ACS AGRICULTURAL SCIENCE & TECHNOLOGY

pubs.acs.org/acsagscitech Article

Analytical Strategy for Identification and Quantification of 13 Steroids in Sole (*Solea senegalensis*) Tissues, Eggs, and Larvae for Application in Aquaculture Studies of Reproduction

Roque Serrano,* Eduardo Beltrán, Vanesa Piquer, Félix Hernández, and Evaristo Mañanós

Cite This: *ACS Agric. Sci. Technol.* 2021, 1, 89−99 Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Knowing the levels of steroids in fish is crucial for understanding the reproductive processes during fish development and for reproductive success in aquaculture facilities. Although some of these compounds are present at very low concentrations, they play a relevant role in reproduction processes. Therefore, a very sensitive and reliable analytical methodology is required for their accurate determination. In this work, ultra-high-performance liquid chromatography coupled to tandem mass spectrometry with a triple quadrupole has been optimized for the identification and quantification of 13 steroids in plasma, muscle, and ovaries of sole (*Solea senegalensis*) and, for the first time, in fish eggs and larvae. This is noteworthy because studying fish development in its early stages could be the key to solving the reproductive problems observed in farmed specimens. Different strategies have been applied to solve analytical challenges such as matrix effects or the small amount of sample available. Compounds selected include progestagens (progesterone, 17α,20β-dihydroxypregnenone, and 17α,20β,21-trihydroxypregnenone), androgens (androstenedione, 11β-hydroxyandrostenedione, 11-ketoandrostenedione, testosterone, 5α-dihydrotestosterone, and 11-ketotestosterone), estrogens (estrone and estradiol), and corticosteroids (cortisol and 11-deoxycortisol). The methodology was validated in terms of accuracy (between 73% and 131%) and precision (relative standard deviations of <24%), at concentrations as low as 0.1 ng/mL for plasma, 0.1 ng/g for muscle and eggs, and 0.5 ng/g for ovaries and larvae. The application of the developed methodology to the analysis of samples from captive fish has allowed the identification and quantification of steroids at sub-nanogram per millilier and sub-nanogram per gram levels.

KEYWORDS: *ultra-high-performance liquid chromatography, tandem mass spectrometry, steroid hormones, fish, sole*

1. INTRODUCTION

Steroid hormones control the reproduction of vertebrates, and therefore, analytical methodologies for their determination in tissues are necessary to study their metabolism. Specifically in fish, the evaluation of steroid concentrations is crucial for understanding the different reproductive processes during fish development. This is an essential tool for optimizing broodstock management and reproductive success in aquaculture facilities. Moreover, fish can be a valid transferable model for research on the endocrine systems of other vertebrates.[1] However, despite scientific and technological efforts in the study of steroid hormones in fish during the past several decades, the specific role of steroids in critical reproductive processes is still unknown in some cases, and it is restricted to a few steroids for which immunoassay techniques are available. Knowledge of the biosynthesis of reproduction-related steroids is essential for understanding the reproductive dysfunctions that many cultured fish exhibit in aquaculture.[2] The Senegalese sole (*Solea senegalensis*), an important aquaculture fish species in Europe and Mediterranean countries, is not an exception, with serious reproductive disorders affecting hatchery-produced sole breeders, which is currently limiting the industrial expansion of the aquaculture of this species.[3,4]

Immunoassays and radioimmunoassays are mostly used for steroid hormone determination[5−7] in aquaculture, as they allow the sensitive determination of steroids with high throughput after automation. However, the lack of specificity, limited dynamic range, crossed reactivity, and matrix effects make their determination problematic at very low concentrations.[8] Analytical methodologies based on liquid chromatography (LC) or gas chromatography (GC) coupled to tandem mass spectrometry (MS/MS) provide improved specificity and allow the simultaneous multiclass determination of several hormones.[5,9] GC-MS/MS has been applied for the determination of steroids in blood,[10] but the laborious sample preparation and low throughput prevent its wide use in laboratories. However, LC-MS/MS-based methods present high sensitivity and specificity, reducing the sample preparation time, and are very appropriate for (medium) highly polar compounds.[5,9] Consequently, LC-MS/MS is widely used for steroid determination in mammalian samples, such as plasma,[11] milk,[12] urine,[13,14] or brain.[15] With regard to fish samples/tissues, LC-MS/MS has been applied for the

Received: December 22, 2020
Revised: February 24, 2021
Accepted: March 3, 2021
Published: March 10, 2021

Downloaded via UNIV JAUME I on July 22, 2022 at 06:58:38 (UTC).
See https://pubs.acs.org/sharingguidelines for options on how to legitimately share published articles.

© 2021 American Chemical Society

determination of steroids in plasma from several fish species,[2,16−20] and to a lesser extent in other fish tissues. Wang et al.[21] determined steroids in whole fish soft tissues with limits of detection (LOD) of ∼0.1 ng/g. Guedes-Alonso et al.[22] analyzed steroids in muscle, viscera, and skin, with LODs between 0.14 and 49 ng/g. Recently, Li et al.[23] used gel permeation chromatography in combination with ultra-high-performance liquid chromatography (UHPLC)-MS/MS to analyze fish tissues with high fat content, reaching LODs of ∼1 ng/g. In fish ovaries, Flores-Valverde and Hill[24] have applied UHPLC-MS/MS to profile the steroid metabolome.

To the best of our knowledge, LC-MS/MS has not been yet applied to the determination of steroids in fish eggs and larvae. Nevertheless, the study of the early stages of fish development could be the key to solving reproduction problems observed in cultured specimens.[25,26]

The aim of this study was to develop and validate a highly sensitive methodology based on UHPLC-MS/MS with a triple quadrupole for the determination of ≤13 steroids that are relevant in the sexual differentiation, maturation, and reproductive cycle of fish.

The steroids under study were selected according to their key roles in fish reproductive processes or their key positions in steroid metabolism.[27] E2, the main fish estrogen, and its precursor, E1, were chosen as representative estrogens (C18 steroids), whereas 11-KT, the main fish androgen, and its precursors, 11OHT and T on one hand and 11KA4 and 11OHA4 on the other, all relevant in fish testicular development, were chosen within the androgenic pathway (C19 steroids).[28,29] Two additional C19 steroids were monitored, A4 as the key entrance for the C18−C19 steroid metabolism and DHT, a well-known major androgen in mammals and a compound recently identified as a potentially relevant steroid in teleost fish.[30] The other selected steroids included the progestogens, progesterone, 17*α*,20*β*-dihydroxypregnenone, and 17*α*,20*β*,21-trihydroxypregnenone, for their well-known role as pheromones and maturation-inducing steroids in fish[31] and the corticosteroids, cortisol and its precursor, 11-deoxycortisol, as key regulators of stress responses and potential interaction with the reproductive axes.[32] The cholesterol-derived metabolite pregnenolone was also monitored.

Plasma, muscle, and ovaries of sole (*S. senegalensis*) were analyzed, in addition to, for the first time, eggs and larvae. The determination of very low steroid concentrations in the matrices being studied requires the application of different strategies to solve analytical challenges such as matrix effects, the small amount of sample available, or the lack of isotopically labeled internal standards (ILIS) for some compounds. Supporting the applicability of the methodology developed implies its validation at realistic low analyte concentrations. The methodology described will allow us to obtain data that can give a response to current challenges in reproduction of cultured sole and, in general, in the study of fish reproduction processes.

2. EXPERIMENTAL SECTION

2.1. Reagents and Materials. Analytical standards (>95% pure) of progestagens [progesterone (P), 17*α*,20*β*-dihydroxypregnenone (17,20*β*-P), and 17*α*,20*β*,21-trihydroxypregnenone (20*β*-S)], androgens [androstenedione (A4), 11*β*-hydroxyandrostenedione (11*β*OHA4), 11-ketoandrostenedione (11KA), testosterone (T), 5*α*-dihydrotestosterone (DHT), and 11-ketotestosterone (11-KT)], estrogens [estrone (E1) and estradiol (E2)], and corticosteroids [cortisol (F) and 11-deoxycortisol (S)], as well as ILIS, cortisol-D4, testosterone-D2, androstenedione-^{13}C3, DHT-D3, 11-deoxycortisol-D5, progesterone-D9, and estradiol-D5, were supplied by Sigma-Aldrich (Barcelona, Spain).

Individual stock solutions were prepared by dissolving the reference standards in methanol (MeOH) (500 mg/L) and stored at −20 °C. The working mixture solution (1 mg/L) was prepared by mixing the individual stock solution of each standard and stored at −20 °C. Working standard solutions for LC-MS/MS analysis and spiking experiments were prepared with MeOH by dilution of the 1 mg/L mixed solution. The ILIS mix solution (2 *μ*g/L) was prepared and used as a surrogate.

LC gradient grade MeOH, acetonitrile (ACN), ammonium acetate (NH_4Ac), and formic acid (p.a.) (HCOOH) were purchased from Scharlab (Barcelona, Spain). HPLC grade water was obtained by purification of demineralized water in a Milli-Q Gradient A10 instrument (Millipore, Bedford, MA). A Homogenizer-Glass vessel (2 mL) was purchased from Scharlab. Polymeric hydrophilic−lipophilic balanced (HLB) solid phase extraction (SPE) cartridges (60 mg/3 mL) were supplied by Waters (Mildford, MA).

2.2. Sampling. Fish used in this study were hatchery-produced cultured Senegalese soles, maintained in the facilities of the Institute of Aquaculture of Torre la Sal (IATS-CSIC, Castellón, Spain). For all matrices, the amount of sample available was rather limited as a consequence of the scarce stock of sole, due to the problems in reproduction, especially in the case of eggs and larvae. For sampling, fish were anesthetized by immersion in phenoxyethanol (0.1 mL/L of water). Blood was collected from the caudal vein in ice-cold heparinized tubes and centrifuged (3000*g*, 15 min, 4 °C), and plasma stored at −20 °C. For sampling of tissues, fish were euthanized, and then, pieces of muscle and ovaries were collected and stored at −20 °C. Eggs were obtained during the spring spawning period from different batches of captive sole broodstock, collected in limited quantities, and stored at −20 °C. Larvae (14−20 days from hatching), obtained from different batches of eggs in the larval culture facilities of the IATS-CSIC, were also collected in small quantities and stored at −20 °C. To obtain the matrix blanks for validation, immature specimens were chosen as often as possible.

The experimental procedures were in accordance with the principles of the European Animal Directive (86/609/EEC) for the protection of experimental animals[33] and were approved by the Ethics Committees of the Spanish National Research Council (CSIC) and the Institute of Aquaculture of Torre la Sal, with permits associated with Project AGL2013-49027-C3-3.

2.3. Sample Preparation. *2.3.1. Plasma.* Acetonitrile (1 mL) and the appropriate volume of the 20 *μ*g/L ILIS mix solution used as a surrogate were added to 0.5 mL of plasma and left to stand in the freezer for at least 2 h, to boost protein precipitation. The mixture was centrifuged (12000 rpm, 5 min), and the supernatant collected and diluted with 8 mL of Milli-Q water, before the cleanup step.

2.3.2. Muscle and Ovaries. Samples were cut with a scalpel and chopped in a homogenizer. A 0.5 g portion was accurately weighed in Eppendorf tubes with the adequate amount of the 20 *μ*g/L isotopically labeled standard mix. Spiked samples were left to stand for 2 h. Then samples were extracted twice with 1 mL of ACN in a blender homogenizer (Ultraturrax T18, IKA) for 2 min. The supernatant was separated, after a centrifugation step (5000 rpm, 5 min). Then, the extract was transferred to a 15 mL polypropylene plastic tube and diluted 5-fold with water to reduce the amount of organic solvent in the extract to <20%, before the subsequent cleanup step.

2.3.3. Eggs and Larvae. Pooled larvae (0.1 g wet sample) were mixed in a homogenizer with 0.1 mL of H_2O HPLC and chopped. The sample was transferred into a 2 mL Eppendorf tube. For eggs, 0.5 g of a homogenized wet sample was accurately weighed into an Eppendorf tube.

Before extraction, 2 *μ*g/L ILIS mix was added to each sample and left to stand for 2 h. That is 25 *μ*L of ILIS, except for ovaries (125 *μ*L of ILIS) at the lowest value of fortification for each matrix.

Table 1. Mass Spectrometry Parameters Used for Quantification and Identification of Target Compounds and Isotopically Labeled Internal Standards

compound	precursor ion (*m*/*z*)	product ion (*m*/*z*)	cone voltage (V)	collision energy (eV)	retention time (min)	q_i/Q
progesterone (P)	315.1	97.0[a]	10	20	3.99	
		109.0	10	20		0.83
		123.0	10	15		0.07
11-deoxycortisol (S)	347.0	109.1[a]	40	40	3.09	
		97.0	40	40		1.00
		317.0	40	10		0.25
cortisol (F)	363.0	121.0[a]	10	25	2.69	
		309.1	10	15		0.28
		267.0	10	20		0.17
17α,20β-dihydroxypregnenone (17α,20β-P)	333.2	97.2[a]	20	20	3.65	
		109.2	20	25		0.92
		253.2	20	15		0.29
17α,20β,21-trihydroxypregnenone (20β-S)	349.2	109.2[a]	20	30	3.06	
		271.2	20	15		0.70
		97.0	20	30		0.78
4-androstenedione (A4)	287.1	97.0[a]	20	20	3.31	
		109.0	20	20		0.68
		123.0	20	25		0.05
testosterone (T)	289.1[b]	97.0[a]	30	25	3.48	
		109.0	30	30		0.46
		123.0	30	30		0.08
5-androstanolone [dihydrotestosterone (DHT)]	291.2	255.2[a]	20	15	3.82	
		159.2	20	20		0.45
		145.2	20	20		0.29
11-hydroxyandrostenedione (11βOHA4)	303.2	267.2[a]	30	15	2.86	
		121.2	30	25		0.89
		145.2	30	25		0.73
11-ketoandrostenedione (11-KA)	301.2	257.2[a]	40	20	2.58	
		242.2	40	35		0.20
		121.2	40	25		0.80
11-ketotestosterone (11-KT)	303.2[b]	259.1[a]	40	20	2.74	
		121.2	40	25		1.98
		241.2	40	25		0.28
estrone (E1)[b]	269.2	145.2[a]	30	40	3.32	
		159.2	30	40		0.13
		183.2	30	40		0.12
17β-estradiol (E2)[b]	271.2	145.2[a]	30	40	3.28	
		183.2	30	35		0.96
		239.2	30	35		0.365
progesterone-D9	324.2	100.1	20	20	3.98	–
cortisol-D4	367.1	121.0	20	20	2.69	–
testosterone-D2	291.2	99.1	20	20	3.47	–
androstenedione-^{13}C3	290.1	100.0	30	20	3.31	–
DHT-D3	294.1	258.1	50	15	3.81	–
11-deoxycortisol-D5	352.1	100.0	40	25	3.09	–
estradiol-D5	276.2	147.2	30	40	3.30	–

[a]Quantification ion. [b]Negative mode.

For all matrices studied, the extraction was performed twice with 1 mL of acetonitrile (ACN) in an Eppendorf tube by homogenization with a blender homogenizer (Ultraturrax T18, IKA) for 2 min. Then, the mixture was centrifuged at 5000*g* (5 min) and the supernatant collected in an Eppendorf tube. The extract was diluted 5-fold with water to reduce the amount of organic solvent in the extract to <20% before the subsequent cleanup step.

Polymeric solid phase extraction cartridges (OASIS HLB, 60 mg, 3 mL) were used for the cleanup, to remove interference, and to preconcentrate steroids in all matrices. The sorbent was preconditioned with 3 mL of MeOH and 3 mL of H_2O. Afterward, the sample was loaded into the cartridge and washed with 2 mL of H_2O. The sorbent of the cartridges was dried under vacuum, and the target compounds were eluted with 2 mL of MeOH. The eluates were evaporated to dryness at 40 °C under a gentle N_2 stream and reconstituted with 100 μL of MeOH.

In the case of eggs and larvae, the washing step was performed with 2 mL of a H_2O/MeOH mixture [70:30 (v/v)]. Cartridges were dried, and targeted compounds were eluted with 2 mL of a H_2O/MeOH mixture [10:90 (v/v)]. The eluates were evaporated to dryness at 40 °C under a gentle N_2 stream and reconstituted with 100 μL of MeOH.

Finally, 10 μL of the final extracts was injected into the UHPLC system for all matrices.

https://dx.doi.org/10.1021/acsagscitech.0c00075
ACS Agric. Sci. Technol. 2021, 1, 89−99

Table 2. Analytical Performance of the Method Developed in This Paper in Comparison with Other Methods Reported for Steroid Determination in Fish Tissues by (U)HPLC-MS/MS[a]

matrix	sample preparation	level (ng/g)	accuracy (%)	precision [RSD (%)]	sample mass (g)	LOD (ng/g)	LOQ (ng/g)	ref
fish plasma	Na_2CO_3, acetonitrile, centrifugation	1−250[b]	83.5−115.4	<19.4		−	1−2.5[b]	16
fish plasma	LLE (ethyl acetate/water), derivatization	10[b]	81−110	2−20		0.16−1.25[b]	−	17
fish plasma	water dilution, SPE	0.1[b]	81−116	<15		−	0.02−0.6[b]	19
fish plasma	ACN/water, TF-SPME	5, 10, 25[b]	85	6−15		0.006−0.15[b]	0.02−0.5	20
fish plasma	ACN, homogenization, centrifugation, water dilution	0.1[b]	73−119	4−21		0.01−0.03[b]	0.1[b]	this work
		1[b]	74−117	2−17				
fish tissues	ACN extraction, dynamic MAE, salting-out LLE	0.5	75.3−92.1	3.3−5.0	3	0.03−0.15	0.11−00.47	21
		5	82.0−95.4					
fish tissues	methanol extraction, MAE-SPE	0.4	range of 50%	<20	0.1[c]	0.14−49.0	−	22
		4						
fish tissues	diethyl ether extraction, GPC	1	82.4−85.4	7.6−10	5	−	0.2−1.5	23
		5	81.7−87.5	5.4−8.8				
		10	85.6−90.8	3.5−8.2				
fish muscle	ACN extraction, SPE (OASIS HLB)	0.1	80−120	5−21	0.5	0.002−0.04	0.1	this work
		1	90−119	2−9				
fish ovary, testis, liver	methanol extraction, SPE			metabolome profiling				24
fish ovary	ACN extraction, SPE (OASIS HLB)	0.5	82−130	3−21	0.5	0.001−0.03	0.5	this work
		2	79−131	3−20				

[a]Abbreviations: SPE, solid phase extraction; GPC, gel permeation chromatography; MAE, microwave-assisted extraction; TF-SPME, thin-film solid phase microextraction. [b]Nanograms per milliliter. [c]Lyophilized.

2.4. Ultra-High-Performance Liquid Chromatography Coupled to Tandem Mass Spectrometry. Compounds were determined with a UPLC system (Acquity, Waters) interfaced to a triple-quadrupole mass spectrometer (TQS, Waters Micromass) equipped with electrospray ionization (ESI). Chromatographic separation was achieved with a UPLC Acquity BEH C18 analytical column (50 mm × 2.1 mm, 1.7 μm, thermostated at 40 °C; Waters) employing as a mobile phase water (A) and MeOH (B). In positive ionization mode, 0.5 mM NH_4Ac and 0.01% HCOOH were added as modifiers in both phases; in negative ionization mode, 0.025% NH_3 was also added in the two phases. In both cases, the flow rate was set to 0.3 mL/min. A linear gradient program was set up as follows: min 0, 30% B; min 4, 90% B and maintained for 1 min at 90% before returning to the initial conditions. Finally, the gradient was held to the initial conditions (30% B) to re-equilibrate the column. The total run time was 6 min. Determination was performed in negative ESI mode (ESI−) for estradiol (E2) and estrone (E1) and in positive ESI mode (ESI+) for the remaining steroids. Target compounds were monitored in selected reaction monitoring (SRM), selecting three SRM transitions per compound. The cone voltage and collision energy were optimized by acquiring full-scan MS and MS/MS spectra of target hormones from chromatography of the 1 μg/mL reference standard in a MeOH/H_2O mixture [50:50 (v:v)] (Table 1).

2.4.1. Identification of Analytes. Confirmation of the identity of the compounds in samples was based on agreement in retention time (maximum deviation of ±0.1 min between the analyte in the sample and the reference standard) and on acquisition of three SRM transitions and the compliance of at least one q/Q ratio with maximum tolerance of ±30%.[25] The q/Q ratio deviation was calculated by comparison of the q/Q ratio in samples (q/Q_s) with the q/Q ratio for the reference standard (q/Q_{st}).

Table 1 reports mass spectrometry parameters for identification and quantification of target compounds and ILIS. Figures S1 and S2 show UHPLC-MS/MS chromatograms corresponding to the quantification transitions for fish tissues, eggs and larvae, respectively, at the lowest concentrations validated.

2.5. Evaluation of the Matrix Effects. To evaluate the effects of the matrix, the variation of the slopes was studied between a calibration prepared with standards in a solvent (standard calibration curves) and matrix-matched calibration.

Matrix effects were estimated by calculating the percentage of signal suppression or enhancement (SSE) using eq 1:

$$\mathrm{SSE} = \frac{\text{matrix calibration slope} - \text{standard calibration slope}}{\text{standard calibration slope}} \times 100 \quad (1)$$

Then, correction factors were estimated for each sample matrix as follows:

$$F = \frac{1}{1 + \frac{\mathrm{SSE}\,(\%)}{100}} \quad (2)$$

The concentration of steroids without ILIS available was then obtained by multiplying the concentration calculated upon applying direct calibration in solvent by the corresponding correction factor (for more details, see ref 34).

2.6. Validation. Due to the absence of Certified Reference Materials containing the steroids and the matrices under study, the method was statistically validated on the basis of the Commission Decision 2002/657/EC (EC, 2002)[35] and Guidance Document SANTE/12682/2019.[36] The accuracy was determined by means of recovery experiments (n = 5) at two levels of concentration for each compound. Plasma was directly fortified with a standard solution. The other matrices were spiked in chopped samples. After being spiked, samples were left to stand for 2 h before extraction. The precision, expressed as the relative standard deviation (percent), was calculated from five replicates processed under the same conditions.

Calibration curves were calculated from five calibration points covering the range of 0.05−25 ng/mL, by taking 100 μL of the corresponding standard mixture, 100 μL of 100 ng/mL ILIS, and 800 μL of MeOH. Matrix-matched calibration curves were prepared with the reconstituted extract after evaporating the SPE eluates to dryness. Pools of samples from different batches of the five sample matrices were used for matrix-matched calibration. Each point of the calibration curve was injected three times. The final extract was injected twice for separate determination of compounds in positive and negative ionization mode.

The linearity of the calibration curves was proven by the study of residuals. Homoscedasticity was verified by means of an F test (α <

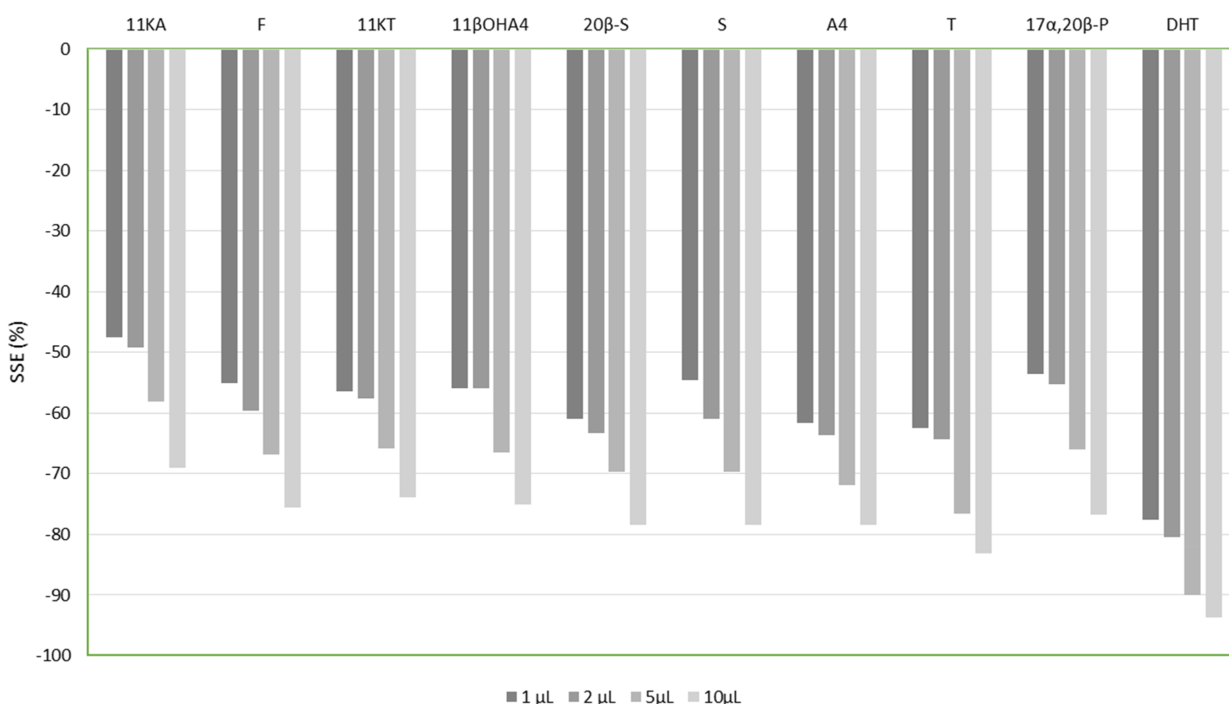

Figure 1. Signal suppression/enhancement caused by matrix effects, depending on the amount of ovary extract injected into the UHPLC-MS/MS system.

0.05). The specificity was evaluated by analyzing a procedural blank, a processed blank sample, and a processed blank sample spiked at the lowest level validated for each matrix, using the quantitative transition (Q). The limits of detection (LODs) were estimated from the chromatograms of sample extracts fortified at the lowest level validated for a signal-to-noise ratio of 3. The lowest level of concentration validated in each matrix was considered as the limit of quantification (LOQ).

2.7. Quality Control. The reliability of the quantitative determination of steroids in samples was assessed by the analysis of two quality control samples (QCs) per matrix, prepared at two concentrations, for each batch of samples. For this purpose, "blank" samples were spiked at 0.1 and 1 ng/mL for plasma, 0.5 and 2 ng/g for ovaries and larvae, and 0.1 and 1 ng/g for muscle and eggs, adding 100 μL of the corresponding standard mixture in MeOH. The "blank" samples used for preparing QCs were also processed and analyzed to subtract the endogenous hormones. Additionally, 100 μL of ILIS was added to each QC and "blank" analyzed. Exactly the same analytical procedure described in the previous section was applied to QCs, blanks, and real-world samples.

2.8. Data Processing. Data processing was carried out using Targetlynx Application Manager (Masslynx, Waters) for peak integration. Calculations and statistical tests were carried out with MS EXCEL 2013.

3. RESULTS AND DISCUSSION

3.1. Sample Preparation. Although some authors add a derivatization step to the whole procedure to minimize the matrix effect and increase the sensitivity of the method,[9,37−39] we did not consider this step in our study, because our goal of determining simultaneously several steroid hormone classes is not compatible with the application of a single derivatization procedure.

3.1.1. Plasma. In the case of fish plasma, we tested different treatments for protein precipitation (see Table 2). The addition of ACN directly into the vial containing plasma followed by the introduction of sample extracts into the freezer (below −18 °C) for at least 2 h favored the elimination of the co-extracted matrix and provided the cleanest chromatograms.

3.1.2. Muscle and Ovaries. With regard to muscle and ovaries, we tested the most commonly used solvents for the extraction of steroids, such as methanol and diethyl ether (see Table 2), and finally, ACN was selected because of its universality. Nevertheless, ACN leads to the co-extraction of a number of nondesired compounds, such as lipids and proteins, potentially causing matrix effects. To achieve the preconcentration of analytes and reduce the amount of co-extracted matrix, an additional step using SPE was included. Polymeric hydrophilic−lipophilic balanced cartridges were selected due to their capability to retain analytes with a wide range of polarities. The previous dilution of extracts was necessary to ensure a complete retention of all the steroids in the sorbent. To prevent losses during the SPE washing step, special attention was paid to the most polar compounds, such as 11-hydroxyandrostenedione and cortisol. The maximum amount of ACN in the extract was found to be 20% to avoid breakthrough when the sample was loaded into the cartridge.

3.1.3. Eggs and Larvae. The elution with methanol of eggs and larvae extracts gave unsatisfactory recoveries for several steroids. Our efforts were focused on improving the SPE process to avoid potential analyte losses in these sample matrices. After several optimization assays, we found that a washing step with 30% methanol, after sample loading, led to the best results. In both sample matrices, steroids were satisfactorily eluted from the cartridge with 2 mL of a H_2O/MeOH mixture (10:90), minimizing undesired matrix interference, such as fatty acids, which elute with pure MeOH.[18] The methodology developed led eventually to rather clean extracts and satisfactory recoveries.

3.2. UHPLC-MS/MS Study. The variety of chemical structures of the target compounds made necessary different strategies for optimizing the mass spectrometry and chromatographic conditions. In general, a better ionization was observed

Table 3. Validation Data of the Proposed Method at Two Levels of Concentration for Each Tissue (n = 5) and Matrix Effect[a]

	recovery (%) ± relative standard deviation (%)									
	plasma		ovaries		muscle		eggs		larvae	
compound	0.1[b]	1[b]	0.5[c]	2[c]	0.1[c]	1[c]	0.1[c]	1[c]	0.5[c]	2[c]
P	116 ± 4	99 ± 2	103 ± 7	101 ± 9	93 ± 12	93 ± 9	102 ± 8	82 ± 5	94 ± 8	95 ± 7
S	–	74 ± 17	112 ± 7	108 ± 8	107 ± 9	118 ± 4	–	98 ± 6	–	95 ± 17
F	–	–	102 ± 14	90 ± 7	80 ± 21	93 ± 2	–	–	–	–
17,20β-P	97 ± 14	90 ± 8	91 ± 19	100 ± 9	110 ± 15	131 ± 6	–	100 ± 10	–	112 ± 8
20β-S	82 ± 11	88 ± 6	82 ± 7	94 ± 8	97 ± 12	108 ± 3	99 ± 7	118 ± 7	107 ± 9	105 ± 15
A4	97 ± 10	89 ± 9	93 ± 3	90 ± 3	87 ± 9	90 ± 2	–	83 ± 3	104 ± 7	96 ± 5
T	117 ± 7	117 ± 1	92 ± 4	92 ± 4	98 ± 7	97 ± 2	99 ± 6	92 ± 6	107 ± 12	99 ± 5
DHT	–	93 ± 17	–	106 ± 8	–	94 ± 6	–	–	–	127 ± 11
11βOHA4	73 ± 15	85 ± 10	100 ± 6	104 ± 6	130 ± 14	116 ± 3	–	86 ± 14	107 ± 7	110 ± 7
11KA	119 ± 9	91 ± 8	111 ± 10	107 ± 8	107 ± 8	119 ± 4	99 ± 12	123 ± 8	116 ± 12	104 ± 7
11KT	74 ± 21	86 ± 8	126 ± 6	121 ± 3	111 ± 6	110 ± 5	92 ± 11	93 ± 10	98 ± 10	91 ± 11
E1	95 ± 8	116 ± 10	105 ± 3	79 ± 17	106 ± 5	104 ± 3	104 ± 15	102 ± 5	111 ± 9	101 ± 8
E2	98 ± 24	97 ± 7	131 ± 9	85 ± 20	120 ± 17	97 ± 7	109 ± 19	101 ± 14	100 ± 20	112 ± 10

[a]Dashes indicate values were not calculated. Bold values are recovery values of >120 or <70. [b]Nanograms per milliliter. [c]Nanograms per gram.

in positive mode for all selected steroids, except for estrogens. In ESI+, all compounds showed their $[M + H]^+$ ions, which were selected as precursor ions. Fragmentation in the collision cell led to the generation of steroid characteristic product ions, such as those at m/z 97, 109, and 121, for most compounds. To improve the analysis performance, three SRM transitions were selected for each compound, which facilitated the reliable identification of the hormones in cases in which one of the transitions could be disrupted.

It is worth noticing that the chromatographic separation was crucial for some pairs of compounds, for which no specific SRM transitions were available. This was the case for 11KT and 11βOHA4, with the same precursor ion (m/z 303.2) and shared SRM transitions. DHT also shared its precursor ion (m/z 291.2) with testosterone-D2. The use of a UHPLC column that performs over narrower peaks and the optimization of the amount of organic solvent to start the chromatographic run (30% organic solvent) allowed the separation of the studied steroids to baseline, and therefore, they could be satisfactorily analyzed.

Estrogens E1 and E2 showed poor ionization in ESI+, hindering their inclusion in multiclass steroid methodologies due to the low sensitivity. Derivatization is an alternative commonly used in these cases.[5,40] In our case, we decided to determine these two estrogens in ESI−, improving ionization and avoiding additional sample treatment.

The chromatographic conditions were studied to enhance the ionization of the analytes. The addition of the modifiers HCOOH and NH_4Ac to the mobile phase improved considerably the sensitivity for several compounds and reduced the level of formation of sodium adducts. However, when NH_4Ac was present in the mobile phase under acidic conditions, E1 and E2 were drastically affected. As a consequence, samples were injected twice, using the selected ionization mode with the optimized chromatographic conditions for each mode. Thus, for estrogens, determined in ESI−, ammonia was added to the mobile phase to promote their deprotonation.[41] With regard to the solvents employed in the mobile phase, the use of ACN and MeOH was compared. MeOH was finally selected, because it provided an improved peak shape for testosterone, androstenedione, and progesterone, while giving similar results for the other analytes.

3.3. Quantification: Study of Matrix Effects.

The determination of hormones in biological samples requires, in addition to the use of sensitive and selective instrumentation, the evaluation and correction of the matrix effect caused by coeluting interfering compounds, which could compromise the quantification process. To study the matrix effect, we tested several injection volumes of the extract (1, 2, 5, and 10 μL). In general, signal suppression was less significant at smaller injection volumes, but decreasing the volume of the extract injected led to an increase in the LODs. Finally, 10 μL was selected as the injection volume as a compromise between matrix effects and LODs obtained. As an example, Figure 1 shows the results obtained at different injection volumes for ovaries.

Table S1 shows the signal suppression observed for steroids in the five matrices studied. All compounds showed a noticeable signal suppression, between −37% and −99%. When matrix effects cannot be minimized, e.g., including a cleanup step, an appropriate correction is necessary for an accurate quantification.[42] In this work, the analyte ILIS was used as a surrogate, which allowed an efficient matrix correction and also a correction of potential errors associated with the sample treatment.[11,15] However, for some hormones, the analyte ILIS was not available. Although other labeled compounds eluting close to the analyte have been used as internal standards for quantification,[22,40] in this work we did not realize accurate quantification when the matrix effect was compensated using an ILIS analogue to the target hormone. Thus, for those compounds without analyte ILIS available, quantification was performed with calibration in the solvent and applying a correction factor based on the percentage of signal suppression (see Experimental Section), obtaining satisfactory results.

3.4. Validation and Analytical Parameters.

Table 3 shows the recoveries (percent) at two levels of concentration for each matrix [0.1−1 ng/mL for plasma and 0.1−1 ng/g for muscle and eggs (i.e., 0.5−5 ng/mL in the extract) and 0.5−2 ng/g for ovaries and larvae (2.5−10 and 0.5−2 ng/mL in the extract, respectively)]. All "blank" samples for validation were prepared by mixing samples from different batches, selecting immature specimens to minimize the amount of endogenous steroids. The concentration of endogenous steroids in "blank"

Table 4. Concentration Ranges (n = 4) Found in Tissues from Experimental Specimens Maintained in Captivity, Eggs,and Larvae[a]

compound	plasma (ng/mL)	ovaries (ng/g)	muscle (ng/g)	eggs (ng/g)	larvae (ng/g)
progesterone (P)	<LOQ	<LOQ	nd−2.31	<LOQ	0.7−7.5
11-deoxycortisol (S)	0.14−1.75	1.4−54	<LOQ	<LOQ	5.0−19
cortisol (F)	5.85−174	1.8−85	nd−30	<LOQ	347
17α,20β-dihydroxypregnenone (17α,20β-P)	0.12	0.7−2.8	nd	<LOQ	<LOQ
17α,20β,21-trihydroxypregnenone (20β-S)	nd	nd	nd	nd	nd−0.8
4-androstenedione (A4)	<LOQ	0.7−81	<LOQ	<LOQ	0.6−6.1
testosterone (T)	0.1−0.26	0.5−15	nd	<LOQ	0.6−5.8
5-androstanolone [dihydrotestosterone (DHT)]	nd	nd	nd	nd	nd
11-hydroxyandrostenedione (11βOHA4)	0.5−0.9	7.0−32	nd−0.5	<LOQ	2.46−19
11-ketoandrostenedione (11-KA)	0.33−2.4	143−367	0.8−4.5	nd	nd−0.5
11-ketotestosterone (11-KT)	0.28−1.80	112−232	nd−0.84	nd	nd
estrone (E1)	0.2−0.8	d−0.8	0.1−0.3	nd	nd
17β-estradiol (E2)	0.9−1.1	d−0.72	0.7−30	nd	nd

[a]nd, not detected; d, detected.

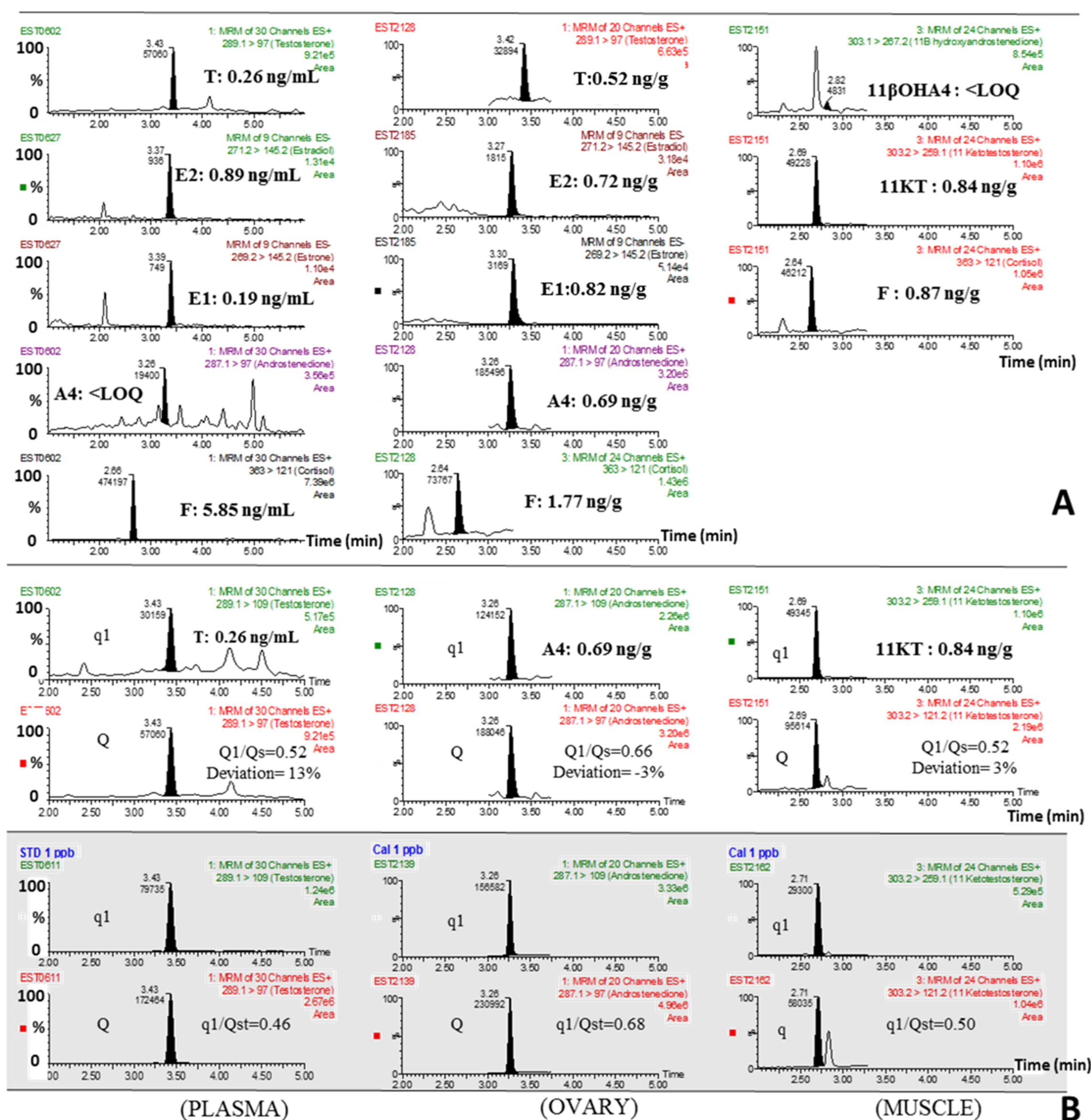

Figure 2. UHPLC-MS/MS chromatograms corresponding to the analysis of plasma, ovary, and muscle from sole. (A) Quantification of SRM transitions for the detected steroids in each sample. (B) Examples of confirmations of steroids by the q/Q ratio (T in plasma, A4 in ovary, and 11KT in muscle).

https://dx.doi.org/10.1021/acsagscitech.0c00075
ACS Agric. Sci. Technol. 2021, 1, 89−99

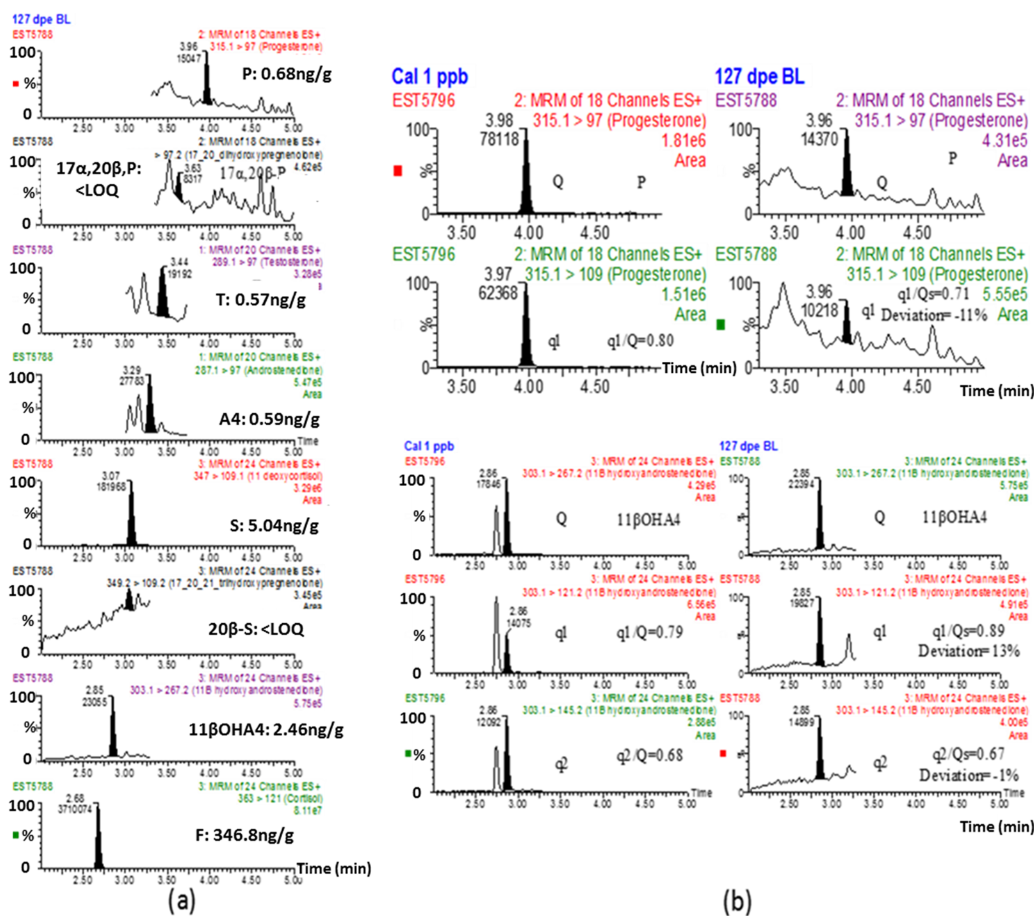

Figure 3. UHPLC-MS/MS chromatograms corresponding to larval samples. (a) Detected steroids in larval samples. (b) Confirmation of positive results by the q/Q ratio for progesterone (P) and 11-hydroxyandrostenedione (11βOHA4).

samples, which were analyzed in triplicate, was subtracted from the spiked samples for the calculation of recovery.

In general, validation data were highly satisfactory with only a few exceptions. Thus, the high endogenous levels in "blank" samples prevented the calculation of recoveries in some cases, such as cortisol (F) in plasma, eggs, and larvae, for which recovery could not be calculated at either of the two levels assayed, or 11-deoxycortisol (S), also in plasma, eggs, and larvae, at the lowest level validated. DHT could not be validated at the lowest level in any of the samples due to its lower ionization efficiency in the ESI source.

Apart from the exceptions mentioned above, most of the recoveries were between 80% and 120% for all matrices, with relative standard deviations of <20%. With regard to ovaries, the lowest concentration level validated was 0.5 ng/g, due to a noisier baseline as consequence of its special complexity, probably due to the composition of the yolk. Despite the high content and variety of lipids and proteins present in eggs, and the additional presence of skin and bones in the larvae, the optimization of the sample preparation procedure in these samples allowed us to obtain good values of recovery and precision for most of the steroids. 17,20β-P, A4, and 11βOHA4 showed low sensitivity, which made validation at the lowest level tested difficult.

With respect to the linearity of calibration curves, residuals did not present any trend and were lower than 5%. Homoscedasticity was proved for all calibration curves by the F-test. Regression coefficients (r^2) were >0.99 for all compounds. Intercept values on the abscissa axis were always below LODs.

Table S2 shows the LODs obtained from the three SRM transitions selected. In general, LODs ranged from <0.1 to 0.01 ng/g for both quantitative and qualitative transitions, except for some specific cases, mainly for qualitative/confirmatory transitions. LODs for cortisol could not be calculated due to its presence in the "blank" samples at high concentrations. Larvae presented the highest LODs for qualitative ions, but they were <0.3 ng/g in most cases.

Table 2 shows a comparison of the performance of the developed analytical method with other LC-MS/MS methods reported in the literature. It is worth noting a significant improvement of the LODs in this study with respect to those previously reported, most of them for fish plasma[16,17] and muscle or whole fish.[22] Although the LODs obtained in this paper for ovaries were similar to those of the other matrices studied, the lack of previous work on this sample matrix prevents comparisons with the work of other authors. In addition, LC-MS/MS has been applied for the first time to the determination of steroids in fish egg and larvae, with good LODs, mostly below 0.3 ng/g, down to 0.01 ng/g.

3.5. Sample Analysis. The validated methodology was applied to the analysis of tissues from captive sole (3 years old), and eggs and larvae (14–20 days) obtained in captivity. In total, 20 samples were analyzed, corresponding to four samples for each matrix. The specimens used belong to

different batches from experimentakl groups, without relation to this work, due to the shortage of samples.

QCs were included in each sample batch, providing recoveries of 87−136%. The acceptance criterion for QC recoveries was 60−140%, according to the SANTE guideline for routine multiresidue analysis.[35]

Confirmation of the identity of the steroids found in the samples was supported by the agreement in retention time (maximum deviation of ±0.1 min) and q_i/Q ratios (±30%) between the compound in the sample and the reference standard.[35] The Q/q ratio is defined as the ratio between the signal obtained from the quantification transition (Q) and the signal obtained from the confirmation transitions (q_i).

As one can see in Table 4, steroids present at sub-parts per billion levels could be quantified in plasma and ovaries. Cortisol was present at levels well above the parts per billion level. Progesterone was detected below the LOQ, while 20β-S and DHT were not detected.

The analysis of larvae also showed the presence of 11-deoxycortisol and 11βOHA4 at sub-parts per billion levels. Like for plasma and ovaries, cortisol presented the highest concentrations in all samples, and A4, T, and progesterone were detected and quantified in samples. 17−20β-P and 20β-S were detected at levels close to the LOQ. DHT, E1, and E2 were not detected in any sample.

In muscle and egg samples, most of the hormones were detected at concentrations close to the LOQ.

Figure 2 shows UHPLC-MS/MS chromatograms for the quantification transitions, for steroids found in different fish tissues. Additionally, some examples of confirmation of the identity of the steroids by the q/Q ratio, according to the SANTE guideline,[35] are shown.

Likewise, Figure 3 shows UHPLC-MS/MS chromatograms corresponding to larval samples and the confirmation of the identity by the q/Q ratio.

As one can see, the sample treatment applied and the optimization of the chromatographic separation and MS/MS measurements allowed us to obtain clean chromatograms of real-world complex samples with the sensitivity and reliability required for the determination of very low concentrations of steroid hormones in different fish tissues. It is remarkable that the low LODs that can be attained with the, commonly less abundant, qualitative transitions allowed the unequivocal identification of these compounds at very low concentrations in the matrices studied.

4. CONCLUSIONS

An analytical methodology based on UHPLC-MS/MS with a triple quadrupole has been successfully developed and validated for the determination of 13 steroids in fish tissues and, for the first time, in eggs and larvae. The different strategies applied have allowed the optimization of sample treatment and instrumental determination reaching limits of detection, including the measurement of both quantitative and qualitative ions, down to picogram per gram levels. Therefore, the method developed can identify and quantify in a reliable way steroids at extremely low concentrations. The applicability of the method was supported by analysis of real-world sole samples, confirming the presence of several steroid hormones in the different matrices studied. The information obtained will be crucial for understanding the reproductive processes during fish development, for optimizing broodstock management, and for realizing reproductive success in aquaculture facilities.

ASSOCIATED CONTENT

Supporting Information

Tables S1 and S2. Figure S1 and S2. The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsagscitech.0c00075.

(PDF)

AUTHOR INFORMATION

Corresponding Author

Roque Serrano − *Environmental and Public Health Analytical Chemistry, Research Institute for Pesticides and Water (IUPA), University Jaume I, 12071 Castellón, Spain; Research Unit of Marine Ecotoxicology (IATS-IUPA), 12595 Cabanes, Castellón, Spain;* orcid.org/0000-0002-0700-8369; Phone: +34 964387358; Email: serrano@uji.es

Authors

Eduardo Beltrán − *Environmental and Public Health Analytical Chemistry, Research Institute for Pesticides and Water (IUPA), University Jaume I, 12071 Castellón, Spain*

Vanesa Piquer − *Institute of Aquaculture Torre de la Sal (IATS-CSIC), 12595 Cabanes, Castellón, Spain*

Félix Hernández − *Environmental and Public Health Analytical Chemistry, Research Institute for Pesticides and Water (IUPA), University Jaume I, 12071 Castellón, Spain; Research Unit of Marine Ecotoxicology (IATS-IUPA), 12595 Cabanes, Castellón, Spain*

Evaristo Mañanós − *Research Unit of Marine Ecotoxicology (IATS-IUPA), 12595 Cabanes, Castellón, Spain; Institute of Aquaculture Torre de la Sal (IATS-CSIC), 12595 Cabanes, Castellón, Spain*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsagscitech.0c00075

Author Contributions

All authors contributed equally to this study.

Funding

The authors acknowledge the financial support of the Programa Estatal de Investigación, Desarrollo e Innovación orientada a los retos de la sociedad (Ministerio de Economia y Competitividad, Spain): Research Project "Ritmos embrionarios y larvarios: papel de los ciclos ambientales en el desarrollo. Determinación/diferenciación sexual y reproducción del lenguado" (AGL2013-49027-C3-3-R). This work has been developed within the framework of the Research Unit of Marine Ecotoxicology (IATS-CSIC/IUPA-UJI). E.B. thanks University Jaume I for the financial support via his postdoctoral contract (POSDOC/2016/19). IUPA authors acknowledge the financial support of Generalitat Valenciana (Research Excellence Group Prometeo/2019/040).

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Zohar, Y. Fish reproductive biology − Reflecting on five decades of fundamental and translational research. *Gen. Comp. Endrocrinol.* **2021**, *300*, 113544.

(2) Mañanós, E.; Duncan, N.; Mylonas, C. C. Reproduction and control of ovulation, spermiation and spawning in cultured fish. In *Methods in Reproductive Aquaculture: Marine and Freshwater Species*; Cabrita, E., Robles, V., Herráez, M. P., Eds.; CRC Press, Taylor & Francis Group: Boca Raton, FL, 2008; pp 3−80.

(3) Beltrán, E.; Piquer, V.; Guzmán, J. M.; Swanson, P.; Mañanós, E.; Serrano, R. Quantification of plasma steroids in sole (*Solea senegalensis*) by ultra high performance liquid chromatography coupled to tandem mass spectrometry. *Adv. Comp. Endocrinol.* **2016**, *7*, 170−2.
(4) Mañanós, E.; Duncan, N.; Sarasquete, C.; García-López, A.; Guzmán, J. M.; Muñoz-Cueto, J. A. Reproductive Physiology and Broodstock Management of Soles. In *The Biology of Sole*; Muñoz-Cueto, J. A., Mañanós-Sánchez, E. L., Sánchez-Vázquez, F. J., Eds.; CRC Press, Taylor & Francis Group: Boca Raton, FL, 2019; pp 90−141.
(5) Wudy, S. A.; Schuler, G.; Sanchez-Guijo, A.; Hartmann, M. F. The art of measuring steroids. Principles and practice of current hormonal steroid analysis. *J. Steroid Biochem. Mol. Biol.* **2018**, *179*, 88−103.
(6) Wheaton, C. J.; Mylniczenko, N. D.; Rimoldi, J. M.; Gadepalli, R. S. V. S.; Hart, R.; O'Hara, B. R.; Evans, A. N. Challenges, pitfalls and surprises: development and validation of a monoclonal antibody for enzyme immunoassay of the steroid 1α-hydroxycorticosterone in elasmobranch species. *Gen. Comp. Endocrinol.* **2018**, *265*, 83−9.
(7) Uraipong, C.; Allan, R. D.; Li, C.; Kennedy, I. R.; Wong, V.; Lee, N. A. 17β-Estradiol residues and estrogenic activities in the Hawkesbury River, Australia. *Ecotoxicol. Environ. Saf.* **2018**, *164*, 363−9.
(8) Kushnir, M. M.; Rockwood, A. L.; Roberts, W. L.; Yue, B.; Bergquist, J.; Meikle, A. W. Liquid chromatography tandem mass spectrometry for analysis of steroids in clinical laboratories. *Clin. Biochem.* **2011**, *44*, 77−88.
(9) Abdel-Khalik, J.; Björklund, E.; Hansen, M. Simultaneous determination of endogenous steroid hormones in human and animal plasma and serum by liquid or gas chromatography coupled to tandem mass spectrometry. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2013**, *928*, 58−77.
(10) Hansen, M.; Jacobsen, N. W.; Nielsen, F. K.; Björklund, E.; Styrishave, B.; Halling-Sørensen, B. Determination of steroid hormones in blood by GC-MS/MS. *Anal. Bioanal. Chem.* **2011**, *400*, 3409−17.
(11) Koren, L.; Ng, E. S. M.; Soma, K. K.; Wynne-Edwards, K. E. Sample preparation and liquid chromatography-tandem mass spectrometry for multiple steroids in mammalian and avian circulation. *PLoS One* **2012**, *7*, e32496.
(12) Regal, P.; Cepeda, A.; Fente, C. Development of an LC-MS/MS method to quantify sex hormones in bovine milk and influence of pregnancy in their levels. *Food Addit. Contam., Part A* **2012**, *29*, 770−9.
(13) Wozniak, B.; Matraszek-Zuchowska, I.; Zmudzki, J. LC-MS/MS fast analysis of androgenic steroids in urine. *Anal. Bioanal. Chem.* **2012**, *403*, 2965−72.
(14) Doué, M.; Dervilly-Pinel, G.; Pouponneau, K.; Monteau, F.; Le Bizec, B. Analysis of glucuronide and sulfate steroids in urine by ultra-high-performance supercritical-fluid chromatography hyphenated tandem mass spectrometry. *Anal. Bioanal. Chem.* **2015**, *407*, 4473−84.
(15) Bertin, J.; Dury, A. Y.; Ke, Y.; Ouellet, J.; Labrie, F. Accurate and sensitive liquid chromatography/tandem mass spectrometry simultaneous assay of seven steroids in monkey brain. *Steroids* **2015**, *98*, 37−48.
(16) Zhang, F.; Bartels, M. J.; Geter, D. R.; Carr, M. S.; McClymount, L. E.; Marino, T. A.; Klecka, G. M. Simultaneous quantitation of testosterone, estradiol, ethinyl estradiol, and 11-ketotestosterone in fathead minnow fish plasma by liquid chromatography/positive atmospheric pressure photoionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 3637−46.
(17) Hala, D.; Overturf, M. D.; Petersen, L. H.; Huggett, D. B. Quantification of 2-hydrazinopyridine derivatized steroid hormones in fathead minnow (*Pimephales promelas*) blood plasma using LC-ESI+/MS/MS. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2011**, *879*, 591−598.
(18) Rai, S.; Szeitz, A.; Roberts, B. W.; Christie, Q.; Didier, W.; Eom, J.; Yun, S. S.; Close, D. A. A putative corticosteroid hormone in Pacific lamprey, Entosphenus tridentatus. *Gen. Comp. Endocrinol.* **2015**, *212*, 178−84.
(19) Guzmán, J. M.; Luckenbach, J. A.; da Silva, D. A. M.; Hayman, E. S.; Ylitalo, G. M.; Goetz, F. W.; Swanson, P. Seasonal variation of pituitary gonadotropin subunit, brain-type aromatase and sex steroid receptor mRNAs, and plasma steroids during gametogenesis in wild sable fish. *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol.* **2018**, *219−220*, 48−57.
(20) Maciążek-Jurczyk, M.; Bessonneau, V.; Ings, J.; Bragg, L.; McMaster, M.; Servos, M. R.; Bojko, B.; Pawliszyn, J. Development of a thin-film solid-phase microextraccion (TF-SPME) method coupled to liquid chromatography and tandem mass spectrometry for high-throughput determination of steroid hormones in whiet sucker fish plasma. *Anal. Bioanal. Chem.* **2020**, *412*, 4183−4194.
(21) Wang, H.; Zhou, X.; Zhang, Y.; Chen, H.; Li, G.; Xu, Y.; Zhao, Q.; Song, W.; Jin, H.; Ding, L. Dynamic microwave-assisted extraction coupled with salting-out liquid-liquid extraction for determination of steroid hormones in fish tissues. *J. Agric. Food Chem.* **2012**, *60*, 10343−51.
(22) Guedes-Alonso, R.; Sosa-Ferrera, Z.; Santana-Rodríguez, J. J. Determination of steroid hormones in fish tissues by microwave-assisted extraction coupled to ultra-high performance liquid chromatography tandem mass spectrometry. *Food Chem.* **2017**, *237*, 1012−20.
(23) Li, P.; Yan, Z.; Sun, X.; Chen, S.; Chen, Y.; Zhang, X. Determination of steroid hormone residues in fish tissues using gel permeation chromatography and ultra-high performance liquid chromatography-tandem mass spectrometry. *J. Ocean Univ. China* **2018**, *17*, 1171−77.
(24) Flores-Valverde, A. M.; Hill, E. M. Methodology for profiling metabolome in animal tissues using ultraperformance liquid chromatography-electrospray-time-of-flight mass spectrometry. *Anal. Chem.* **2008**, *80*, 8771−879.
(25) Blanco-Vives, B.; Vera, L. M.; Ramos, J.; Bayarri, M. J.; Mañanós, E.; Sánchez-Vázquez, F. J. Exposure of larvae to daily thermocycles affects gonad development, sex ratio, and sexual steroids in Solea senegalensis. *J. Exp. Zool., Part A* **2011**, *315A*, 162−169.
(26) Guzmán, J. M.; Bayarri, M. J.; Ramos, J.; Zohar, Y.; Sarasquete, C.; Mañanós, E. L. Follicle stimulating hormone (FSH) and luteinizing hormone (LH) gene expression during larval development in Senegalese sole (Solea senegalensis). *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol.* **2009**, *154*, 37−43.
(27) Tokarz, J.; Möller, G.; Hrabě de Angelis, M.; Adamski, J. Steroids in teleost fishes: A functional point of view. *Steroids* **2015**, *103*, 123−144.
(28) Guzmán, J. M.; Luckenbach, A.; da Silva, D. A. M.; Hayman, E. S.; Ylitalo, G. M.; Goetz, F. W.; Swanson, P. Seasonal variation of pituitary gonadotropin subunit, brain-type aromatase and sex steroid receptor mRNAs, and plasma steroids during gametogenesis in wild sablefish. *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol.* **2018**, *219−220*, 48−57.
(29) Borg, B. Androgens in teleost fishes. *Comp. Biochem. Physiol., Part C: Pharmacol., Toxicol. Endocrinol.* **1994**, *109*, 219−45.
(30) Margiotta-Casaluci, L.; Courant, F.; Antignac, J. P.; Le Bizec, B.; Sumpter, J. P. Identification and quantification of 5a-dihydrotestosterone in the teleost fathead minnow (Pimephales promelas) by gas chromatography−tandem mass spectrometry. *Gen. Comp. Endocrinol.* **2013**, *191*, 202−209.
(31) Scott, A. P.; Sumpter, J. P.; Stacey, N. E. The role of the maturation-inducing steroid, 17,20b-dihydroxypregn-4-en-3-one, in male fish: a review. *J. Fish Biol.* **2010**, *76*, 183−224.
(32) Ganesh, C. B. The stress − Reproductive axis in fish: The involvement of functional neuroanatomical systems in the brain. *J. Chem. Neuroanat.* **2021**, *112*, 101904.
(33) The European Commission. COUNCIL DIRECTIVE of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. 86/609/ECC(L358/1), 1986.

(34) Botero-Coy, A. M.; Marín, J. M.; Serrano, R.; Sancho, J. V.; Hernández, F. Exploring matrix effects in liquid chromatography-tandem mass spectrometry determination of pesticide residues in tropical fruits. *Anal. Bioanal. Chem.* **2015**, *407*, 3667−81.

(35) The European Commission. Analytical Quality Control and Method Validation Procedures for Pesticide Residues Analysis. Sante/12682/2019; 2020; pp 1−49.

(36) The European Commission. Commission Decision. 2002/657/EC; 2002.

(37) Yuan, T.-F.; Le, J.; Cui, Y.; Peng, R.; Wang, S.-T.; Li, Y. An LC-MS/MS analysis for seven sex hormones in serum. *J. Pharm. Biomed. Anal.* **2019**, *162*, 34−40.

(38) Ke, Y.; Gonthier, R.; Simard, J.-N.; Labrie, F. A validated LC-MS/MS method for the sensitive quantitation of serum 7alpha hydroxy-, 7beta hydroxy- and 7keto-dehydroepiandrosterone using a novel derivatization resgent. *Steroids* **2016**, *108*, 112−7.

(39) Szterk, A.; Sitkowski, J.; Bocian, W. UPLC-MS/MS determination of steroid hormones via a novel reaction based on derivatization by a cyclic-organophosphate. *Talanta* **2019**, *204*, 415−423.

(40) Boggs, A. S. P.; Bowden, J. A.; Galligan, T. M.; Guillette, L. J.; Kucklick, J. R. Development of a multi-class steroid hormone screening method using Liquid Chromatography/Tandem Mass Spectrometry (LC-MS/MS). *Anal. Bioanal. Chem.* **2016**, *408*, 4179−90.

(41) Ripollés, C.; Ibáñez, M.; Sancho, J. V.; López, F. J.; Hernández, F. Determination of 17β-estradiol and 17α-ethinylestradiol in water at sub-ppt levels by liquid chromatography coupled to tandem mass spectrometry. *Anal. Methods* **2014**, *6*, 5028−37.

(42) Panuwet, P.; Hunter, R. E.; D'Souza, P. E.; Chen, X. Y.; Radford, S. A.; Cohen, J. R.; Marder, M. E.; Kartavenka, K.; Ryan, P. B.; Barr, D. B. Biological Matrix Effects in Quantitative Tandem Mass Spectrometry-Based Analytical Methods: Advancing Biomonitoring. *Crit. Rev. Anal. Chem.* **2016**, *46*, 93−105.