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Application of a robust analytical method for quantifying progestins in environmental samples from three Portuguese Estuaries

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ABSTRACT

In the last years, progestins have raised special concerns for their documented negative effects on aquatic species, yet little is known about their environmental levels in surface waters and bioaccumulation in the trophic web. This study aimed to 1) adapt an extraction method for quantifying progestins in freeze-dried matrices, 2) validate the analytical procedure for three matrices: bivalve, polychaete, and crustacean, and 3) characterize levels of the four most prescribed synthetic progestins in key species across three Portuguese estuaries. Through the validated method, progestins were only quantifiable for the crustacean. Values were generally low, peaking with drospirenone values in Ria de Aveiro (1.33 ± 0.26 ng/g ww) and Tagus estuary (1.42 ± 0.55 ng/g ww), while Ria Formosa exhibited the lowest progestin concentrations (< 1 ng/g ww). This study enabled the development of a precise extraction and analytical method for quantifying steroid hormones in three distinct biological matrices.

1. Introduction

Coastal ecosystems, essential components of the Earth's biosphere, have been increasingly threatened by multiple stressors, among which the contamination from pharmaceuticals is an emerging pressing concern, due to increasing use and persistence in the environment. Within this pharmacological spectrum, steroid hormones, due to their impact even at trace concentrations (ng/L) within aquatic ecosystems, have garnered significant scrutiny (Cardoso, 2021). Among steroids, progestins are a subset of hormones with progestogenic activity, which have received little attention compared to other hormones (e.g., estrogenic compounds) by environmental scientists. Progestins are potent endocrine-disrupting compounds widely used in human medicine and contraceptive realms for utilization in livestock (Fent, 2015). The metabolism of progestins, though incompletely understood, has revealed that a proportion is excreted by humans (in the urine and faeces) in unchanged forms, entering directly into the wastewater treatment plants (WWTPs) (Bick et al., 2021). Despite treatment efforts, many WWTPs fall short of totally eliminating these compounds, resulting in a constant discharge into the aquatic systems, at low concentrations (ng/L) (Fent, 2015). In the last years, a great number of publications have emerged on these contaminants proving their negative effects, across different aquatic species, at different levels (e.g., reproduction, behaviour, thyroid functioning). These effects span from invertebrates (e.g. *Gammarus locusta*, Cardoso et al., 2018; *Nucella lapillus*, Morais et al., 2023a) to vertebrates (zebrafish *Danio rerio*, Cardoso et al., 2017; *Cyprinus carpio*, Steinbach et al., 2023; *Oryzias latipes*, Watanabe et al., 2023). Simultaneously, there is a growing concern about the amplified deployment of these synthetic compounds, projected to rise by approximately 13 % in the next 5 years (according to "Progesterone market" report (https://www.mordorintelligence.com/industry-reports/progesterone-market). This increase corresponds with

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population growth and an augmented focus on target disorders (e.g., abnormal uterine bleeding, menopause, endometrial cancer, hyperplastic precursor lesions). So, based on the evidence, it is of real interest to monitor the levels of synthetic progestins in various environmental matrices, encompassing water, sediment, and biota.

Some works have confirmed the presence of progestins in the environment at concentrations close to the WWTPs' influent (e.g., up to 1000 ng/L in West Malaysia (Tan et al., 2015) and 2000 ng/L in Beijing, China (Shen et al., 2019) and effluents (< 50 ng/L) (King et al., 2015; Shen et al., 2019). In surface waters, the concentrations have generally been much lower (average values <10 ng/L) (Golovko et al., 2018; Wang et al., 2021). However, there is almost no reference to the levels of progestins along the trophic web, particularly within estuarine systems. A recent study by Liu et al. (2023) identifies, for the first time, evidence of the occurrence of progestins and other steroids within an estuarine food web, demonstrating their potential for bioaccumulation. Attending to this potential of bioaccumulation of the steroid hormones along the trophic web, we expect to observe that the deposit feeder *Scrobicularia plana* and the omnivore *Hediste diversicolor* would present lower levels than the predator *Carcinus maenas* (Hypothesis).

Moreover, the integration of advanced, high-resolution instrumentation and refined analytical methods offers the prospect of deepening our investigation of these areas. Quadrupole Time-of-Flight Mass Spectrometry (QTOF-MS), recognized for its high resolution and precision, emerges as a pivotal tool for environmental samples (Xian et al., 2012). The technology facilitates the screening and identification of compounds, with high accuracy and sensitivity, allowing the quantification of compounds, in biological matrices, at low concentrations (Murao et al., 2007; Li et al., 2021).

Additionally, the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction method has demonstrated its utility across a wide range of matrices and analytes; this includes progestins analysis in fish (Tan et al., 2016; Luo et al., 2018; Gu et al., 2019), milk (Tan et al., 2016; Decheng et al., 2021), rabbit plasma (Tran et al., 2020), wastewater (Peysson and Vulliet, 2013) and water (Yang et al., 2015).

In this work, we employ this rapid extraction technique in conjugation with an analytical Ultra-High Performance Liquid Chromatography (UHPLC) coupled to a QTOF-MS/MS to detect and quantify progestins in different biological matrices from three key estuaries along the Portuguese coast. Consequently, the primary objectives encompass the following:

1) Adapting the QuEChERS extraction protocol for freeze-dried matrices;
2) Validate the quantification method of 4 main progestins from target biological models (*Hediste diversicolor, Scrobicularia plana,* and *Carcinus maenas*);
3) Do a temporal profiling of the prevailing progestin levels focusing on the most frequently prescribed progestins in Portugal (i.e., drospirenone (DRO), desogestrel (DSG), gestodene (GST) and levonorgestrel (LNG)), based on the list of authorized contraceptives for each EU member state, aligned with the Directive 2001/83/CE. This investigation is conducted across three distinct estuaries: Ria de Aveiro, Tagus Estuary and Ria Formosa.

2. Materials and methods

2.1. Chemicals and solvents

Target progestins: desogestrel (CAS n°: 54024-22-5; >98 %), drospirenone (CAS n°: 67392-87-4; >98 %), gestodene (CAS n°: 60282-87-3; >98 %) and levonorgestrel (CAS n°: 797-63-7; >97 %), were obtained from TCI chemicals (Japan). The internal standard (IS) used was progesterone-d9 (CAS n° 15775-74-3, 97 %, Steraloids, USA).

Stock solutions of individual compounds were prepared, with concentrations set at 1000 μg/L in methanol, and stored at −20 °C for a maximum of 1 month. Stock solutions were appropriately mixed and diluted to create the working solutions. Chemicals reagents: methanol hypergrade (CAS 67-56-1) for liquid chromatography-mass spectrometry (LC-MS), from Supelco (Germany), ultrapure water (CAS 7732-18-5, resistivity 18.2 MΩ.cm 25 °C, Milli-Q® Advantage A10 Water Purification System, Millipore, USA), formic acid (CAS 64–18-6, Sigma Aldrich, >95 %, USA), isopropanol (CAS 67–63-0, Fisher Chemicals, 99.9 %, USA) and sodium hydroxide (CAS 1310-73-2, Fisher Chemicals, USA).

For QuEChERS: magnesium sulphate (Alfa Aesar, > 99 %, Germany), sodium chloride (VWR Chemicals, 99.9 %, USA), citrate sodium (ChemCruz, > 99 %, USA) and disodium citrate sesquihydrate (> 99 %, Sigma Aldrich, USA), acetonitrile (ACN, CAS 75–05-8, Honeywell, >99.9 %, USA), and n-hexane (CAS 110–54-3, Honeywell, > 95 %, USA).

2.2. Study sites

Three Portuguese estuaries along the coast underwent a seasonal sampling (Ria de Aveiro: November of 2019 (au) and July (su) of 2020; Tagus estuary: October (au) of 2019 February (wi), March (sp), July (su) of 2020; Ria Formosa: October (au) of 2019, March (sp) and July (su) of 2020). The lack of data in some temporal points in Ria de Aveiro and Ria Formosa was due to some logistic issues related to COVID-19 constraints.

Ria de Aveiro: Ria de Aveiro is a shallow coastal lagoon located on the north-west Portuguese coast (40° 38′N, 8° 45 W) separated from the sea by a sand bar (Dias et al., 1999) with an artificial connection to the Atlantic Ocean. The lagoon has four main branches: Mira, S. Jacinto, Ílhavo and Espinheiro channels covering an area of approximately 75 Km^2. Small rivers or streams discharge into the channels (Dias et al., 1999). The area is surrounded by small and medium-sized cities, with approximately 370 K inhabitants (Fidélis et al., 2019). Population pressure and industrialization have increased over the past decades, increasing the contamination of this lagoon. Municipal WWTPs are important sources of synthetic progestins (Chang et al., 2009). Aveiro has three main WWTPs: Cacia, Ilhavo and S. Jacinto. Cacia WWTP is prepared to receive domestic effluents from 272 K inhabitants (https://www.aguasdocentrolitoral.pt/aveiro/); Ilhavo treats domestic effluents from around 160 K inhabitants (https://www.adp.pt/pt/?id=61&img=90&bl=6) and S. Jacinto is the smallest designed to serve a population of around 9.4 K inhabitants (http://www.simria.pt/gca/popup_2.php?id=97). The S. Jacinto effluent is sent to a submarine outfall (S. Jacinto outfall) and discharged into the Atlantic Ocean a few kilometres from the entrance to the estuary (Jonkers et al., 2010). However, due to sea currents, it can participate in the contamination of the lagoon.

Five sampling stations (i.e., Ovar, 40°52′00″N 8°39′11″W; Torreira, 40°46′14″N 8°42′09″W, Murtosa, 40°44′08″N 8°38′31″W, Gafanha do Carmo, 40°35′18″N 8°44′52″W and Gafanha de Boa Hora, 40°31′48″N 8°46′42″W) were selected along a transect over the coast (see Fig. 1).

Tagus estuary: Tagus estuary is one of the biggest in Europe, covering an area of approximately 320 km^2 (Álvarez-Muñoz et al., 2015). The surrounding area is the most populated area of Portugal with approximately 1.77 million inhabitants. In the past decades, the estuary has been receiving agricultural and urban effluents from the large metropolitan area of Lisbon (Brito et al., 2018). WWTPs are dispersed in the margins. There are several WWTPs that discharge treated effluent into the Tagus estuary. Some of them that are close to our study area are: Alcântara, Beirolas, Chelas, Afonsoeiro, Seixal and Seixalinho. Alcântara is one of the largest WWTPs and was designed to treat domestic effluents from 756 K inhabitants (https://www.aguasdotejoatlantico.adp.pt/content/alcantara). Beirolas is prepared to receive effluents from approximately 214 K inhabitants (https://www.aguasdotejoatlantico.adp.pt/content/beirolas). Chelas is a little smaller with the capacity to receive effluent from approximately 211 K inhabitants (https://www.aguasdote

Fig. 1. Schematic representation of the study sites in the three estuaries on the Portuguese coast. A) Ria de Aveiro; B) Tagus estuary; C) Ria Formosa.

joatlantico.adp.pt/content/chelas). Afonsoeiro receives effluent for 48 K inhabitants (https://www.simarsul.adp.pt/content/afonsoeiro), Seixal for 156 K inhabitants (https://www.simarsul.adp.pt/content/seixal) and Seixalinho for 64.5 K inhabitants (https://www.simarsul.adp.pt/content/seixalinho).

Five sampling stations were selected along the estuary, two in the North Margin (Alhandra, 38°55'51 N 9°00′16″W and Trancão, 38°48'09 N 9°05′39″W), two in the South margin (Seixal, 38°38'48 N 9°06′31″Wand Samouco, 38°44'03 N 9°00′51″W) and one in the mouth (Trafaria, 38°40′52″N 9°14′10″W).

Ria Formosa: Ria Formosa is a mesotidal coastal lagoon with 180 Km^2 of area, in permanent connection with the sea through six channels from the sand barrier. Located in the South of Portugal, represents the largest lagoon of the Portuguese coast (Said et al., 2019) and extends for 55 km, with a total surface area of 10 000 ha. Water renovation in the lagoon is about 50–75 % in each tidal cycle. The population living in the surrounding area varies throughout the year, increasing especially in summer due to intense tourism. There are 3 WWTPs that discharge into this lagoon system: Almargem, Faro Noroeste and Faro/Olhão. Almargem has the capacity to treat the effluent of a maximum population of 48 K inhabitants (https://www.aguasdoalgarve.pt/content/etar-de-almargem-0). Faro Noroeste was designed for a treatment capacity of approximately 45 K inhabitants (https://www.aguasdoalgarve.pt/content/etar-de-faro-noroeste). The Faro/Olhão WWTP has a maximum capacity to receive effluent equivalent to approximately 113 K inhabitants (https://www.aguasdoalgarve.pt/content/etar-de-faroolhao). In the surrounding areas, there are two main WWTPs: Albufeira-Poente and Vila Real de Sto António. Albufeira-Poente serves 134 K inhabitants () and Vila Real de Sto António serves approximately 58 K inhabitants (https://www.aguasdoalgarve.pt/content/etar-de-vila-real-de-santo-antonio).

Five sampling stations were selected along the lagoon (i.e., Aeroporto de Faro, 37°01′10″N 7°59′14″W; Faro, 37°00′31″N7°55′33″W; Olhão, 37°01′35″N 7°50′51″W; Fuseta, 37°03′21″N 7°44′40″W and Tavira, 37°07′07″N 7°37′51″W).

2.3. Sampling procedure

Macrobenthic samples were collected with a core (141 cm^2 surface area) to a depth of 20 cm. Samples were, later, washed with estuarine water through a 500 μm mesh bag, placed into plastic bags and transported in a cool box. In the laboratory, organisms were separated into the main species and kept in filtered seawater (adjusted to field salinity), with air bubbling for 24 h to eliminate sediment particles. Afterwards, they were separated under a dissecting microscope, identified to the lowest possible taxon and frozen at −20 °C. All the samples were freeze-dried (to guarantee sample stability and reduce sample variability) and homogenised for later quantification. To ensure adequate biomass for progestins' quantification, composite samples (of 0.5 g dry weight (dw) each) were prepared. These samples consisted of individuals from the same species, collected across different study sites within each estuary and categorized by season. Three main species per estuary were chosen as representative of the trophic web: the bivalve *Scrobicularia plana* (detritivore and suspension feeder), the polychaete *Hediste diversicolor* (carnivore) and the crustacean *Carcinus maenas* (predator).

2.4. QuEChERS extraction method

The same extraction procedure was applied to the three species. For validation and quantification purposes, we used the edible part (soft body) of *S. plana*, and the whole-body of *H. diversicolor* and *C. maenas*.

The progestins' extraction procedure was adapted from Berlioz-Barbier et al. (2014), with modifications to accommodate our freeze-dried samples. In our approach, reagent quantities were adjusted proportionally based on the equivalent wet weight of the samples.

Briefly, we added 1) distilled water (3000 μL) to the sample (0.5 g dw) and let it soak for 15 min. Sequentially, we added 2) the organic solvents (ACN (3000 μL), followed by n-hexane (1200 μL)), and 3) a mixture of salts containing: sodium citrate (0.3234 g), disodium citrate (0.1617 g), sodium chloride (0.3234 g), and magnesium sulphate (1.2936 g). Then, after 4) vigorous vortexing and immediate shaking to prevent salt agglomeration (for 2 min), a 5) centrifugation step (5478 *g*, 4 °C, 2 min, Thermo Scientific, USA) facilitated phase separation. Subsequently, 6) 2 mL of ACN (located below the hexane fraction) was collected and then added 6 μL of internal standard (30 μgL^{-1} in the final extract), followed by 7) a filtration process (0.22 μm, non-sterile, Clarify-PTFE 13 mm, USA) before injection. Samples were stored at −20 °C. For validation purposes, we utilized commercial *S. plana*, while *H. diversicolor* along with *C. maenas*, were collected from the Mondego estuary (reference site). The same reference matrices were used for quantification purposes (i.e., matrix-matched calibration curves) of the environmental samples.

2.5. Analytical instrument setup and mass spectrometer conditions

Samples were injected via an UHPLC Focused Dionex ultimate 3000 (Thermo Fisher, USA) coupled to a quadrupole time-of-flight (QTOF) instrument Impact II (Bruker Daltonics, USA), equipped with electrospray ionisation (ESI) source. The ion polarity used was in positive mode generating $[M + H]^+$ precursor and product ions. The UHPLC conditions for the separation were as follows: mobile phase A was ultrapure water 0.1 % formic acid and mobile phase B was methanol 0.1 % formic acid. The gradient program (*v*/v) started with 55 % B (v/v), held at 55 % B (v/v) for 4 min, increased to 95 % B (v/v) at 4 min, held at 95 % B (v/v) for 1 min and then decreased at 55 % B (v/v) for 2 min. For each run, there was a separation of 10 μL (volume injected) of the sample through an Acclaim™ 120 C18 column (ref: 059143, 100 mm × 2.1 mm, id 2.2 μm, Thermo Scientific, USA), at a flow rate of 0.35 mL/min and a column temperature of 55 °C. Gas temperature and capillary voltage were maintained at 200 °C and 4500 V, respectively. The flow was 4 L/min and the nebulization gas pressure was 0.3 bar. The endplate offset potential was 500 V and the dry temperature was 200 °C. Internal calibration reagent used to calibrate the masses in each injection consisted of 12.5 mL ultrapure water, 12.5 mL isopropanol, 50 μL formic acid (95 %), 250 μL sodium hydroxide. Detection using MS/MS was executed with specific parameters detailed in Table 1; each compound was characterized by its retention time and at least two ions (precursor and product ions). To process data, we used the Compass Data Analysis ver. 5.0 software.

2.6. Method validation

The validation procedure for the identification and quantification of drospirenone, gestodene, desogestrel and levonorgestrel, followed the ICH Guidelines (EMEA (European Medicines Agency), 2022).

For each target compound, we evaluated linearity using three (n = 3) independent calibration matrix curves, done in three independent days, each with seven nominal concentrations, ranging from 10 ng/L to 200 ng/L in the final extract (equivalent to 0.20 to 3.99 ng/g ww). For plotting the mathematical curves, we used the ratio between the standard and the IS areas (denominated as A_{ratio}) and the relationship was evaluated based on the statistical F-test and the R^2 value. The limits of detection (LOD) and quantification (LOQ) were determined using the following equations (eq.):

$$LOD = 3.3 \times \frac{\alpha}{S}, \tag{1}$$

$$LOQ = 10 \times \frac{\alpha}{S}, \tag{2}$$

where α is the standard deviation of the response, and S is the average slope of the calibration curves.

For calculating absolute recoveries (%), matrix effect (%), precision (%RSD), accuracy (%), and stability, we used three independent replicates (n = 3) of a quality control (QC): 10 x higher than the average LOQ. To check recoveries, we used control samples (blank) spiked with the mixture of target compounds at 10 x LOQ concentration, as mentioned in Eq. (3).

$$Recoveries\ (\%) = \left(\frac{A_{ratio\ 10xLOQ\ in\ matrix\ spiked\ before}}{A_{ratio\ 10xLOQ\ in\ matrix\ spiked\ after}}\right) \times 100 \tag{3}$$

Matrix effect (ME) was calculated by spiking the matrix after extraction ($A_{standard}$ in matrix) and compared it with those of injected standards ($A_{standards}$), as mentioned in Eq. (4):

$$ME\ (\%) = -\left[\left(\frac{Aratio\ standard - Aratio\ standard\ in\ matrix}{Aratio\ standard}\right)\right] x\ 100 \tag{4}$$

Intra- and inter-day precision (both expressed as the relative standard deviation (% RSD)) were calculated using a quality control (10 x LOQ). Intra-day precision was calculated according to Eq. (5).

$$Intra-day\ precision\ (\%RSD) = \left(\frac{SD\ A_{ratio\ 10xLOQ\ in\ matrix\ (n=8)}}{Average\ A_{ratio\ 10xLOQ\ in\ matrix\ (n=8)}}\right) \times 100 \tag{5}$$

Inter-day precision was calculated, using the information from 3 different days, through the Eq. (6).

Table 1
Molecular mass (g/mol), retention time (RT, min), precursor and product ions $[M + H]^+$ of progestins and IS, as well as their collision energies (eV) used in UHPLC-ESI-QTOF-MS/MS analyses. * Quantification ions.

Target compounds	Molecular mass (g/mol)	RT (min)	Precursor ion $[M + H]^+$	→	Product ions	Collision energy (eV)
Gestodene	310.1933	3.3	311.2095	→	109.0596 183.1169 293.1903*	20
Drospirenone	366.2195	3.4	367.2270	→	97.0598 257.1539*	20
Levonorgestrel	312.2089	3.7	313.2167	→	109.0650 245.1904 295.2059*	20
Desogestrel	310.2297	6.1	311.2370	→	121.1014 159.1169 213.1636*	20
Progesterone -d9 (IS)	325.5300	4.2	324.2762	→	100.0837 113.0899 306.2776*	20

$$Inter-day precision\,(\%RSD) = \left(\frac{Mean\,of\,the\,SDA_{ratio\,10xLOQ\,in\,matrix\,(n=3)}}{Mean\,o\,the\,means\,A_{ratio\,10xLOQ\,in\,matrix\,(n=3)}}\right) \times 100 \quad (6)$$

Accuracy was evaluated as indicated in Eq. (7).

$$Accuracy\ (\%) = \left(\frac{Calculated\ concentration}{Nominal\ concentration}\right) \times 100 \quad (7)$$

For assessing the stability of the progestins in the extracts, samples were stored at rack sampler temperature (10 °C) and were considered several time points, including immediately after preparation (0 h), as well as after 24 h, 48 h, and 7 days. Stability was calculated according to Eq. (8).

$$Stability\ (\%) = \left(\frac{A_{ratio\ 10xLOQ\ in\ matrix\ (hours\ after)}}{A_{ratio\ 10xLOQ\ in\ matrix\ (0\ hour)}}\right) \times 100 \quad (8)$$

To assure the quality of the run, we injected simultaneously with each batch of injections, a 10 x LOQ as QC together with 2 solvent samples (one at the beginning and another at the end of the run).

2.7. Quantification of target compounds

To achieve accurate quantification, a matrix-matched calibration method was used, employing either commercial or environmental-reference matrices, as previously specified. For each series of analyses, a seven-point calibration curve (0, 5, 10, 20, 30, 60 and 75 ng/L equivalent to 0.1, 0.2, 0.4, 0.6, 1.2 and 1.499 ng/g ww) was generated using the same approach as outlined in the sample extraction part. Blank matrices were spiked with the mix standard solution before performing the extraction. Following the extraction process, the IS (6 μL) was added, resulting in a final concentration of 30 μg/L. Trace residues were subtracted from the final environmental samples, when necessary.

2.8. Statistical and data analyses

The performance of the matrix-matched calibration curves was assessed using an F-test for linear regression, with a significance level of 5 %. The determination coefficients (R^2) and adjusted R^2 were employed to evaluate the fitting quality.

Regarding the stability of the samples, for each progestin, a 1-Way ANOVA (factor: time) was applied to check for statistical differences among times.

2-Way ANOVAs (factors: hormone and sampling time) were applied to evaluate differences in *C. maenas* concentrations between times and hormones, for each estuarine system.

For all data, we first checked for normality using the Kolmogorov–Smirnov test and for homogeneity of variances using the Levene's test (Zar, 1999). All the analyses were done using Statistica 7.0 software.

Bioaccumulation factors (BAF) were determined by dividing the total concentration of each progestin in the organism by the concentration of that element in the water. Progestin concentrations in the water column were previously published in Morais et al. (2023b).

3. Results & discussion

3.1. QuEChERS extraction and method validation

Sample extraction was successfully optimised for the extraction of three hormones: drospirenone, gestodene and levonorgestrel; the validation parameters are indicated in Table 2 and Fig. 2. Desogestrel was excluded from the subsequent validation steps because it exhibited a significant lack of stability within the targeted concentration range, where concentrations below 10 ng/L were even undetectable. QuEChERS salt quantities were kept identical for the three species to facilitate the extraction procedure. However, the salt mixture was applied in excess for *H. diversicolor* and *C. maenas* due to their slightly lower water content compared to *S. plana*. This approach ensures a consistent extraction procedure for all samples, minimizing potential errors.

To accurately identify and quantify each target hormone, we considered target peaks with a deviation of <0.01 min and required at least two target ions for quantification. This stringent criterion was implemented to ensure the accuracy and reliability of the final results.

The seven-point calibration curves proved to have good fits, with r^2 values ranging from 0.990 to 0.998 and the target compounds passed successfully the F-test ($p < 0.001$). Based on our preliminary tests involving the pooling of multiple samples from each estuary, we narrowed the concentration range to 5 to 75 ng/L (equivalent to 0.1–1.5 ng/g ww). This adjustment was made to better align with the characteristics of the target environmental samples across the three matrices. Moreover, this concentration range remains applicable to other environmental samples (including different matrices), such as those concentrations found in the crab *Clibanarius striolatus* in the study of Liu et al. (2023).

Intra-day precision (%RSD) results ranged from 4.56 to 7.40 %, for the target progestins and were 4.26 %, for the IS. Regarding inter-day precision, the results were calculated from 3 days (24 h, 48 h and 7 days) and ranged between 8.22 and 15.00 %. Therefore, it successfully achieved the selectivity, specificity, and precision criteria.

LOD values ranged from 0.08 to 0.16 ng/L (0.003 to 0.006 ng/g) and LOQ (0.009 to 0.017 ng/g) values ranged from 0.24 to 0.48 ng/L (Table 2). Due to the lack of analytical methods suitable for the quantification of progestins in biological matrices, it is difficult to compare our results with other studies. Nevertheless, a lower LOQ for levonorgestrel (0.24 ng/L equivalent to 0.009 ng/g ww) was obtained when compared with the LOQ value registered for levonorgestrel in *Chironomus riparius* matrix (4.2 ng/g ww) (Berlioz-Barbier et al., 2014). This indicates a higher sensitivity for the current method, which can be an advantage, especially when dealing with trace levels of substances in complex biological matrices. A lower LOQ allows for the detection and quantification of analytes at lower concentrations, providing a method with better performance in terms of its ability to measure low-level concentrations accurately.

The optimised extraction method yielded recovery rates varying from 72.70 % to 96.40 %, proving the viability of this QuEChERS

Table 2
Calibration curves and their respective coefficient of determination (R^2), limits of detection (LODs) and limits of quantification (LOQs) (ng/L and ng/g ww), average recovery (%, n = 3), accuracy (%, n = 3), intra-day precision (% RSD, n = 8), inter-day precision (% RSD, n = 3), matrix effect (%, n = 3) data for the selected synthetic progestins; average ± SD.

Progestin	Calibration curve	R^2	F-test	LOD	LOQ	Recoveries	Accuracy	Intra-day precision	Inter-day precision	Matrix effect
	Mathematical equation	%	(*p*-value)	ng/L (ng/g ww)		Av (%) ± SD				
Drospirenone	y = 0.0050x - 0.0564	0.999	0.0005	0.16 (0.006)	0.48 (0.017)	75.50 ± 1.26	88.00 ± 4.36	7.40 ± 0.98	8.22 ± 1.66	21.50 ± 6.70
Gestodene	y = 0.0066x + 0.0102	0.998	0.0002	0.15 (0.005)	0.45 (0.016)	72.70 ± 7.17	89.30 ± 1.38	6.84 ± 3.18	15.00 ± 3.30	48.90 ± 13.30
Levonorgestrel	y = 0.0114x + 0.0772	0.990	0.0003	0.08 (0.003)	0.24 (0.009)	96.40 ± 6.23	84.50 ± 3.00	4.56 ± 0.97	9.45 ± 0.13	51.06 ± 8.90

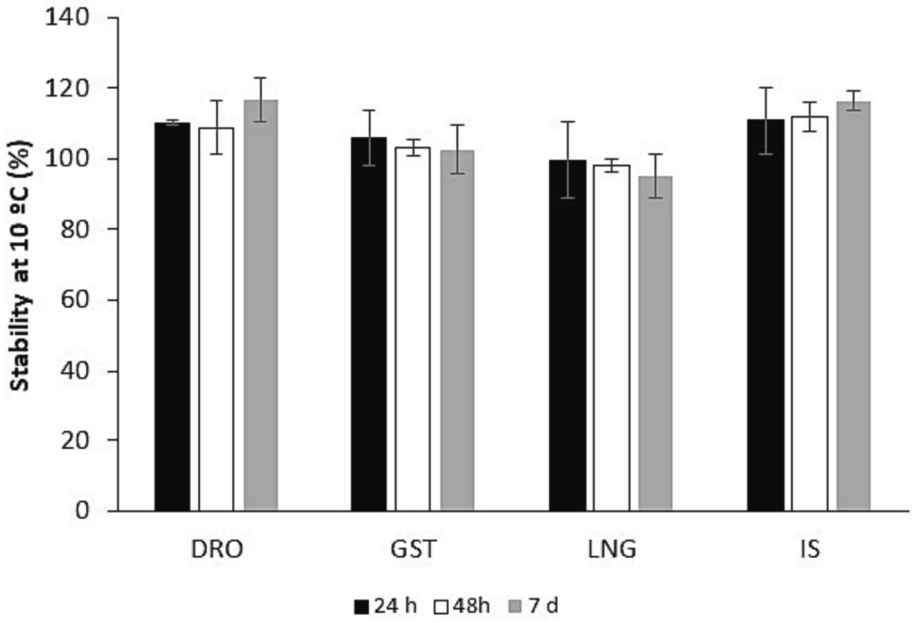

Fig. 2. Stability of the three synthetic progestins and the internal standard (IS) in the extracted matrix over time (n = 3); Values represent mean ± SD. DRO – drospirenone; GST – gestodene; LNG – levonorgestrel; IS – internal standard.

procedure for the extraction of the synthetic progestins. The fact that matrices used have an elevated water content, ranging from 70 % for *C. maenas* to 83–88 % for *H. diversicolor* and *S. plana*, respectively (Cardoso PG, *personal data*), and a low lipid profile (< 5 % for *C. maenas*, <19.3 % for *H. diversicolor,* and < 1.5 % for *S. plana*) (Luis & Passos, 1995; Mendes de Oliveira, 2012; Naczk et al., 2004), have contributed to the efficacy of this protocol's recoveries. Some studies reported similar average recoveries for comparable matrices, such as mussels (93 % for levonorgestrel) (Chafi and Ballesteros, 2022) or fish (87.77–117.08 % for 10 progestins) (Gu et al., 2019). Comparing the present study with the original of Berlioz-Barbier et al. (2014), we could obtain higher recoveries for levonorgestrel (96.40 %) compared to them (62.30 %).

The stability of the extracted progestins was evaluated by comparing the initial results of the quality controls with those obtained after 24 h, 48 h and 7 days. According to Fig. 2, the solutions of the three progestins and IS were kept stable during 7d at 10 °C (rack sampler temperature), indicating that it is possible to still use the extracted samples for quantification purposes, within this time frame. No statistical differences were observed among times (1-Way ANOVA, $p > 0.05$).

Environmental sample analyses often highlight matrix effects (ME), where residual matrix components may cause ion enhancement or suppression in the electrospray source, leading to potential inaccuracies (Berlioz-Barbier et al., 2014; George et al., 2018).

Since the IS was used after extraction to control the equipment's precision between replicates, we used matched-matrix calibration curves to correct for variations adequately. Indeed, our ME results indicated a signal enhancement (above 20 %) for the three compounds (Table 2), showing the importance of using matrix-matched calibration curves for biological samples. Using both approaches (IS and matched-matrix) can offer a more robust analytical validation strategy, ensuring accurate and precise results across various sample matrices.

3.2. Progestins quantification in environmental samples

Seasonal samples, from the three selected species, were analysed to assess the progestin levels in three aquatic environments. The targeted progestins were not detected in samples from *S. plana* and *H. diversicolor*, so our data is limited to the crab *C. maenas*. In general, the values were relatively low, with the highest values found for drospirenone (DRO) in Ria de Aveiro (1.33 ± 0.26 ng/g ww) and Tagus estuary (1.42 ± 0.55 ng/g ww), during autumn and spring, respectively.

In Ria de Aveiro, significantly higher concentrations emerged during autumn compared to summer (2-Way ANOVA, $F_{(1,11)} = 87.22$, $p < 0.05$). Notably, DRO presented significantly higher values than gestodene (GST) (2-Way ANOVA, $F_{(2,11)} = 4.88$, $p < 0.05$) (Fig. 3A).

For the Tagus estuary, we found significantly higher concentrations of progestins during spring campaigns (2-Way ANOVA, $F_{(1,15)} = 12.39$, $p < 0.05$) (Fig. 3B). However, no significant differences were noted among progestins in this estuary.

In contrast, the Ria Formosa system registered the lowest progestin concentrations (< 1 ng/g ww of DRO). A noteworthy interaction between progestins and time was evident (2-Way ANOVA, $F_{(2,12)} = 31.66$, $p < 0.05$) (Fig. 3C), where the highest concentrations were generally observed during spring campaigns. Detailed information on the concentrations of progestins in biological data can be assessed in supplementary material (Tables S1 and S2).

An explanation for higher DRO levels may be related to its greater log Kow value of 4.02 compared to LNG and GST with values of 3.48 and 3.26, respectively (Kumar et al., 2015), suggesting that DRO has a slightly higher affinity for adsorption onto the organic matter in sediments. This characteristic potentially leads to a heightened capacity for bio-concentration within macrobenthic species, as opposed to LNG and GST. This distinction could explain the rationale behind the elevated concentrations observed in most study sites.

The progestins levels found in our study align with those reposted by Liu et al. (2023), in the Pearl River Estuary (China), showing the widespread occurrence of these hormones in aquatic environments. In their research, they found norgestrel concentrations (which is equivalent to levonorgestrel) for the crab *Clibanarius striolatus,* ranging from 4.1 ng/g ww (with non-enzymatic hydrolysis, i.e. in free forms) to 5.6 ng/g ww (with enzymatic hydrolysis, i.e., in free and conjugated forms).

The exclusive quantification of progestins in the crab *Carcinus maenas* can be related to its position within a higher trophic level (i.e., predator), conferring greater capacity to accumulate pollutants compared to species like *Scrobicularia plana* (detritivore) and *Hediste diversicolor* (carnivore). These results support our hypothesis. In addition, according to Liu et al. (2023), crabs have the highest bioaccumulation factor (log BAF) of synthetic steroids, ranging from 2.3 to

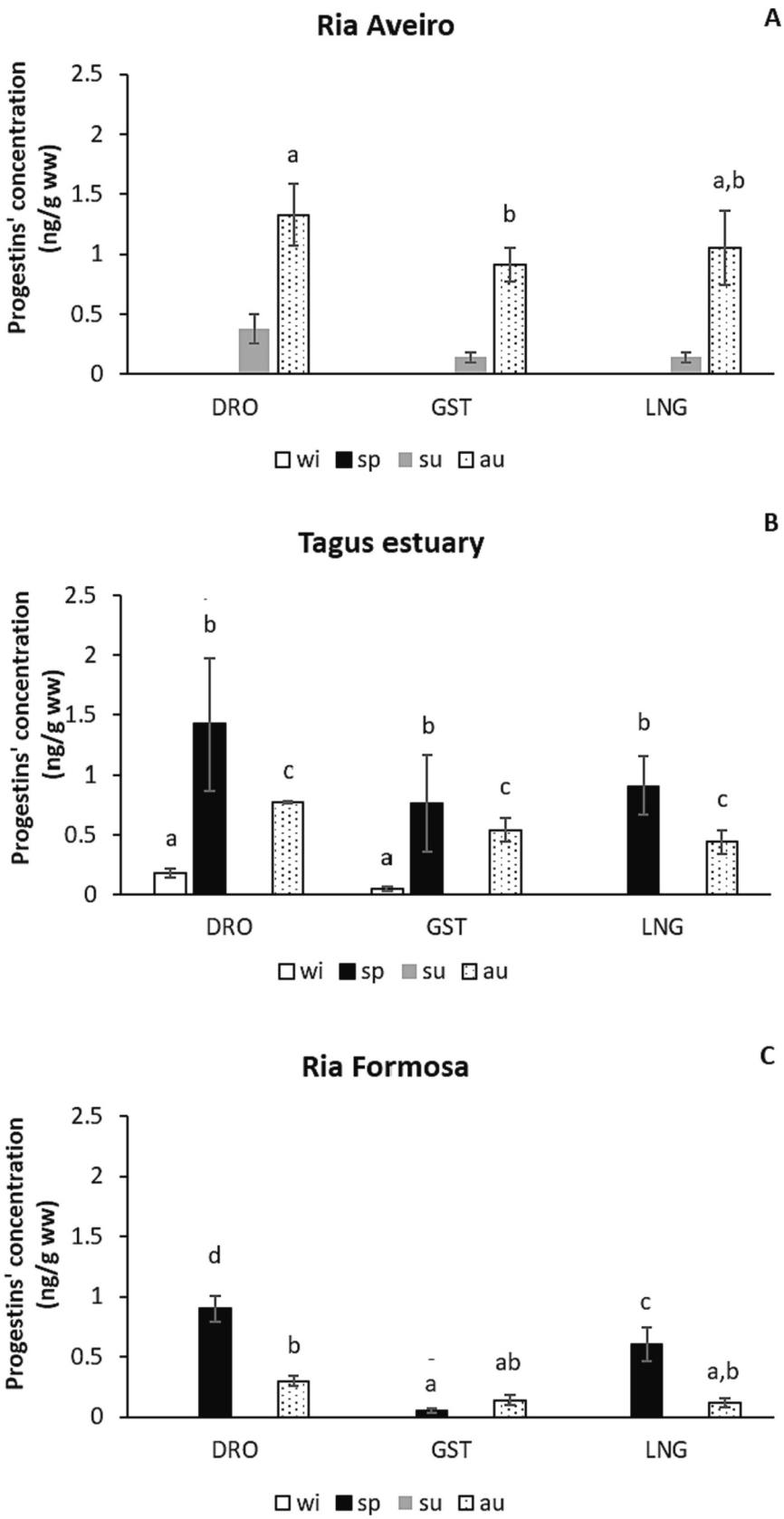

Fig. 3. Progestins' concentrations (ng/g ww) in *Carcinus maenas* in the three estuaries at different sampling periods. A) – Ria de Aveiro; B) – Tagus estuary; C) Ria Formosa. Values represent mean ± SD. Sp – spring; su – summer; au – autumn; wi – winter. Nd – non-detected. DRO – drospirenone; GST – gestodene; LNG – levonorgestrel. Different letters indicate significant differences among treatments.

6.4, followed by fish, snails, and shrimps, whose log BAF values range from 2.1–4.3, 1.8–4.3, and 2.4–4.1, respectively. In the present study, the bioaccumulation factors (BAFs) were calculated whenever it was possible, revealing values predominantly lower than 1, indicating a biodilution rather than an accumulation of pollutants from the water matrix to biological tissues. Only for LNG, in the Tagus estuary during autumn, exhibited a slightly higher BAF (1.16). This is in accordance with Liu et al. (2023), who found the highest BAF for norgestrel. According to them, this compound can be more stable and resistant to metabolism in crabs.

On the other hand, the BAF values found in our study can be related to multiple factors, such as environmental concentrations, sex, lipid content, size, and water content, which can regulate the concentrations of exogenous contaminants (Liu et al., 2017), or even the stability of the freeze-dried samples.

4. Conclusions

This study allowed the development of an accurate extraction and analytical method able to quantify other steroid hormones (i.e., progestins like drospirenone, gestodene, and desogestrel) besides the ones mentioned in the original paper of Berlioz-Barbier et al. (2014). This allowed us to advance with an accurate methodology to quantify different progestins in three different biological matrices, at an important environmental range of concentrations. Furthermore, utilizing freeze-dried samples could enhance their long-term stability. However, it is crucial to quantify these samples within a limited timeframe, such as within one year, to ensure their integrity.

CRediT authorship contribution statement

V.E. Amorim: Writing – original draft, Methodology, Investigation, Data curation. **H. Morais:** Writing – review & editing, Methodology. **A. C. Silva Ferreira:** Writing – review & editing, Supervision. **M.A. Pardal:** Writing – review & editing, Validation, Methodology. **C. Cruzeiro:** Writing – review & editing, Validation, Supervision, Methodology. **P.G. Cardoso:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Patricia Cardoso reports financial support was provided by FCT. Patricia Cardoso reports financial support was provided by NORTE2020. If there are other authors, they 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2023.115967.

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