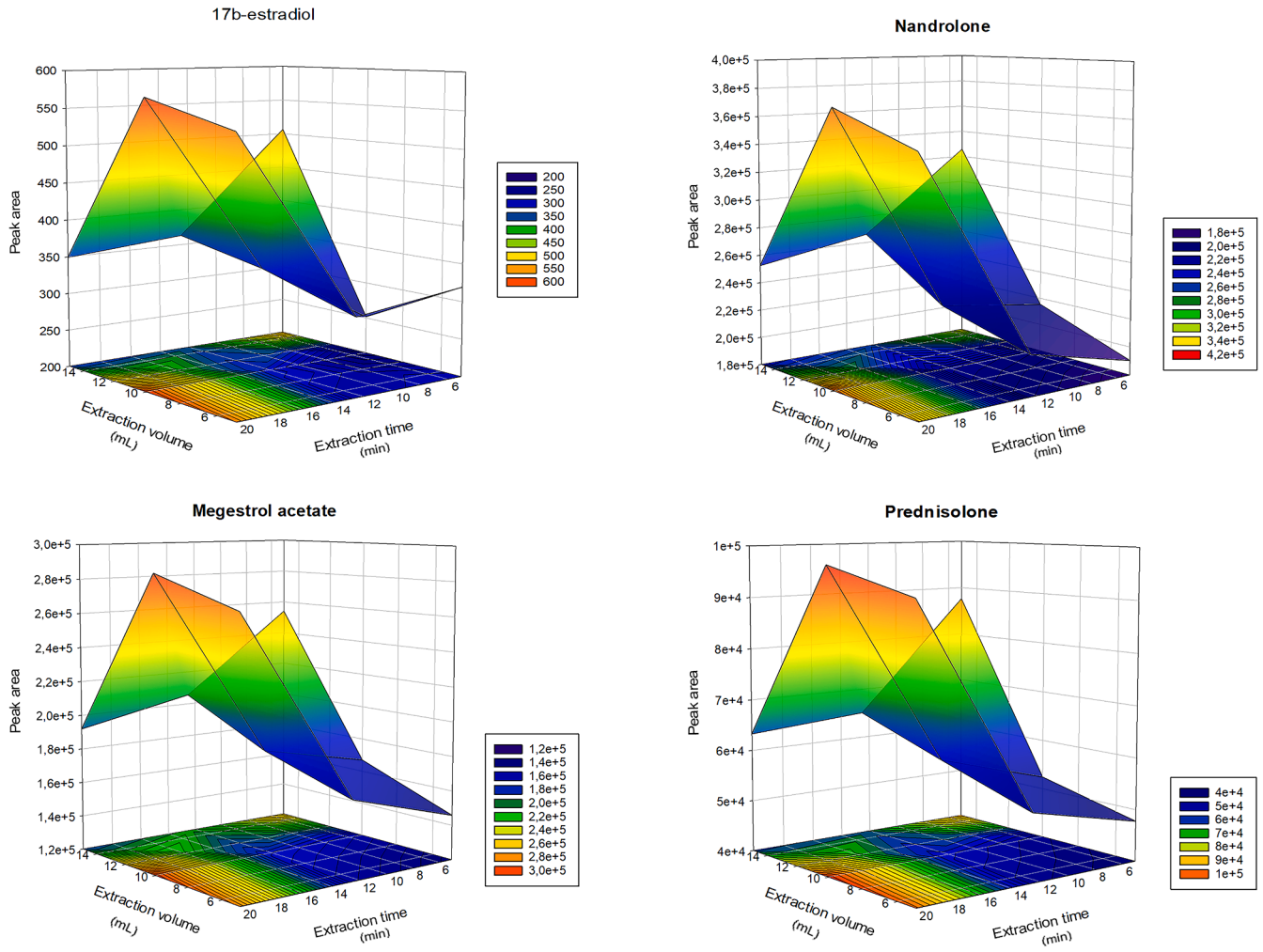


Fig. 3. Response surfaces of the different families of steroid hormones for the optimisation of the best extraction time and volume values.

3.2. *Analytical parameters and methodology validation*

The validation process was carried out to demonstrate reliable and fit-for-purpose results for the optimised analytical method by evaluating linearity, LODs and LOQs, recoveries and intra-/interday precisions (Tables 2 and 3). The last two parameters were evaluated for the 50 ng·g^{-1}, 200 ng·g^{-1}, and 500 ng·g^{-1} concentrations to ensure the method's reliability and accuracy across a range of concentrations.

External calibration curves were built using methanol as a solvent with nine points ranging from 1 to 800 µg·L^{-1}, which corresponded to the concentrations in the solid samples between 5 and 4000 ng·g^{-1} of the target analytes. Very good linearity was obtained, with correlation coefficients (r^2) higher than 0.99 for all the target analytes.

The LODs and LOQs of the whole extraction method were evaluated as the concentration that caused signal-to-noise ratios of 3 and 10 during the quantification ion transition of each compound. The results showed LODs from 0.21 to 2.81 ng·g^{-1} for androgens, progestogens and glucocorticoids (except for prednisone). For the oestrogenic family, LODs were slightly higher and went from 2.80 to 20.4 ng·g^{-1}. Furthermore, the LOQs or androgens, progestogens and glucocorticoids (except for prednisone) ranged from 0.71 to 9.38 ng·g^{-1}f, and from 9.34 to 67.8 ng·g^{-1} for the oestrogenic family. The higher LODs and LOQs for oestrogens could be due to the negative ionisation mode applied in the mass spectrophotometer, which tends to be less sensitive than ionisation in the positive mode. Indeed Cathurn and Sabik determined steroids (E1, E2 and TES) in mussels by a method based on the derivatisation of the compounds containing hydroxyl groups with pentafluorobenzyl bromide, followed by GC–MS [25]. A microwave extraction technique was used for mussels, and values of 3 ng·g^{-1} were obtained as LODs [26]. When comparing these results to those in this study, we find that the LODs were slightly higher for E1 and E2 and lower for TES. Furthermore, LODs lower than 1 ng·g^{-1} have been recorded with a method proposed by Wang et al. based on Dynamic MAE coupled with salting-out liquid--liquid extraction for the determination of hormones in fish tissues [26]. In addition, Dévier et al. developed a method that combined MAE, SPE and detection by GC–MS to determine steroids in aquatic molluscs, and obtained LODs of 0.1–0.4 ng·g^{-1} [15]. Similarly, Guedes-Alonso et al. followed a methodology for the determination of steroid hormones in fish tissues by MAE coupled with UHPLC-MS/MS, which gave significant results in hormone detection [19]. These close-related approaches highlight the versatility and efficiency of MAE combined with advanced chromatographic techniques for environmental sample analyses.

To evaluate the extraction efficiency of the proposed MAE methodology, relative recoveries were studied at three concentration levels (50, 200 and 500 ng·g^{-1}) by comparing the signals from the spiked samples to those of the spiked extracts (Eq. 1), which gave adequate recoveries for all the evaluated concentrations that ranged from 55 % to 86 %. As shown in Table 2, no significant differences were observed for the extraction efficiencies between the evaluated concentration levels, which implies that the MAE methodology can extract both low and high concentrations of the target steroid hormones without affecting extraction yields.

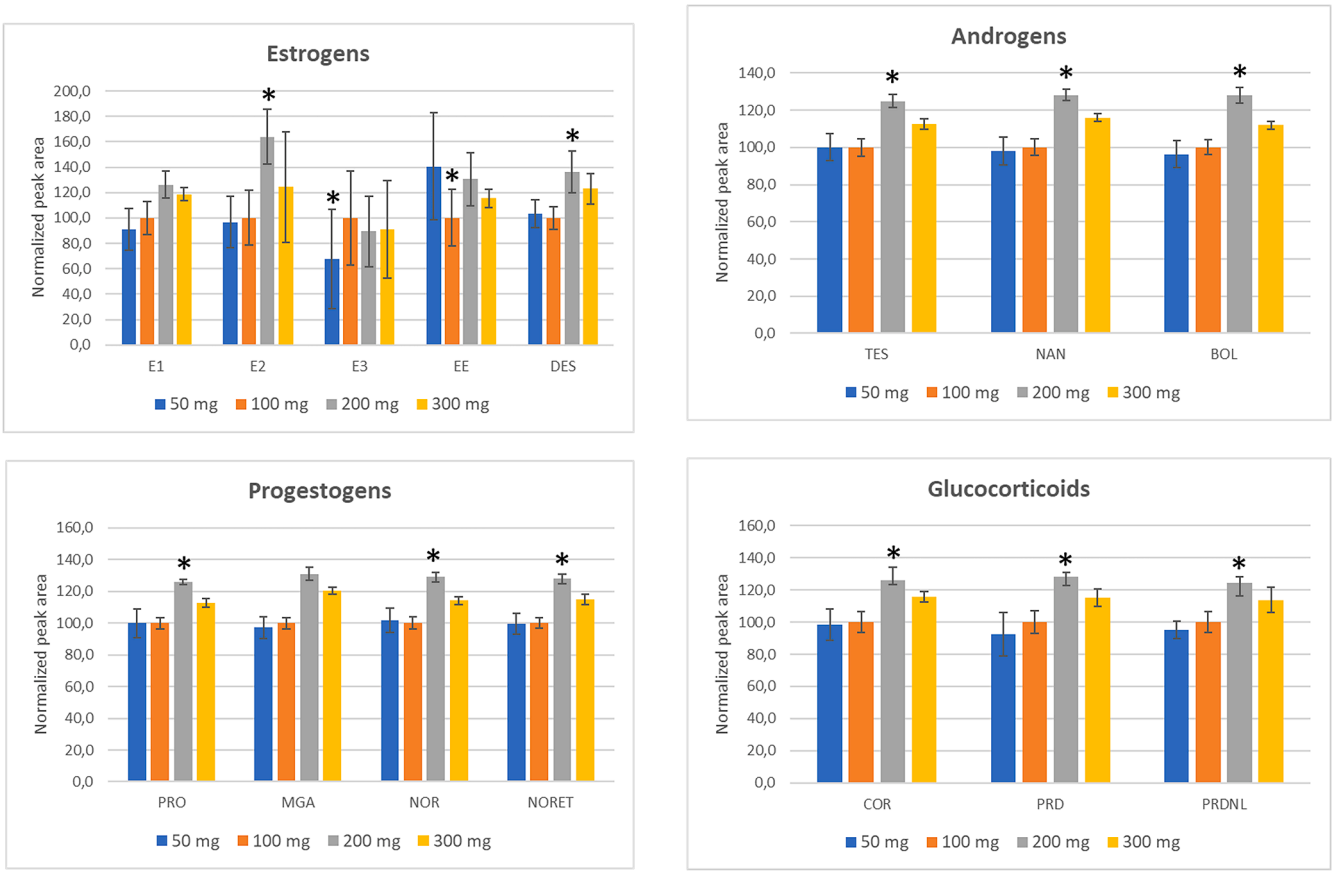


Fig. 4. Normalised peak areas (100 mg=100) obtained in the MAE extractions using different amounts of sample for the different families. * indicates that the value was statistically significant according to the *t*-test with a 90 % confidence level.

Table 2
Detection and quantification limits and recoveries of the method.

Compound	Calibration range ($ng \cdot g^{-1}$)	LOD ($ng \cdot g^{-1}$)	LOQ ($ng \cdot g^{-1}$)	Recovery (%)		
				50 $ng \cdot g^{-1}$	200 $ng \cdot g^{-1}$	500 $ng \cdot g^{-1}$
E1	25–4000	4.75	15.8	74.6	68.4	68.6
E2	50–4000	7.59	25.3	75.8	62.0	85.2
E3	50–4000	14.6	48.7	–	66.2	79.5
EE	125–4000	20.4	67.8	–	66.1	85.6
DES	25–4000	2.80	9.34	66.1	54.8	75.3
TES	5–4000	0.31	1.04	69.8	62.7	80.7
NAN	5–4000	0.65	2.18	75.5	59.8	78.6
BOL	5–4000	0.21	0.71	72.5	66.9	83.0
PRO	5–4000	0.63	2.09	78.0	56.7	85.5
MGA	5–4000	0.36	1.20	70.5	59.8	83.3
NOR	5–4000	1.02	3.40	67.7	58.6	84.7
NORET	5–4000	1.32	4.40	67.0	60.8	83.7
COR	25–4000	2.81	9.38	70.7	60.5	74.6
PRD	125–4000	17.8	59.2	–	74.3	78.8
PRDNL	5–4000	1.36	4.52	69.4	64.7	78.3

Table 3
Intra-day and inter-day precisions of the method.

Compound	Intra-day precision (RSD %) $n = 5$			Inter-day precision (RSD %) $n = 3 \times 5$		
	50 $ng \cdot g^{-1}$	200 $ng \cdot g^{-1}$	500 $ng \cdot g^{-1}$	50 $ng \cdot g^{-1}$	200 $ng \cdot g^{-1}$	500 $ng \cdot g^{-1}$
E1	4.43	4.69	4.83	30.7	21.7	23.1
E2	17.6	10.9	6.70	41.6	26.7	27.5
E3	–	6.22	3.94	–	24.3	23.6
EE	–	16.9	6.64	–	35.4	29.9
DES	5.89	5.14	5.85	18.1	22.4	24.2
TES	1.04	2.58	2.22	25.7	16.4	15.6
NAN	2.97	3.51	1.57	15.5	16.5	15.3
BOL	3.42	3.18	2.71	20.1	16.4	17.2
PRO	5.09	4.31	1.89	26.7	17.7	12.0
MGA	4.59	3.64	2.48	25.6	16.9	13.2
NOR	3.86	2.10	2.31	24.4	16.6	13.2
NORET	2.80	2.50	3.32	14.8	15.0	13.9
COR	4.89	2.91	1.91	19.5	16.2	17.4
PRD	–	4.71	3.70	–	16.9	16.1
PRDNL	4.78	3.04	2.31	22.4	17.6	17.2

$$Recovery\ (\%) = \frac{Peak\ area\ spiked\ sample}{Peak\ area\ spiked\ extract} 100 \quad (1)$$

These results were much higher than those described by Cathurn and Sabik, who obtained recoveries in spiked mussels ranging from 21 % to 48 % [25], but were similar to those obtained by Wang et al., with values from 79 % to 94 % [26].

Table 3 shows the intra- and interday precisions, which were evaluated using five samples per day at all three assessed concentrations. The highest relative standard deviations were calculated at the lowest spiked level due to the presence of some interferences. Nevertheless, both intra- and interday reproducibility were satisfactory for practically all the studied hormones, especially those determined in the positive mode (androgens, progestogens and glucocorticoids). Intra- and interday precisions were lower than 18 % and 42 %, respectively, for oestrogens and lower than 5.1 % and 27 %, respectively, for the other steroid hormone families.

3.3. Evaluation of the greenness of the optimised MAE method

The sustainability and environmental impact of the extraction method were key considerations in developing it. To assess the sustainability of the proposed MAE technique, the AGREEprep greenness tool was used, yielding a score of 0.36 (Fig. 5). Ten criteria, weighted by their impact, contributed to the score. The lowest scoring criteria (0.00) were related to the sample preparation site, which could not be online or in situ, and the use of 10 ml of methanol as a hazardous material. In contrast, criteria linked to microwave extraction, such as sample mass (0.1 g), number of samples per hour (up to 18), and automation, scored favorably. Other criteria with intermediate scores are related to using methanol as an extractant, such as waste generation (Criterion 4) and operator safety (Criterion 10). However, these scores are higher than those obtained with traditional extraction methods for solid samples using bigger volumes and more solvents that pose higher risks. Furthermore, the analytical greenness values are much higher than those obtained for the methodologies that employ a microwave as a sample digestion method, which obtain a score of 0.2 [24]. The specifications of the score value obtained per criterion appear in the Supplementary Material (Table S7).

3.4. Applying the method to real samples

After optimising and validating the method, it was applied to real samples. This made it possible to assess the contamination caused by hormones to evaluate the use of sea urchins as bioindicators. The optimised method was applied to 30 samples of three different species collected in five locations around the Gran Canaria island, sampled in September 2020 and March 2021.

The results showed the detection of some of the studied hormones on an *ad hoc* basis, with more compounds detected during the September 2020 sampling than during the March 2021 sampling (Table 4).

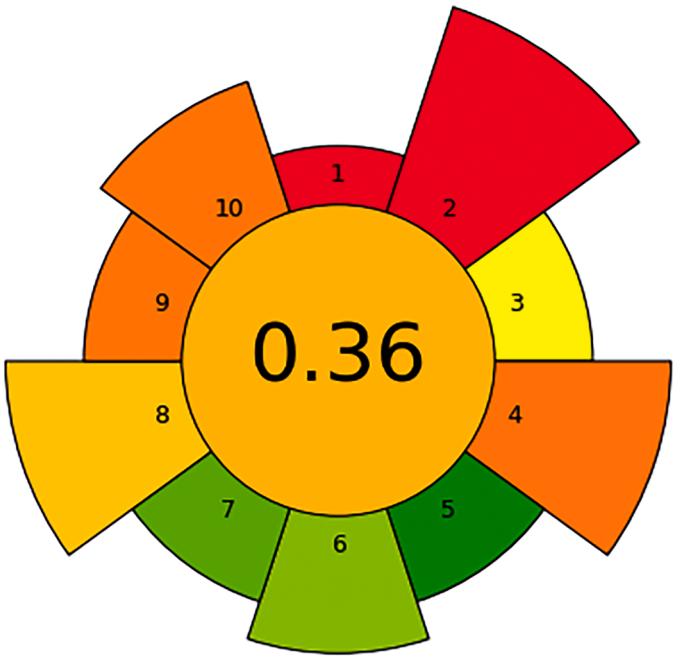


Fig. 5. Results of the AGREEprep assessment of the proposed MAE methodology. Overall impact score in the centre, surrounded by 10 performance criteria relating to: (1) sample preparation placement; (2) hazardous materials; (3) sustainability, renewability and reusability of materials; (4) waste; (5) size economy of the sample; (6) sample throughput; (7) integration and automation; (8) energy use; (9) postsample preparation configuration for the analysis; (10) operator's safety. The length of each criterion represents weight (on the final score) and colour depicts performance.

Table 4
Detected hormones in each site and month.

Month	Site	Species	Detected hormones
September 2020	A: Bañaderos	*A. lixula*	nd
		P. lividus	BOL: 124.6 ± 37.2 ng·g^{-1}
		S. granularis	nd
	B: San Cristóbal	*A. lixula*	TES: <LOQ
		P. lividus	BOL: 118.5 ± 10.7 ng·g^{-1}
			PRO: <LOQ
		S. granularis	nd
	C: Arguineguín	*A. lixula*	nd
		P. lividus	nd
		S. granularis	BOL: 258.1 ± 11.4 ng·g^{-1}
	D: Tasartico	*A. lixula*	BOL: 152.3 ± 22.0 ng·g^{-1}
			PRDNL: 5.6 ± 2.2 ng·g^{-1}
			NORET: <LOQ
		P. lividus	BOL: 127.4 ± 11.6 ng·g^{-1}
		S. granularis	BOL: 181.8 ± 4.9 ng·g^{-1}
	E: La Aldea	*A. lixula*	nd
		P. lividus	nd
		S. granularis	BOL: 143.6 ± 13.0 ng·g^{-1}
March 2021	A: Bañaderos	*A. lixula*	nd
		P. lividus	nd
		S. granularis	nd
	B: San Cristóbal	*A. lixula*	PRDNL: 7.4 ± 1.8 ng·g^{-1}
		P. lividus	nd
		S. granularis	nd
	C: Arguineguín	*A. lixula*	BOL: 162.8 ng·g^{-1}
			MGA: <LOQ
		P. lividus	nd
		S. granularis	nd
	D: Tasartico	*A. lixula*	PRDNL: <LOQ
			TES: <LOQ
			MGA: <LOQ
		P. lividus	nd
		S. granularis	nd
	E: La Aldea	*A. lixula*	nd
		P. lividus	nd
		S. granularis	nd

Specifically, 50 % of the *Arbacia lixula* samples had detectable concentrations of at least one of the target hormones, while only 30 % of the *Paracentrotus lividus* and *Sphaerechinus granularis* samples had positive detections of the target analytes. Two compounds were quantified in the different analysed samples: boldenone (BOL) and prednisolone (PRDNL). The former is an anabolic substance used only in veterinary medicine and as a doping substance. PRDNL is employed to treat patients with low corticosteroid levels by replacing the steroids that the body normally produces. It is typically utilised for certain types of arthritis, severe allergic reactions or multiple sclerosis. Regarding sampling sites, there was no correlation between the results obtained during the two sampling campaigns and sampling sites. In fact the *A. lixula* samples from Tasartico presented the highest detection of the target hormones. However, this sampling site is the least impacted by human activity because it is in an uninhabited area of the Gran Canaria island with no nearby marine outfalls of treated wastewater. However, studies conducted on this island have identified significant BOL levels in untreated wastewater. This may suggest contamination from unregulated wastewater discharges in the region [27,28].

Regarding differences among species, *A. lixula* had more and larger spines than the other species, which provide a bigger adsorption surface. This suggests that the other two studied sea urchin species were not significantly impacted by steroid hormone contamination, at least not their shells. This fact contrasts with previous studies in which measurable concentrations of steroid hormones have been detected in fish exposed to wastewater discharges in some of the herein studied locations [29]. However, it is worth noting that there analyses were more positive during the warmer season (September 2020) than during the colder season sampling (March 2021). This could be attributed to the

higher seawater temperatures in summer and autumn months, which increase animals' metabolic rate [30]. As a result, the sea urchin algal ingestion rate and the ingestion of pollutants associated with algae [31] could have also increased. In September, not only were there more positive analyses (53 %) than in March (20 %), but the detected concentrations were also slightly higher.

4. Conclusions

This study optimised an MAE method for the determination of steroid hormones in sea urchin tissues by UHPLC-MS/MS. The optimised method gave satisfactory recovery rates (55 % to 86 %) and LODs (0.21 to 20.4 $ng \cdot g^{-1}$) across the four families of analysed steroid hormones.

The optimised method was applied to real samples of three sea urchin species (*Arbacia lixula, Paracentrotus lividus* and *Sphaerechinus granularis*) from five different locations around the Gran Canaria island. Three of these areas were directly affected by contamination sources, such as submarine outfalls and WWTPs, while the other two were affected by a scarce anthropic influence. However, only some of the studied hormones, BOL and PRDNL, were quantified on a timely basis in some of the analysed sea urchin species. This may indicate that at least these marine organisms' shells and spines do not greatly adsorb these emerging contaminants. Nevertheless, the detection in some sea urchin samples of certain steroid hormones typically detected in untreated wastewater could indicate the possibility of using these organisms as bioindicators of both wastewater pollution and the health of marine ecosystems.

CRediT authorship contribution statement

Irene Rodríguez-de Cos: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Raibel Núñez-González:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Rayco Guedes-Alonso:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **Sarah Piaugeard:** Methodology, Investigation, Data curation. **María Esther Torres-Padrón:** Writing – review & editing, Validation, Supervision, Conceptualization. **José Juan Castro-Hernández:** Writing – review & editing, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Zoraida Sosa-Ferrera:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **José Juan Santana-Rodríguez:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors thank the Regional Canary Islands Government for permission to collect samples (No.: 168/2020 – Vol.: 1, Book: 401, Date: 10/06/2020). In addition, Raibel Nuñez González wishes to thank all the collaborators during the sampling campaigns. Rayco Guedes Alonso thanks the Spanish Ministry of Science and Innovation for partially funding this research through Grant PID2020–118966RJ-I00, funded by MCIN/AEI /10.13039/501100011033.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sampre.2024.100132.

References

[1] V.K. Parida, D. Saidulu, A. Majumder, A. Srivastava, B. Gupta, A.K. Gupta, Emerging contaminants in wastewater: a critical review on occurrence, existing legislations, risk assessment, and sustainable treatment alternatives, J. Environ. Chem. Eng. 9 (5) (2021) 105966, https://doi.org/10.1016/j.jece.2021.105966.
[2] B. Kasprzyk-Hordern, R.M. Dinsdale, A.J. Guwy, The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters, Water. Res. 43 (2) (2009) 363–380, https://doi.org/10.1016/j.watres.2008.10.047.
[3] N.H. Tran, M. Reinhard, K.Y-H. Gin, Occurrence and fate of emerging contaminants in municipal wastewater treatment plants from different geographical regions-a review, Water. Res. 133 (2018) 182–207, https://doi.org/10.1016/j.watres.2017.12.029.
[4] S.D. Richardson, Water analysis: emerging contaminants and current issues, Anal. Chem. 81 (12) (2009) 4645–4677, https://doi.org/10.1021/ac9008012.
[5] S. Lecomte, D. Habauzit, T. Charlier, F. Pakdel, Emerging estrogenic pollutants in the aquatic environment and breast cancer, Genes. (Basel) 8 (9) (2017) 229, https://doi.org/10.3390/genes8090229.
[6] T.T. Schug, A. Janesick, B. Blumberg, J.J. Heindel, Endocrine disrupting chemicals and disease susceptibility, J. Steroid. Biochem. Mol. Biol. 127 (3–5) (2011) 204–215, https://doi.org/10.1016/j.jsbmb.2011.08.007.
[7] S.K. Khanal, B. Xie, M.L. Thompson, S. Sung, S.-K. Ong, J. (hans) van Leeuwen, Fate, transport, and biodegradation of natural oestrogens in the environment and engineered systems, Environ. Sci. Technol. 40 (21) (2006) 6537–6546, https://doi.org/10.1021/es0607739.
[8] M. Adeel, X. Song, Y. Wang, D. Francis, Y. Yang, Environmental impact of oestrogens on human, animal and plant life: a critical review, Environ. Int. 99 (2017) 107–119, https://doi.org/10.1016/j.envint.2016.12.010.
[9] J.M. Lawrence, Sea urchins: Volume 38: Biology and Ecology, Academic Press, San Diego, CA, USA, 2013 third ed.
[10] M. Sugni, D. Mozzi, A. Barbaglio, F. Bonasoro, M.D. Candia Carnevali, Endocrine disrupting compounds and echinoderms: new ecotoxicological sentinels for the marine ecosystem, Ecotoxicology. 16 (1) (2007) 95–108, https://doi.org/10.1007/s10646-006-0119-8.
[11] C. Qin, P. Chen, G. Sarà, B. Mo, A. Zhang, X. Li, Ecological implications of purple sea urchin (Heliocidaris crassispina, Agassiz, 1864) enhancement on the coastal benthic food web: evidence from stable isotope analysis, Mar. Environ. Res. 158 (104957) (2020) 104957, https://doi.org/10.1016/j.marenvres.2020.104957.
[12] M. Parra-Luna, L. Martín-Pozo, F. Hidalgo, A. Zafra-Gómez, Common sea urchin (Paracentrotus lividus) and sea cucumber of the genus Holothuria as bioindicators of pollution in the study of chemical contaminants in aquatic media. A revision, Ecol. Indic. 113 (106185) (2020) 106185, https://doi.org/10.1016/j.ecolind.2020.106185.
[13] Z. Sosa-Ferrera, C. Mahugo-Santana, J.J. Santana-Rodríguez, Analytical methodologies for the determination of endocrine disrupting compounds in biological and environmental samples, Biomed. Res. Int. 2013 (2013) 1–23, https://doi.org/10.1155/2013/674838.
[14] C. Picot-Allain, M.F. Mahomoodally, G. Ak, G. Zengin, Conventional versus green extraction techniques — a comparative perspective, Curr. Opin. Food Sci. 40 (2021) 144–156, https://doi.org/10.1016/j.cofs.2021.02.009.
[15] M.-H. Dévier, P. Labadie, A. Togola, H. Budzinski, Simple methodology coupling microwave-assisted extraction to SPE/GC/MS for the analysis of natural steroids in biological tissues: application to the monitoring of endogenous steroids in marine mussels Mytilus sp, Anal. Chim. Acta 657 (1) (2010) 28–35, https://doi.org/10.1016/j.aca.2009.10.023.
[16] D.T-T. Nguyen, D. Guillarme, S. Rudaz, J.-L. Veuthey, Fast analysis in liquid chromatography using small particle size and high pressure, J. Sep. Sci. 29 (12) (2006) 1836–1848, https://doi.org/10.1002/jssc.200600189.
[17] R. Guedes-Alonso, S. Montesdeoca-Esponda, Z. Sosa-Ferrera, J.J. Santana-Rodríguez, Liquid chromatography methodologies for the determination of steroid hormones in aquatic environmental systems, Tren. Environ. Anal. Chem. 3–4 (2014) 14–27, https://doi.org/10.1016/j.teac.2014.10.001.
[18] W.J. Langston, G.R. Burt, B.S. Chesman, C.H. Vane, Partitioning, bioavailability and effects of oestrogens and xeno-oestrogens in the aquatic environment, J. Mar. Biol. Assoc. u K. 85 (1) (2005) 1–31, https://doi.org/10.1017/s0025315405010787h.
[19] R. Guedes-Alonso, Z. Sosa-Ferrera, J.J. Santana-Rodríguez, Determination of steroid hormones in fish tissues by microwave-assisted extraction coupled to ultra-high performance liquid chromatography tandem mass spectrometry, Food Chem. 237 (2017) 1012–1020, https://doi.org/10.1016/j.foodchem.2017.06.065.
[20] C. Fernandez, C.F. Boudouresque, Evaluating artificial diets for small *Paracentrotus lividus* (Echinodermata: echinoidea), in: R. Mooi, M. Telford (Eds.), Echinoderms, San Francisco, Balkema, Rotterdam, Netherlands, 1998.
[21] D. Vafidis, C. Antoniadou, V. Ioannidi, Population density, size structure, and reproductive cycle of the comestible sea urchin sphaerechinus granularis (echinodermata: echinoidea) in the pagasitikos gulf (Aegean Sea), Animals. (Basel) 10 (9) (2020) 1506, https://doi.org/10.3390/ani10091506.

[22] W. Wojnowski, M. Tobiszewski, F. Pena-Pereira, E. Psillakis, AGREEprep – analytical greenness metric for sample preparation, Trends Anal. Chem. 149 (2022) 116553, https://doi.org/10.1016/j.trac.2022.116553.
[23] W. Wojnowski, AGREEprep analytical greenness metric for sample preparation, mostwiedzy. pl/AGREEprep, (accessed July 2024).
[24] F. Pena-Pereira, M. Tobiszewski, W. Wojnowski, E. Psillakis, A tutorial on AGREEprep an analytical greenness metric for sample preparation, Adv. Sample Prep. 3 (2022) 100025, https://doi.org/10.1016/j.sampre.2022.100025.
[25] S. Cathum, H. Sabik, Determination of steroids and coprostanol in surface water, effluent and mussel using gas chromatography-mass spectrometry, Chromatographia 53 (S1) (2001) S394–S399, https://doi.org/10.1007/bf02490364.
[26] H. Wang, X. Zhou, Y. Zhang, H. Chen, G. Li, Y. Xu, et al., Dynamic microwave-assisted extraction coupled with salting-out liquid–liquid extraction for determination of steroid hormones in fish tissues, J. Agric. Food Chem. 60 (41) (2012) 10343–10351, https://doi.org/10.1021/jf303124c.
[27] R. Guedes-Alonso, L. Ciofi, Z. Sosa-Ferrera, J.J. Santana-Rodríguez, M. Del Bubba, A. Kabir, K.G. Furton, Determination of androgens and progestogens in environmental and biological samples using fabric phase sorptive extraction coupled to ultra-high performance liquid chromatography tandem mass spectrometry, J. Chromatogr. A. 1437 (2016) 116–126, https://doi.org/10.1016/j.chroma.2016.01.077.
[28] R. Guedes-Alonso, S. Montesdeoca-Esponda, J.A. Herrera-Melián, R. Rodríguez-Rodríguez, Z. Ojeda-González, V. Landívar-Andrade, Z. Sosa-Ferrera, J.J. Santana-Rodríguez, Pharmaceutical and personal care product residues in a macrophyte pond-constructed wetland treating wastewater from a university campus: presence, removal and ecological risk assessment, Sci. Total. Environ. 703 (2020) 135596, https://doi.org/10.1016/j.scitotenv.2019.135596.
[29] M.E. Torres-Padrón, S. Montesdeoca-Esponda, S. Santana-Viera, R. Guedes-Alonso, J.A. Herrera-Melián, Z. Sosa-Ferrera, et al., An update of the occurrence of organic contaminants of emerging concern in the Canary Islands (Spain), Water. (Basel) 12 (9) (2020) 2548, https://doi.org/10.3390/w12092548.
[30] R.J. Ulbricht, A.W. Pritchard, Effect of temperature on the metabolic rate of sea urchins, Biol. Bull. 142 (1) (1972) 178–185, https://doi.org/10.2307/1540254.
[31] J. Roma, K. Schertenleib, P. Ramalhosa, I. Gestoso, J. Canning-Clode, M. Lenz, Moderately elevated temperatures increase macroalgal food consumption in two sea urchin species from coastal waters of Madeira, J. Exp. Mar. Bio Ecol. 542–543 (151603) (2021) 151603, https://doi.org/10.1016/j.jembe.2021.151603.