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Determination of steroid hormones in grey seal (*Halichoerus grypus*) blood plasma using convergence chromatography tandem mass spectrometry

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ABSTRACT

A hybrid solid phase extraction (HybridSPE) protocol tailored to ultra-performance convergence chromatography tandem mass spectrometry (UPC^2-MS/MS) was developed for the determination of 19 steroid hormones in grey seal (*Halichoerus grypus*) blood plasma. In this study, the protocol demonstrated acceptable absolute recoveries ranging from 33 to 90%. The chromatographic separation was carried out using a gradient elution program with a total run time of 5 min. For most target analytes, the method repeatability ranged from 1.9 to 24% and the method limits of quantification (mLOQs) ranged from 0.03 to 1.67 ng/mL. A total of 9 plasma samples were analysed to demonstrate the applicability of the developed method, and 13 steroid hormones were quantified in grey seal pup plasma. The most prevalent steroids: cortisol, cortisone, corticosterone, 11-deoxycortisol, progesterone and 17α-hydroxyprogesterone were detected at concentrations in the range of 12.6–40.1, 7.10–24.2, 0.74–10.7, 1.06–5.72, 0.38–4.38 and <mLOQ - 1.01 ng/mL, respectively. To our knowledge, this is the first study to determine steroid hormones in the plasma of pinnipeds using convergence chromatography.

1. Introduction

Steroid hormones play a crucial role in regulating reproduction, sex differentiation, growth, metabolism, and immune function in vertebrates [1]. A rapid and reliable method for the quantification of steroid hormones is essential in clinical and research environments. Endocrine disrupting chemicals (EDCs) can interfere with steroidogenesis and are recognised as an increasing health threat to biota [2]. As such, there is a need for the multi-residue determination of several steroid hormone analogues for an improved mechanistic overview of how contaminants can potentially disrupt steroidogenesis. Steroid hormones are considered highly stable, allowing for retrospective analysis in samples [3–5].

For over 30 years, the predominant technique used to quantify circulating concentrations of steroids were conventional immunoassay-based methods (e.g., radioimmunoassays or enzymatic immunoassays) [6]. While these immunological methods offer good sensitivity, they often lack specificity due to cross-reactivity with other steroids and their metabolites [7,8]. Both the high inter-laboratory variability and lack of specificity has led to difficulties in interpreting and comparing steroid concentrations among laboratories [9–11].

Chromatography has helped to address some of these limitations, allowing for higher specificity as well as simultaneous measurement of steroid hormones. Over the last decade, significant advances in chromatographic methods, including ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS), have alleviated some of these challenges. In recent years, supercritical fluid chromatography (SFC) has emerged as an efficient 'green' analytical technique, due to its reduced organic solvent consumption [12,13]. Carbon dioxide (CO_2) is often employed in SFC as it is readily available, low cost, non-toxic and can reach its supercritical state under relatively mild conditions together with an organic modifier, such as methanol. Ultra-performance convergence chromatography (UPC^2) combines the advantages of SFC and UPLC to enhance chromatographic efficiency and reduce analysis time. Consequently, UPC^2 combined with tandem mass spectrometry is

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becoming a powerful technique for separating challenging analytes, including steroid hormones [14,15].

Pinnipeds and other marine mammals are exposed to environmental contaminants. As top predators, seals are particularly vulnerable to bioaccumulation and biomagnification of organic contaminants [2]. Many legacy and emerging persistent organic contaminants (POPs) are either known or suspected endocrine disruptors [16,17]. In the Baltic Sea, populations of grey seals (*Halichoerus grypus*) underwent massive decline in the 1970s due to almost a century of high culling rates, in addition to decades of reduced fertility, reproductive failure and immunosuppression caused by persistent environmental pollution [18,19]. Synthetic chemicals (contaminants) are shown to interfere with steroidogenesis affecting circulating concentrations of steroid hormones [20,21]. Therefore, bioanalytical method development and application are deemed necessary for the reliable quantification of those in wildlife and the successful biomonitoring of animal health.

With this background, the present study aimed to develop a methodology for the multiresidue determination of 19 steroid hormones in grey seal blood plasma. The objectives were to: (1) develop a rapid and accurate methodology for the analysis of steroid hormones in blood media; and (2) to investigate the occurrence profiles of steroid hormones in grey seal pup plasma. To our knowledge, this is the first study to quantify steroid hormones in the plasma of pinnipeds using convergence chromatography.

2. Materials and methods

2.1. Chemicals and materials

Analytical standards of the target analytes (TAs): dehydroepiandrosterone (DHEA, 99.8%), androstenedione (AN, 99.7%), testosterone (TS, 99.7%), 5α-dihydrotestosterone (DHT, 99.9%), 11-deoxycorticosterone (DOC, 99.1%), 11-deoxycortisol (11-deoxyCOR, 98.6%), aldosterone (ALDO, 99.1%), corticosterone (COS, 99.5%), 17α-hydroxyprogesterone (17α-OHP, 98.2%), cortisol (COR, 98.9%), cortisone (CORNE, 97.8%), pregnenolone (P5, 99.6%), 17α-hydroxypregnenolone (17αOH-P5, 96.6%), progesterone (P4, 99.5%), estrone (E1, 99.1%), and 17β-estradiol (17β-E2, 99.7%) were purchased from Cerilliant (Texas, USA). The remaining analytical standards of TAs, 11-ketotestosterone (11-KetoTS, ≥ 98%) and 17α-estradiol (17α-E2, ≥ 98%), were purchased from Sigma–Aldrich (Steinheim, Germany). Androstenediol (A5) was purchased from Toronto Research Chemicals, Inc (North York, ON, Canada). Isotopically labelled internal standards (ISs), cortisone-$^{13}C_3$ (2,3,4–$^{13}C_3$-CORNE), dihydrotestosterone-$^{13}C_3$ (2,3,4–$^{13}C3$-DHT) and 17α-hydroxyprogesterone-$^{13}C_2$ (2,3,4–$^{13}C_2$-17α-OHP) were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA) with purity ≥ 98%.

Individual stock solutions were prepared in methanol (MeOH) and stored at −20 °C, expect for DHT which was stored at −80 °C. Ethyl acetate (EtOAc), methyl-*t*-butyl ether (MTBE), heptane, ammonium formate ($HCOONH_4$), hydrochloric acid (HCl, 37% v/v), HPLC grade MeOH, isopropanol (IPA), acetonitrile (ACN) and acetone were purchased from VWR International AS (Oslo, Norway). Ammonium acetate (NH_4CH_3COO) and formic acid (HCOOH, 98% v/v) were purchased from Merck (Damstadt, Germany). Ultrapure carbon dioxide (CO_2) was purchased from AGA industrial gases (Lidingö, Sweden). Ultrapure water was prepared via a water purification system (Qoption, Elga Labwater, Veolia Water Systems LTD, U.K.).

The solid phase extraction (SPE) cartridges, Oasis HLB 60 mg/3 mL (Waters; Milford, MA, USA), Thermo Scientific HyperSep C18 50 mg/1 mL (Thermo Scientific; Waltham, MA, USA) and HybridSPE 30 mg/1 mL (Sigma–Aldrich; Steinheim, Germany) were assessed for the extraction of steroid hormones. Polypropylene (PP) tubes (15 mL) and amber glass LC vials (1.5 mL) were purchased from VWR International AS (Oslo, Norway). A 12-port Visiprep DL (Disposable Liners) SPE vacuum manifold was purchased from Supelco, Inc (Bellefonte, PA, USA). Radioimmunoassay (RIA) kits ^{125}I were purchased from MP Biomedicals (California, USA) for total unconjugated 17β-estradiol (Catalogue No. 238101) and testosterone (Catalogue No. 189101) concentrations. A γ-scintillation counter (Cobra Auto-Gamma, model 5003, Packard Instrument Co., Dowers Grove, IL, USA) was used to measure reactivity from the RIA test kits.

2.2. Sample collection

For the method development and validation, matrix-matched standards were prepared from bovine plasma samples, as previously described [22]. Blood from live-captured grey seal (n = 9) pups were collected from Vesitukimaa island, Saaremaa, Estonia (57° 53′N, 22° 03′E) in the Baltic Sea (n = 8) and from the Isle of May, Scotland (56°11′N, 02°33′W) in the East Atlantic Ocean (n = 1). The blood samples were drawn from the hind flipper vein (animals from Estonia) or extradural vein (animal from Scotland) using 19-gauge needles and collected directly into 4–10 mL ethylenediaminetetraacetic acid (EDTA) vacutainers. Permission and ethical approval to conduct the studies was provided by the Estonian and UK authorities (see permissions section). After collection, the samples were centrifuged at 2100 rpm (700 g-force) for 10 min and the obtained plasma was divided into 500 μL aliquots. Samples from Estonia were stored initially at −20 °C for 2 days before being transferred to −80 °C until further sample preparation and analysis. The sample from Scotland was transferred to −80 °C immediately after sampling. For further information regarding grey seal samples see Table S1.

2.3. LLE, SPE and HybridSPE-based sample preparation protocols

Three different sample preparation techniques were assessed for the extraction of steroid hormones: liquid-liquid extraction (LLE), common solid-phase extraction (SPE) and HybridSPE. Procedural blanks were used to monitor background contamination during sample preparation and instrumental analysis. For the LLE, a generic protocol was used with minor modifications [22,23]. Aliquots of 150 μL of matrix were transferred to 15 mL PP tubes, and 20 ng ISs were added. Samples were fortified with 300 μL of 1.0 M ammonium acetate. Subsequently, the samples were extracted 3 consecutive times with 3 mL of ethyl acetate each time (3 × 3 mL). For each extraction, the mixture was ultrasonicated for 45 min, and thereafter centrifuged (4000 rpm) for 10 min. The supernatants were combined, and 1 mL of ultrapure water was added to remove any residual salt. The mixture was centrifuged again, and the supernatant was collected and concentrated to near dryness under a gentle stream of nitrogen (N_2) gas. Finally, 500 μL of MeOH: ultrapure water (1:1 v/v) were added, vortex mixed, and transferred for UPC^2-MS/MS analysis.

For common SPE, the extraction of steroid hormones was assessed by both, Oasis HLB (60 mg) and HyperSep C18 (50 mg) cartridges. The SPE with Oasis HLB cartridges was performed according to previous work with minor modifications [22]. The Oasis HLB cartridges were conditioned with 3 mL MeOH and 3 mL acidified ultrapure water (adjusted to pH < 3 with 37% v/v HCl). Subsequently, aliquots of 150 μL per sample were fortified with 20 ng ISs followed by 300 μL ammonium acetate and were passed through the cartridge. Thereafter washed with 3 mL of ultrapure water, and dried under vacuum for ~10 min. The target steroid hormones were eluted with 3 mL of MeOH. Eluents were concentrated to near dryness under a gentle N_2 stream. The solvents were reconstituted with 1 mL of MeOH:ultrapure water (1:1 v/v), vortex-mixed, and transferred for UPC^2-MS/MS analysis.

The extraction with HyperSep C18 SPE cartridges was performed according to Weisser et al. [24] with minor modifications. HyperSep C18 (50 mg) cartridges were conditioned consecutively with 3 mL heptane, 3 mL acetone, 3 mL MeOH and 3 mL ultrapure water. Aliquots of 150 μL pooled matrix were transferred to 15 mL PP tubes, where they were fortified with 20 ng of ISs. The cartridges were equilibrated with 3 mL

ultrapure water followed by 3 mL ultrapure water:MeOH (75:25% v/v). The samples were loaded onto the cartridges and eluted with 3 mL ultrapure water: MeOH (20:80% v/v), followed by evaporation to 500 μL under a gentle stream of N_2 at 45 °C, and transferred for UPC^2- MS/MS analysis.

For the HybridSPE, two precipitation agents: ACN with 0.1% formic acid (v/v), and MeOH with 0.1% ammonium formate (w/v), were assessed according to our previous work [22,25] with minor modifications. In each instance, aliquots of 150 μL of pooled sample were transferred into a 15 mL PP tube and 450 μL of the respective precipitation agent were added. Samples were vortex-mixed and centrifuged (4000 rpm, 10 min) at room temperature. The HybridSPE cartridges were initially conditioned with 1 mL MeOH before the supernatants were passed directly through at a flow rate of approx. 1 mL/min and analysed with UPC^2- MS/MS.

2.4. Radioimmunoassay analysis of 17β-estradiol and testosterone

To compare with chromatographic results, testosterone and 17β-estradiol were analysed with commercial radioimmunoassay kits (MP Biomedicals, California, USA) in duplicates according to the manufacturer protocols (Catalogue number: 07–189105, LOT# RTK2108 and 07–238102, LOT#E2K2109 A).

Briefly, for the analysis of 17β-estradiol, aliquots of 100 μL of plasma were added to ImmunoChem antibody-coated tubes followed by the addition of 1 mL of radioactive estradiol ^{125}I tracer. The samples were incubated in a water bath (Grant Instruments LTD, Cambridge, UK) for 60 min at 37 °C. The solution was decanted, and the tubes were allowed to air dry prior to analysis with the γ-scintillation counter. For testosterone analysis, aliquots of 50 μL of plasma were added to the polypropylene test tubes, with 100 μL of sex binding globulin inhibitor (SBGI) solution and 500 μL of radioactive testosterone ^{125}I tracer. Thereafter, 500 μL of anti-serum were added and the samples were vortex-mixed, and eventually incubated in the water bath at 37 °C for 120 min. After incubation, 100 μL of a secondary antibody were added to all samples, to allow precipitation of the bound primary antibody [26], vortex-mixed and incubated at 37 °C for an additional 60 min. After this second incubation, all samples were centrifuged for 15 min (2500 rpm), the supernatant was decanted and the antibody–antigen complexes were measured with the γ-scintillation counter.

Calibration curves and concentrations of 17β-estradiol and testosterone in the samples were calculated with the embedded software (SpectraWork Spectrum Analysis Software, Packard Instrument Company, Connecticut, USA) of the γ-scintillation counter. The quality control/quality assurance (QA/QC) of the measurements was established by the analysis of the control material, Lyphochek Immunoassay Plus Control Levels 1, 2 and 3 (BioRad; California, USA, LOT# 40380). The QA/QC were run in triplicates (n = 3) and the repeatability (RSD %) was 12.6 and 8.5% for testosterone and 17β-estradiol, respectively. The limits of detection (LODs) were 0.02 ng/mL for testosterone and 23.9 pg/mL for 17β-estradiol.

2.5. UPLC-MS/MS

Method development was carried out using an Acquity UPLC I-Class system (Waters, Milford, U.S.) coupled to a triple quadrupole mass analyser (QqQ; Xevo TQ-S) with a ZSpray ESI ion source (Waters, Milford, U.S.). Initial method development was carried out on a Phenomenex Kinetex C18 column (30 × 2.1 mm, 1.3 μm, 100 Å) connected to a Phenomenex C18 guard column (2.1 mm). However, with this column, the coelution of peaks was unavoidable and due to crosstalk in the multiple reaction monitoring (MRM) channels, chromatographic separation was not deemed sufficient (Fig. S1). Optimal separation was achieved with an Acquity UPLC Charged Surface Hybrid (CSH) Fluoro Phenyl (2.1 × 100 mm, 1.7 μm) column. The mobile phase consisted of solvents: (A) ultrapure water containing 0.1% v/v formic acid and (B) acetonitrile containing 0.1% v/v formic acid. The flow rate was 100 μL min^{-1} and the injection volume was 2 μL. The gradient elution was initiated with 40% B, held for 2.4 min and then increased to 100% B over 12.6 min, held for 1.6 min and decreased back to 40% B over 0.5 min with a hold of 2.1 min for a separation time of 20 min. Electrospray ionisation was conducted under electrospray positive ionisation mode (ESI+) in MRM mode. Optimal source settings were the following: source temperature 150 °C, capillary voltage 2.8 kV, desolvation temperature 500 °C and nebulizer gas pressure 6.0 bar.

2.6. UPC^2-MS/MS

Method development and chromatographic separation using convergence chromatography was carried out on a Waters ACQUITY UltraPerformance Convergence chromatographic (UPC^2) system (Milford, MA, USA) coupled to a triple quadrupole (QqQ; Xevo TQ-XS) mass spectrometer (UPC^2-MS/MS) with a Zspray ESI ion source (Waters, Milford, U.S). The UPC^2 system was equipped with a binary solvent manager; a sample manager maintained at 10 °C; a 10 μL injection loop; a temperature-controlled column manager; a convergence manager to regulate backpressure; and an isocratic solvent manager for delivering make-up solvent (a simple overview of the UPC^2-MS/MS configuration is shown in Fig. S2). Two columns were tested for the initial method development: an Acquity BEH UPC^2 (100 × 3.0 mm, 1.7 μm) and a Viridis CSH Fluoro-Phenyl UPC^2 column (100 × 2.1 mm, 1.7 μm, 130 Å). The CSH Fluoro-Phenyl column showed improved peak shape relative to the BEH column (Fig. S3) and as such the final separation of steroid hormones was performed on the Viridis CS Fluoro-Phenyl UPC^2 column (100 × 2.1 mm, 1.7 μm, 130 Å) equipped with a CSH Fluoro-Phenyl VanGuard guard column (Viridis; 5 mm × 2.1 mm) in ESI positive mode for progestogens, androgens and corticosteroids and in ESI negative mode for estrogens (Table 2).

For the analysis of progestogens, androgens and corticosteroids, the gradient program was according to de Kock et al. [14]: modifier 2% (0.5 min), 2–17% (2.5 min), hold 0.5 min, back to 2% modifier (0.5 min) and hold 1 min for a separation time of 5 min, where the modifier was MeOH: IPA, 1:1 v/v containing 0.1% v/v formic acid. The injection volume was 1 μL (partial needle overflow mode) with a flow rate of 1 mL/min. The column oven temperature was set at 40 °C and the automatic backpressure regulator at 2000 psi. A post column make-up flow (0.2 mL/min) of MeOH containing 0.1% v/v formic acid was used to enhance ESI+ ionisation. For the analysis of estrogens (negative mode), elution was carried out under the same gradient conditions with a different organic modifier and make-up solvent to enhance ESI- ionisation; MeOH: IPA (1:1 v/v) was employed as the organic modifier (B) with a post column make-up flow (0.2 mL/min) of MeOH: IPA (1:1 v/v) containing 0.1% v/v ammonium hydroxide.

The specific MS/MS parameters are presented in Table 2. The electrospray ionisation voltage was +2.8 kV in positive mode and −2.5 kV in negative mode. N_2 was used as both the desolvation and cone gas, at a flow rate of 1000 and 150 L/h, respectively. The desolvation and source temperatures were 500 and 150 °C, respectively. The calibration standard solutions were prepared in MeOH containing 0.1% w/v ammonium formate.

2.7. Data analysis and statistical treatment

All chromatographic data were acquired with Intellistart and MassLynx v4.1 software, while quantification processing was performed with TargetLynx (Waters, Milford, USA). Excel (Microsoft, 2021) was used for general descriptive statistics. Data analysis did not include non-quantifiable data (<mLOQ) unless stated otherwise. Concentrations were reported as ng/mL.

Table 1
Baseline concentrations of steroid hormones present in plasma (ng/mL) from grey seals (n = 9) relative to other studies using various determination methods.

Steroids	Present study (ng/mL)	Species (sample number, life stage, sex, other relevant information)	Other studies (Range, ng/mL)	Method	Reference
Corticosteroids					
COR	13–40	Grey seal, *Halichoerus grypus* (n = 3, Adult, M)	36–354	DIDA	[27]
		Australian fur seal, *Arctocephalus pusillus doriferus* (n = 114, pre-weaned pups, M/F)	17–344	ELISA	[28]
		Harbor seal, *Phoca vitulina* (n = 5, Adult, M/F)	Mean ± SE Summer: 51.5 ± 20.3 Winter: 28.5 ± 17.4	RIA	[29]
		Southern elephant seals, *Mirounga leonine* (n = 63, Adult & weaned pups, M/F)	Mean ± SD 77.9 ± 36.0 (F, n = 28) 59.7 ± 40.5 (M, n = 35) 98.9 ± 39.9 (pups, n = 24)	RIA	[30]
		Grey seal, *Halichoerus grypus* (n = 71, pups, M/F)	16.6-161	ELISA	[10]
COS	0.7–11	Grey seal, *Halichoerus grypus* (n = 3, Adult, M)	46.3–82.0	DIDA	[27]
CORNE	7.1–24	Grey seal, *Halichoerus grypus* (n = 3, Adult, M)	19.6–58.9	DIDA	[27]
11-deoxyCOR	1.1–5.7	Grey seal, *Halichoerus grypus* (n = 1, Adult, M)	2.7	DIDA	[27]
ALDO	<mLOQ–2.4	Grey seal, *Halichoerus grypus* (n = 3, Adult, M)	1.4–3.3	DIDA	[27]
		Southern elephant seals, *Mirounga leonine* (n = 63, Adult & weaned pups, M/F)	Mean ± SD 0.217 ± 0.159 (F, n = 28) 0.260 ± 0.233 (M, n = 35) 0.381 ± 0.232 (pups, n = 24)	RIA	[30]
DOC	<mLOQ–0.94	–	–	–	
Androgens					
TS	<mLOQ	Grey seal, *Halichoerus grypus* (n = 3, Adult, M)	3.1–7.45	DIDA	[27]
		Northern fur seals, *Callorhinus ursinus* (n = 98, M/F)	<0.004–0.038 (F, n = 93) 0.028–1.70 (M, n = 5)	RIA	[31]
		Grey seal, *Halichoerus grypus* (n = 71, pups, M/F)	0.05–1.26	ELISA	[10]
11-KetoTS	n.d	Grey seal, *Halichoerus grypus* (n = 3, Adult, M)	n.d.	DIDA	[27]
DHT	<mLOQ	–	–	–	–
DHEA	<mLOQ–7.4	Northern fur seals, *Callorhinus ursinus* (n = 98, M/F)	<0.004–1.40 (F, n = 93) 0.21–0.81 (M, n = 5)	RIA	[31]
AN	<mLOQ–0.65	Northern fur seals, *Callorhinus ursinus* (n = 98, M/F)	<0.004–4.83 (F, n = 93) <0.004–2.35 (M, n = 5)	RIA	[31]
A5	n.d.	Northern fur seals, *Callorhinus ursinus* (n = 98, M/F)	<0.004–1.26 (F, n = 93) <0.004–0.17 (F, n = 93)	RIA	[31]
Progestagens					
P4	0.38–4.4	Northern fur seals, *Callorhinus ursinus* (n = 98, M/F)	3.7–101 (F, n = 93) 0.2–4.9 (M, n = 5)	RIA	[31]
		South American fur seal, *Arctophoca australis* (n = 11, Adult, F, Breeding season)	0.6–55.4	RIA	[32]
		Stellar sea lions, *Eumetopias jubatus* (n = 3, Adult, F, pregnant and non-pregnant)	13.8–24.4	LC-MS/MS	[33]
P5	<mLOQ–30	–	–	–	
17α-OHP	<mLOQ–1.0	–	–	–	
17αOH-P5	1.3–5.6	–	–	–	
Estrogens					
E1	<mLOQ–4.7	Northern fur seals, *Callorhinus ursinus* (n = 98, M/F)	<0.004–0.25 (F, n = 93) <0.004–0.04 (M, n = 5)	RIA	[31]
17β-E2	n.d.	Northern fur seals, *Callorhinus ursinus* (n = 98, M/F)	<0.004–0.093 (F, n = 93) <LOD (M, n = 5)	RIA	[31]
		Grey seal, *Halichoerus grypus* (n = 71, pups, M/F)	0.02–0.39	ELISA	[10]
17α-E2	n.d.	–	–	–	

DIDA Direct isotopic diluton analysis, ELISA Enzyme-linked immunosorbent assay, RIA Radioimmunoassy, n.d not detected. SE standard error. SD standard deviation. COR Cortisol, COS Corticosterone, CORNE Cortisone, 11-deoxyCOR 11-deoxycortisol, ALDO Aldosterone, DOC 11-deoxycorticosterone, TS Testosterone, 11-KetoTS 11-Ketotestosterone, DHT 5α-dihydrotestosterone, DHEA Dehydroepiandrosterone, AN Androstenedione, A5 Androstenediol, P4 Progesterone, P5 Pregnenolone, 17α-OHP 17α-hydroxyprogesterone, 17α–OH–P5 17α-hydroxypregnenolone, E1 Estrone, 17β-E2 17β-estradiol, and 17α-E2 17α-estradiol.

3. Results and discussion

3.1. UPLC- vs. UPC^2-MS/MS analysis

To attain sufficient chromatographic separation in UPLC for coeluting steroid hormones, especially the corticosteroids: cortisol, cortisone, and aldosterone, a chromatograph of 20 min was acquired (Fig. S4). In comparison, the UPC^2 achieved chromatographic separation of the same 19 steroid hormones within 5 min (Fig. S5). It is noteworthy that chromatographic separations can be performed faster in UPC^2 relative to UPLC, due to the low viscosity of supercritical CO_2, the high analyte diffusivity, and the applicability of higher flow rates in the former [12]. A CSH fluoro phenyl stationary phase with pentafluoro phenyl groups was employed for the separation of steroid hormones in both UPLC and UPC^2. Fluoro phenyl stationary phases operate via multiple retention mechanisms including dipole-dipole, aromatic (π - π), hydrogen bonding and hydrophobic interactions. The improved separation of structural isomers and structurally similar steroids in UPC^2 relative to UPLC were not solely attributed to the stationary phase (since the same column was used in both chromatographic techniques), but rather to its unique interaction with the hydrophobic supercritical CO_2 and the organic modifier in the former. In UPC^2, a mixed elution order of the target analytes was observed when compared to UPLC, which agreed with previous studies [34,35]. However, most steroid hormones appeared to demonstrate greater retention with increased polarity as it would be expected in normal phase chromatography; a combination of –OH and π - π interactions could be a contributing factor towards the unique selectivity observed in UPC^2 [36,37].

Table 2
Name, abbreviation, molecular formula, partition coefficient (logP), retention time (RT) and multiple reaction monitoring (MRM) parameters for steroid hormones used in UPLC- and UPC^2-MS/MS analysis.

Steroid Class, Name, (Name IS)	Molecular Formula	LogP[†]	MRM Transitions	RT	CV (V)	CE (eV)
Androgens						
Dehydroepiandrosterone, DHEA (DHT-$^{13}C_3$)	$C_{19}H_{28}O_2$	3.2	253 > 197[#a]	1.76	20	18
					20	18
Androstenedione, AN (DHT-$^{13}C_3$)	$C_{19}H_{26}O_2$	2.7	287 > 97[#]	1.97	20	22
			287 > 109		20	26
Androstenediol, A5 (DHT-$^{13}C_3$)	$C_{19}H_{30}O_2$	3.5	255 > 159[#a]	1.73	20	18
					20	18
Testosterone, TS (DHT-$^{13}C_3$)	$C_{19}H_{28}O_2$	3.3	289 > 97[#]	2.45	20	20
			289 > 109		20	26
5α-dihydrotestosterone, DHT (DHT-$^{13}C_3$)	$C_{19}H_{30}O_2$	3.7	291 > 255[#]	1.69	24	24
			291 > 159		24	16
11-Ketotestosterone, 11-KetoTS (CORNE-$^{13}C_3$)	$C_{19}H_{26}O_3$	2.0	303 > 121[#]	2.89	18	26
			303 > 267		18	18
Corticosteroids						
11-deoxycorticosterone, DOC (17α-OHP-$^{13}C_3$)	$C_{21}H_{30}O_3$	2.9	331 > 97[#]	2.15	20	20
			331 > 109		20	22
11-deoxycortisol, 11-deoxyCOR (CORNE-$^{13}C_3$)	$C_{21}H_{30}O_4$	2.5	347 > 347[#]	2.70	20	32
			347 > 109		20	10
Aldosterone, ALDO (CORNE-$^{13}C_3$)	$C_{21}H_{28}O_5$	1.1	361 > 315[#]	3.26	20	20
			361 > 325		20	18
Corticosterone, COS (CORNE-$^{13}C_3$)	$C_{21}H_{28}O_5$	1.9	347 > 91[#]	2.97	20	46
			347 > 97		20	22
Cortisol, COR (CORNE-$^{13}C_3$)	$C_{21}H_{30}O_5$	1.6	363 > 121[#]	3.29	20	24
			363 > 327		20	14
Cortisone, CORNE (CORNE-$^{13}C_3$)	$C_{21}H_{28}O_5$	1.5	361 > 163[#]	2.83	20	30
			361 > 121		20	24
Progestogens						
Pregnenolone, P5 (DHT-$^{13}C_3$)	$C_{21}H_{32}O_2$	4.2	299 > 159[#b]	1.74	20	20
			299 > 281[b]		20	20
Progesterone, P4 (DHT-$^{13}C_3$)	$C_{21}H_{30}O_2$	3.9	315 > 109[#]	1.93	34	20
			315 > 97		34	24
17α-hydroxyprogesterone, 17α-OHP (17α-OHP-$^{13}C_3$)	$C_{21}H_{30}O_3$	3.2	331 > 97[#]	2.37	20	26
			331 > 109		20	24
17α-hydroxypregnenolone, 17αOH-P5 (17α-OHP-$^{13}C_3$)	$C_{21}H_{32}O_3$	3.1	297 > 159[#a]	2.22	20	18
			297 > 279[a]		20	18
Estrogens						
Estrone, E1 (17αOHP-$^{13}C_3$)	$C_{18}H_{22}O_2$	3.1	271 > 197[#]	1.98	22	35
			271 > 133		22	35
17β-Estradiol, 17β-E2 (17αOHP-$^{13}C_3$)*	$C_{18}H_{24}O_2$	4.0	271 > 171[#]	2.54	22	20
					22	20
17α -Estradiol, 17α-E2 (17αOHP-$^{13}C_3$)*	$C_{18}H_{24}O_2$	4.0	271 > 171[#]	2.45	22	20
					22	20
Internal standards						
2,3,4-Cortisone-$^{13}C_3$, CORNE-$^{13}C_3$	$C_{18}{}^*C_3H_{28}O_5$	–	364 > 166[#]	2.83	20	30
			364 > 123		20	24
2,3,4-Dihyrdotestosterone-$^{13}C_3$, DHT-$^{13}C_3$	$C_{16}{}^*C_3H_{30}O_2$	–	294 > 258[#]	1.68	24	24
			294 > 162		24	16
2,3,4-17α-hydroxyprogesterone-$^{13}C_2$, 17α-OHP-$^{13}C_2^{\neq}$	$C_{18}{}^*C_3H_{30}O_3$	–	334 > 100[#]	2.37	20	26
			334 > 112		20	24

IS internal standard, *RT* retention time, *CV* cone voltage, *CE* collision energy, [a]Neutral loss of 2×H_2O, [b]Neutral loss of H_2O, *Analysed in ESI(−) mode, [#]Quantification ion,[†]LogP data sourced from PubChem. [≠] Was also used as the IS for 17β-E2 and 17α-E2 in polarity switching mode.

One major challenge of steroid hormones analysis is crosstalk, which refers to when target analytes obtain common MRM transitions. This results in giving signals at their expected retention time (RT) in other MRM channels or signals at the RT of other compounds. Therefore, crosstalk needs to be effectively addressed to avoid false positive findings either through the selection of specific mass transitions for the target analytes or via sufficient chromatographic resolution. Upon the individual injection of each target steroid hormone, most steroids (except for progesterone, pregnenolone and 17α-hydroxypregnenolone) experienced crosstalk to some degree manifesting often as a peak (at its RT) in the MRM channels of one or more steroid hormones (Fig. 1). An extensive investigation was performed into the crosstalk of 52 steroids analysed with UPC^2 -MS/MS by du Toit et al. [38], and they similarly found crosstalk for all corticosteroids, progestogens, and androgens. Additionally, the authors found that several analytical standards of steroid hormones were contaminated with their precursors, including corticosterone (with cortisol) and androstenedione (with testosterone), further complicating analysis. However, the use of unique MRMs compensated for steroid crosstalk in most instances. Similarly to du Toit et al. [38], the same unique MRM transitions were employed for 7 out of 16 steroid hormones (cortisol, cortisone, aldosterone, corticosterone, pregnenolone, 5α-dihydrotestosterone and dehydroepiandrosterone) in this study. For the remaining 9 steroid hormones (androstenedione, androstenediol, testosterone, 11-ketotestosterone, 11-deoxycorticosterone, 11-deoxycortisol, 17α-hydroxypregnenolone, progesterone and 17α-hydroxyprogesterone), different transitions were chosen due to increased sensitivity compared to those employed by du Toit et al. [38]. Generally, very few studies address the critical issue of crosstalk when analysing steroid hormones, which may lead to an overestimation of some steroids and false positive findings. Moreover, instrumental background contamination was neither observed in UPLC or UPC^2. Overall, the UPC^2 method demonstrated improved separation efficiency (resolution) that was critical for effectively addressing crosstalk, significantly reducing analysis time and consuming less solvent(s)

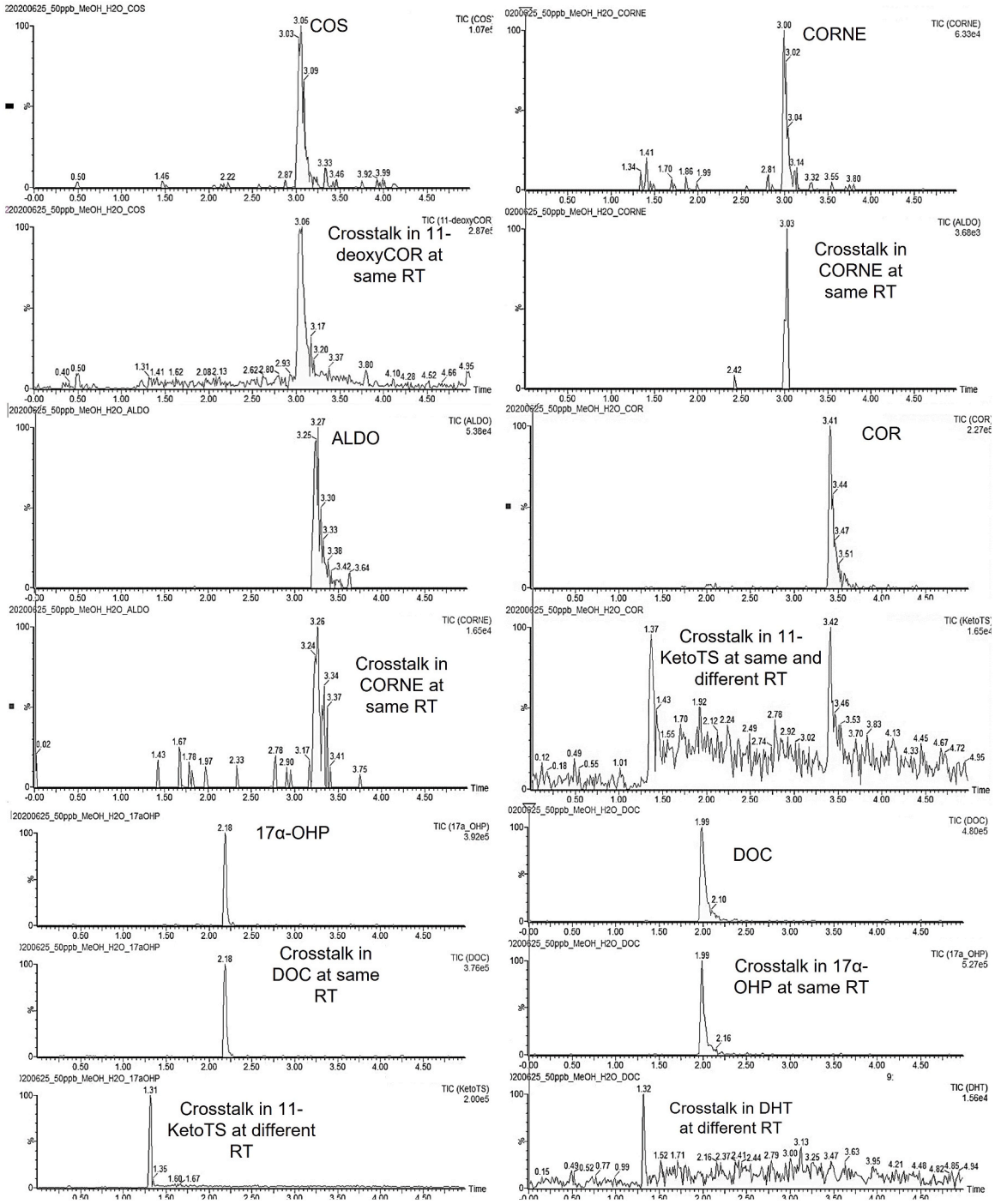

Fig. 1. Chromatograms of crosstalk observed in UPC^2-MS/MS upon individual injection of steroids. Where: COR Cortisol, COS Corticosterone, CORNE Cortisone, 11-deoxyCOR 11-deoxycortisol, ALDO Aldosterone, DOC 11-deoxycorticosterone, TS Testosterone, 11-KetoTS 11-Ketotestosterone, DHT 5α-dihydrotestosterone, DHEA Dehydroepiandrosterone, AN Androstenedione, A5 Androstenediol, 17α-OHP 17α-hydroxyprogesterone, E1 Estrone, 17β-E2 17β-estradiol, and 17α-E2 17α-estradiol.

relative to UPLC.

3.2. Extraction efficiency of the sample preparation protocols

The extraction efficiency of the applied sample preparation protocols (LLE, SPE and HybridSPE) are presented through the calculation of the absolute recoveries of the target analytes in both bovine and grey seal plasma (Table S2) [39]. The LLE protocol demonstrated recoveries ranging from 46 to 107% and from 45 to 98% in bovine and grey seal plasma, respectively. Results agreed with previous studies for most target analytes [40,41]. The lower recoveries (<50%) observed for some steroids (progesterone, pregnenolone an 11-ketotestosterone) could be specifically attributed to the solvent choice, where improved recoveries were previously reported when using n-hexane: EtOAc (70:30% v/v) or methyl-*t*-butyl ether (MTBE) as the extraction solvent [40,41]. Although the LLE protocol demonstrated acceptable absolute recoveries, the values had higher relative standard deviations (RSD %) when compared to those from the SPE based protocols; with more than half of all target

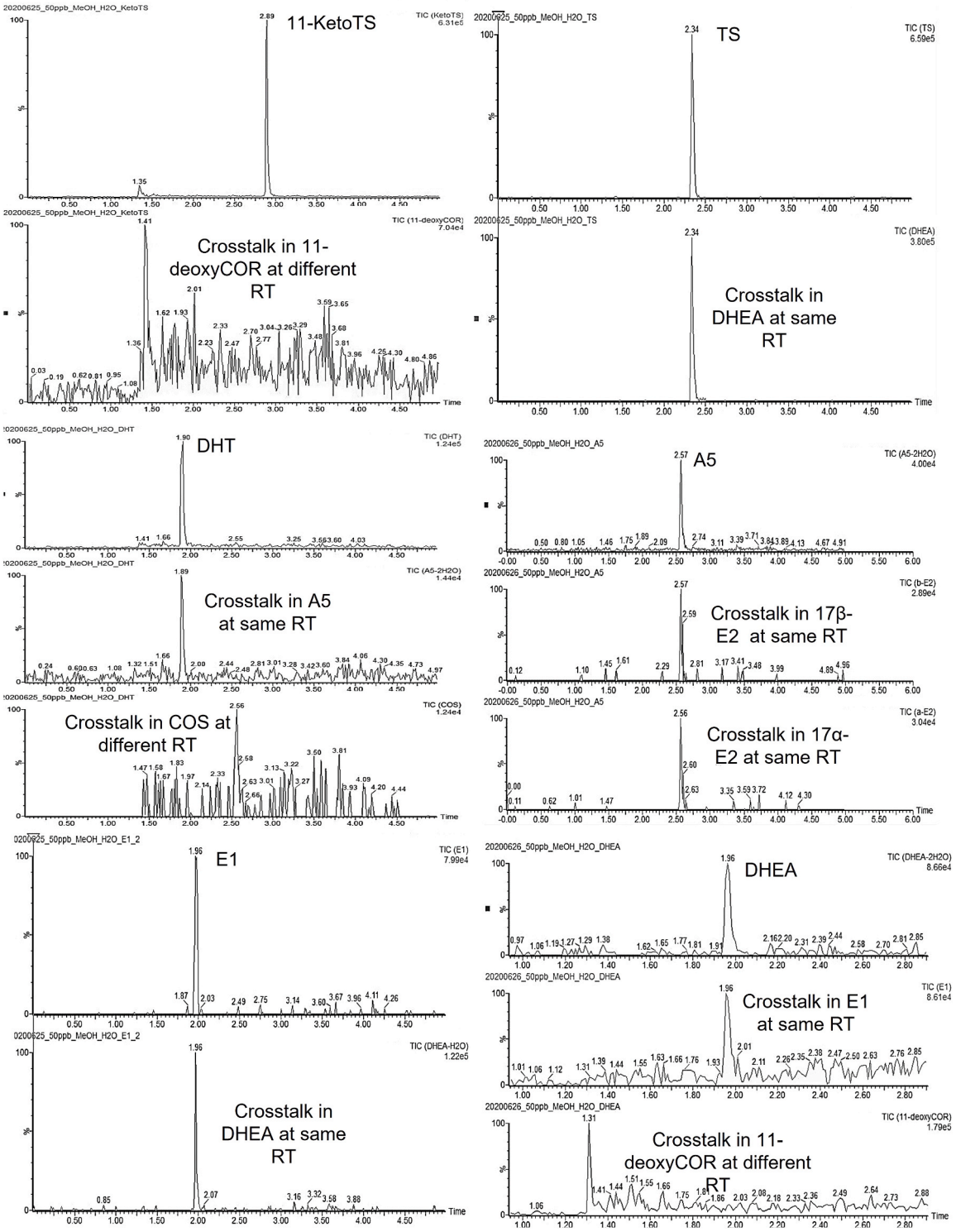

Fig. 1. (*continued*).

analytes demonstrating RSDs % > 15% with the LLE protocol. Additionally, the LLE protocol was more time consuming and required a high volume of organic solvent than the SPE based protocols.

In the SPE based protocols, the recoveries of the TAs, when using the Oasis HLB cartridges, ranged from 46 to 113% in bovine and from 42 to 69% in grey seal plasma (Table S2), and were consistent with previous studies [42,43]. The recoveries of the TAs from the HyperSep C18 cartridges ranged from 19 to 90% in bovine and from 56 to 116% in grey seal plasma. Interestingly, the recoveries varied based on the species, which indicated that both species and individual matrix composition (e.g., % phospholipids, % lipids) can impact extraction efficiencies. Generally, the HyperSep C18 method showed extraction efficiencies consistent with previous works [24,44]. However, some steroids (Table S2: progesterone, pregnenolone, androstenediol and dehydroepiandrosterone) demonstrated lower recoveries in this study, which can be attributed to either relative recoveries being reported in other studies (rather than absolute) or to the inclusion of additional clean up step(s) prior to analysis. Overall, both SPE cartridges provided

acceptable recoveries for the analysis of steroid hormones in plasma.

The HybridSPE technique was eventually employed for the extraction of the steroid hormones. HybridSPE cartridges consist of a porous silica sorbent coated with zirconia (Zr). This sorbent is designed for the effective removal of endogenous protein and phospholipid interferences from plasma and serum [45]. For the HybridSPE protocol, two different precipitation agents were tested. Acetonitrile containing 0.1% v/v formic acid showed recoveries ranging from 50 to 99% and from 32 to 98% in bovine and grey seal plasma, respectively. Methanol containing 0.1% w/v ammonium formate demonstrated recoveries from 33 to 90% and from 33 to 107% in bovine and grey seal plasma, respectively. Although, the HybridSPE protocol with MeOH containing 0.1% w/v ammonium formate did not display the highest recoveries as other tested extraction protocols, it was the preferred extraction method predominately due to the ease of extraction, good reproducibility (RSDs %: 1.9–23%), low cost, and a lack of an evaporation and reconstitution step during extraction.

It should be noted that in addition, the steroid hormones protocol could be used for the simultaneous extraction of other classes of organic contaminants (e.g., perfluoroalkyl and polyfluoroalkyl substances (PFAS), and bisphenols and benzophenones UV-filters) [25,46]. Thus, the present protocol offers a rapid and accurate method for the analysis

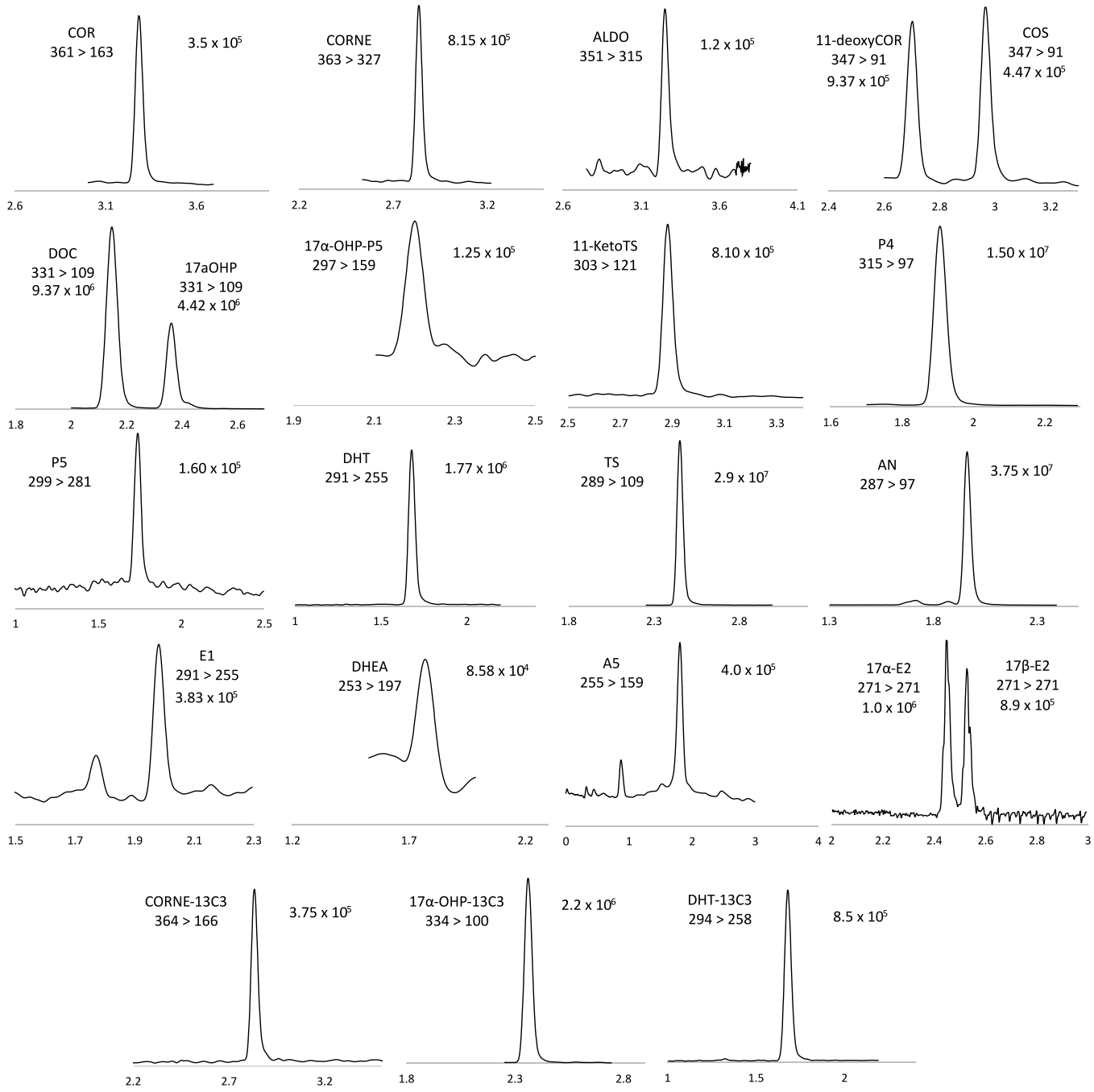

Fig. 2. MRM chromatograms of a (pre-extraction) spiked plasma sample analysed with the HybridSPE-UPC^2-MS/MS method. Where: COR Cortisol, COS Corticosterone, CORNE Cortisone, 11-deoxyCOR 11-deoxycortisol, ALDO Aldosterone, DOC 11-deoxycorticosterone, TS Testosterone, 11-KetoTS 11-Ketotestosterone, DHT 5α-dihydrotestosterone, DHEA Dehydroepiandrosterone, AN Androstenedione, A5 Androstenediol, P4 Progesterone, P5 Pregnenolone, 17α-OHP 17α-hydroxyprogesterone, 17αOH-P5 17α-hydroxypregnenolone, E1 Estrone, 17β-E2 17β-estradiol, and 17α-E2 17α-estradiol.

of both plasma concentrations of steroid hormones and the concentrations of certain groups of suspected EDCs.

3.3. Method performance of HybridSPE-UPC^2-MS/MS

Target analyte quantification was accomplished using ISs and with matrix-matched calibration standards prepared by fortifying target analytes into the matrix prior to extraction (Fig. 2) [39]. A 12-point calibration curve ranging from 0.01 to 50.0 ng/mL (0.01, 0.02, 0.05, 0.10, 0.20, 0.50, 1.00, 2.00, 5.00, 10.0, 20.0 and 50.0 ng/mL) was prepared and demonstrated a satisfactory regression coefficient for every target analyte ($R^2 \geq 0.99$; Table S3). Additionally, one procedural blank was analysed with every batch of 5 samples to monitor background contamination. In all cases, the concentrations in procedural blanks were < the instrumental limits of detection (iLODs).

The lowest standard on the calibration curve that was identifiable and discrete in solvent matrix was accepted as the instrumental limit of quantification (iLOQ) [39,47]. The method limit of detection (mLOD) and quantification (mLOQ) for each target analyte were estimated from the respective iLOD and iLOQ by accounting for the dilution factor. The mLOQs for the majority of the steroid hormones ranged from 0.03 to 1.67 ng/mL (Table S3). Three steroids, namely dehydroepiandrosterone, 17α-hydroxypregnenolone, and androstenediol displayed higher mLOQs at 3.33, 6.67 and 16.6 ng/mL, respectively, than presented in other studies [14]. While the majority of mLOQs were consistent with literature [14,24], a few studies have demonstrated lower mLOQs when using derivatisation. For instance, de Kock et al. [14] determined mLOQs between 0.05 and 1 ng/mL when derivatising with methoxyamine hydrochloride, while Qin et al. [48] determined mLOQs for a range of steroid hormones between 3 and 15 pg/mL when derivatising with methoxyamine hydrochloride and dansyl chloride. In literature, various approaches were used for the determination of LODs and LOQs for steroid hormones, but often the lack of details concerning the methodology used for estimating those, renders interstudy comparison difficult.

Intra- and inter-day instrumental precision (n = 5, 20 ng/mL, k = 5 days) were presented (as RSDs %) and were deemed satisfactory for most steroid hormones with a range between 4 and 20% (Table S3). Higher inter-day precision (>25%) was documented for P5 (27%), A5 (28%), 17α- (39%) and 17β-estradiol (29%). Estradiol and other estrogens suffer from poor ionisation in electrospray (e.g., unstable Taylor Cone formation) and as such are often derivatised when analysed chromatographically; this can explain the higher RSDs % [49]. Derivatisation would likely increase sensitivity for several steroid hormones analysed as indicated by other studies [14,48]. However, the use of a derivatising agent increases extraction time, cost of analysis, and can disrupt the simultaneous extraction of other compounds of interest, but it can also lead to the formation of multiple derivatives from a single target analyte, impacting the accuracy of analysis [50,51]. Additionally, multiple derivatising agents are required for the derivatisation of steroid hormones, further increasing the complexity of the task, the cost and extraction time [48]. Other ionisation techniques, such as atmospheric pressure chemical ionisation (APCI) and atmospheric pressure photoionisation (APPI) demonstrated improved sensitivity of estrogens, when compared to electrospray ionisation [52]. On the contrary, other studies indicated that ESI remains the most robust technique for the analysis of estrogens [53].

The retention time (RT) and relative retention time (RRT), matrix effects (ME %) and ion ratio (IR %) of all steroid hormones were presented in Table S4. The matrix effects were quantified by comparing the response of each target analyte in post-extraction fortified standards (20 ng/mL) with a reference calibration standard prepared in solvent (MeOH containing 0.1% w/v ammonium formate) (Table S4). Ionisation enhancement was evident in most steroid hormones in the range of 4 to 32%. Ionisation suppression occurred in the range of –1 to –70% for five steroid hormones, namely androstenedione, androstenediol, estrone, 5α-dihydrotestosterone and pregnenolone. Matrix effects in this study were of an acceptable level or even reduced relative to the matrix effects documented in UHPLC-MS/MS [14,15,52,54,55]. Although, few studies have compared matrix effects between ultra-high performance supercritical fluid chromatography tandem mass spectrometry (UHPSFC-MS/MS) and UHPLC-MS/MS; the former often exhibited lower matrix effects relative to the latter in urine analysis [56,57]. Interestingly, when analysing serum the matrix effect relative to UHPLC-MS/MS was documented to be highly dependent on the column stationary phase and sample extraction protocol, with lower matrix effects observed after an additional SPE clean up step [56]. The lower matrix effects observed in UHPSFC-MS/MS could be attributed to different retention mechanisms, where separation is accomplished primarily by hydrogen bonding and dipole-dipole interactions. Conversely, the retention in UHPLC-MS/MS is mainly based on partitioning; the same mechanism exploited during common extraction protocols (e.g., LLE, SPE, etc), which may explain the higher occurrence of coeluting compounds, and as such, higher matrix effects in UHPLC- relative to UHPSFC-MS/MS [57,58]. Nonetheless, matrix effects in UHPSFC-MS/MS relative to UHPLC-MS/MS need further investigation, especially in complex matrices.

The extraction efficiency was evaluated through the calculation of absolute recovery and relative recovery for each target analyte at 20 and 2 ng/mL [39]. Extraction efficiencies ranged from 60 to 90% for most steroid hormones at the fortification concentration of 20 ng/mL (Table 3). A total of 18 steroid hormones demonstrated acceptable precision (repeatability) with RSD % (20 ng/mL, n = 3) ranging between 1.9 and 23%. Only androstenediol demonstrated a low recovery together with low precision (33 ± 50.3%), it is unclear why androstenediol presented such poor recovery and as such was classified as semi-quantifiable. At a low fortification level (2 ng/mL, n = 3), precision remained acceptable with most target analytes having an RSD % < 25%. Higher RSDs % were present for androstenediol (due to mLOQ < 2 ng/mL), aldosterone (due to peak instability), pregnenolone (due to mLOQ < 2 ng/mL) and 17α-hydroxypregnenolone (due to mLOQ < 2 ng/mL), which ranged between 47.8 and 86.1%. Lower relative

Table 3
Mean absolute and relative recoveries (%, n = 3 ± relative standard deviation, RSD %) at low (2 ng/mL) and high (20 ng/mL) fortification level.

Target analytes	Absolute recovery % (± RSD %)		Relative recovery, % (± RSD %)	
	20 ng/mL	2 ng/mL	20 ng/mL	2 ng/mL
COR	75.9 (±1.9)	52.3 (±26.9)	61.4 (±27.2)	n.d.
CORNE	59.9 (±5.1)	39.8 (±13.6)	98.9 (±19.8)	84.1 (±10.7)
ALDO	51.2 (±18.1)	20.8 (±62.0)	83.5 (±5.9)	44.3 (±77.7)
COS	60.4 (±18.9)	44.1 (±13.5)	96.7 (±3.4)	94.5 (±26.7)
11-deoxyCOR	64.5 (±7.5)	33.6 (±12.5)	107 (±11.4)	82.3 (±11.7)
17α-OHP	69.3 (±11.3)	30.2 (±15.9)	111 (±9.4)	65.8 (±16.6)
DOC	70.3 (±14.4)	43.3 (±15.6)	111 (±7.1)	93.0 (±12.7)
17αOH-P5	76.5 (±8.6)	25.5 (±86.1)	110 (±2.6)	n.d.
11-KetoTS	65.5 (±13.2)	47.3 (±22.0)	105 (±7.8)	86.8 (±25.0)
P4	73.7 (±10.1)	73.3 (±8.7)	100 (±16.2)	86.7 (±12.4)
P5	90.4 (±20.3)	50.9 (±76.2)	96.8 (±19.5)	n.d.
DHT	89.6 (±6.0)	58.6 (±11.0)	129 (±15.1)	88.0 (±2.65)
TS	66.8 (±23.1)	53.6 (±26.4)	91.3 (±34.7)	82.3 (±22.8)
AN	81.4 (±7.2)	54.3 (±11.7)	115 (±21.6)	83.1 (±17.1)
E1	81.0 (±7.8)	48.1 (±23.5)	114 (±24.5)	48.4 (±44.3)
DHEA	80.8 (±12.1)	45.8 (±25.2)	107 (±19.0)	39.0 (±29.7)
A5	33.0 (±50.3)	65.6 (±47.8)	7.9 (±306)	21.5 (±197)
17α-E2	61.9 (±15.8)	59.5 (±7.0)	104 (±20.3)	61.8 (±39.7)
17β-E2	71.2 (±10.6)	52.8 (±19.8)	120 (±6.7)	53.8 (±15.3)

*n.d.: not determined. COR Cortisol, COS Corticosterone, CORNE Cortisone, 11DeoxyCOR 11-Deoxycortisol, ALDO Aldosterone, DOC 11-Deoxycorticosterone, TS Testosterone, 11-KetoTS 11-Ketotestosterone, DHT 5α-dihydrotestosterone, DHEA Dehydroepiandrosterone, AN Androstenedione, A5 Androstenediol, P4 Progesterone, P5 Pregnenolone, 17α-OHP 17α-hydroxyprogesterone, 17α–OH–P5 17α-hydroxypregnenolone, E1 Estrone, 17β-E2 17β-estradiol, and 17α-E2 17α-estradiol.

recovery compared to absolute recovery was observed for cortisol and androstenedione; this may indicate that the selected ISs are not the optimal choice for compensating for extraction losses. Nonetheless, this did not affect the actual performance of the method. Overall, the HybridSPE technique demonstrated acceptable recoveries and precision for most target steroid hormones, which were in accordance with previous literature [24,59].

3.4. Application of method in actual seal plasma samples

The developed HybridSPE-UPC^2-MS/MS method was applied in grey seal pup plasma samples (Table 4). A total of 13 steroid hormones were determined with concentrations > mLOQ. Cortisol, corticosterone, cortisone, 11-deoxycortisol and progesterone were detected in all individuals with median concentrations of 15.2, 15.5, 3.50, 3.26 and 2.30 ng/mL, respectively. Testosterone, 17α-estradiol, 17β-estradiol and androstenediol were not quantified in grey seal pup plasma, which was expected since these target analytes are often present in the pg/mL concentration range [10,49,60].

Very few studies have been performed with multiple steroid hormones analysed in pinnipeds, and particularly in suckling and/or recently weaned pups as reported herein. Survelienė et al. [10] reported plasma concentrations of 17β-estradiol, testosterone and cortisol in suckling and recently weaned grey seals from Scotland analysed with immunoassays (0.02–0.39, 0.05–1.26, and 16.7–161 ng/mL, respectively). In the present study, concentrations of cortisol were considerably lower at 12.6–40.1 ng/mL, and both testosterone and 17β-estradiol were < mLOD (Table 4). The lower concentrations, particular of cortisol, agreed with previous literature, where chromatographic techniques consistently reported lower concentrations than immunoassays for the analysis of steroid hormones [61]. This may be attributed to cross reactivity in immunological based methods which can overestimate blood steroid concentrations; and this is extensively documented for cortisol [62–65]. Table 1 provides an overview of circulating steroid hormone concentrations in species of suckling and weaned seals, and juvenile and adult seals. In general, concentrations in juvenile and adult pinnipeds appear to be higher than reported in the present grey seal pups [10,27–33,66,67]. The observed differences are most likely due to developmental (age) and sex related to maturation of the animals, and the result of differences in the applied analytical techniques [33,66,67].

In light of the lower concentrations of testosterone and 17β-estradiol in the present pups as compared to those reported by Survilienė et al. [10] using immunoassay, the analysis of testosterone and 17β-estradiol was further cross-array compared with radioimmunoassay (RIA). The concentration of testosterone in grey seal plasma using RIA was consistent with our results for UPC^2-MS/MS analysis; in both techniques testosterone was not quantifiable in the samples. This was attributed to the analysis being carried out on sexually immature pups, where a low level of circulating testosterone is anticipated, as shown in the hair of immature Northern fur seals [67]. Immunological assays, such as RIA are however designed for human tissues and thus may not be calibrated for concentrations present in wildlife. Therefore, for a valid cross-array comparison, a standard material of human-based serum (Lyphochek®Immunoassay Plus Control) at three different fortification levels was analysed with both UPC^2-MS/MS and RIA. A significant correlation coefficient ($r = 0.93$; $p < 0.00003$) for testosterone suggests that UPC^2-MS/MS can be used for the measurement of testosterone in biological serum (Fig. 3). For testosterone, RIA demonstrated a higher degree of precision relative to UPC^2-MS/MS. However, further cross-array comparison should be performed with other steroid hormones and

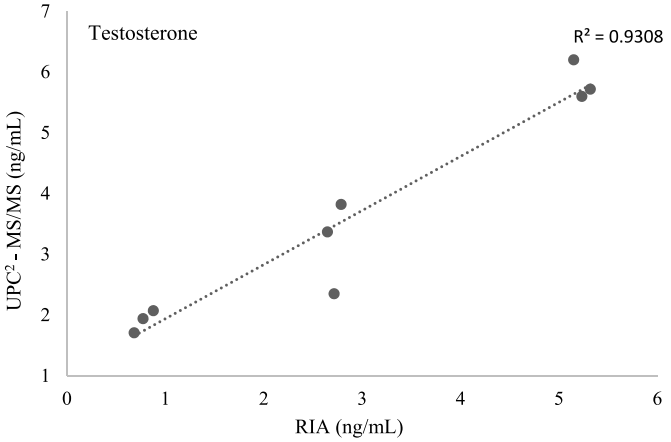

Fig. 3. Cross array comparison of testosterone concentration between UPC^2-MS/MS and RIA in standard control material, Lyphochek Immunoassay Plus Control Levels 1, 2 and 3 (BioRad, California, USA). The samples were run in triplicate (n = 3).

Table 4
Detection frequency (DR) and concentrations of steroid hormones in grey seal pup plasma (n = 9).

Target analytes	DR (%)	Geometric mean (ng/mL)	Median (ng/mL)	Min (ng/mL)	Max (ng/mL)
COR	100	17.6	15.2	12.6	40.1
CORNE	100	14.7	15.5	7.10	24.2
COS	100	3.73	3.50	0.74	10.7
11-deoxyCOR	100	2.95	3.26	1.06	5.72
P4	100	2.11	2.30	0.38	4.38
17α-OHP	100	0.74	0.67	<mLOQ	1.01
DOC	100	0.38	0.33	<mLOQ	0.94
AN	66.7	0.28	0.39	<mLOQ	0.65
E1	55.6	2.00	1.99	<mLOQ	4.65
P5	44.4	10.3	18.9	<mLOQ	29.5
17αOH-P5	22.2	3.87	4.14	1.34	5.59
DHEA	77.8	<mLOQ	<mLOQ	<mLOQ	7.35
ALDO	66.7	<mLOQ	<mLOQ	<mLOQ	2.44
TS	88.9	<mLOQ	<mLOQ	<mLOQ	<mLOQ
DHT	66.7	<mLOQ	<mLOQ	<mLOQ	<mLOQ
A5	n.d.	n.d.	n.d.	n.d.	n.d.
17β-E2	n.d.	n.d.	n.d.	n.d.	n.d.
17α-E2	n.d.	n.d.	n.d.	n.d.	n.d.
11-KetoTS	n.d.	n.d.	n.d.	n.d.	n.d.

<mLOQ: Concentration below the method limit of quantification. n.d.: not detected. Note: DR% calculated based on values > mLOD. COR Cortisol, COS Corticosterone, CORNE Cortisone, 11-deoxyCOR 11-deoxycortisol, ALDO Aldosterone, DOC 11-deoxycorticosterone, TS Testosterone, 11-KetoTS 11-Ketotestosterone, DHT 5α-dihydrotestosterone, DHEA Dehydroepiandrosterone, AN Androstenedione, A5 Androstenediol, P4 Progesterone, P5 Pregnenolone, 17α-OHP 17α-hydroxyprogesterone, 17αOH–P5 17α-hydroxypregnenolone, E1 Estrone, 17β-E2 17β-estradiol, and 17α-E2 17α-estradiol.

wildlife media to test both the robustness of the chromatographic method and the applicability of the immunoassay in wildlife. For 17β-estradiol, grey seal pup plasma was quantified using RIA with values ranging between 75.1 and 143 pg/mL (0.075–0.143 ng/mL), similar to previously reported values in suckling and weaned grey seal pups from Scotland [10].

4. Conclusions

This is the first study quantifying steroid hormones in seals using convergence chromatography. The method was applied to the analysis of blood plasma from grey seal pups (n = 9). Cortisol, cortisone, corticosterone and 11-deoxycortisol were detected in plasma samples at concentrations ranging from <mLOQ - 40.1 ng/mL. The HybridSPE-UPC^2-MS/MS method is reliable, less labour intensive and cost competitive compared to other common extraction protocols, while additionally allowing for the simultaneous extraction of various EDCs (e.g., PFAS and bisphenols) [25,46]. The developed Hybrid-SPE-UPC^2-MS/MS technique allows for further in-depth studies on the presence and role of various steroid hormones in wildlife.

Permissions

Permission to conduct the studies was provided by the Estonian authorities and was performed by a trained personal licence holder (Laboratory animal science certificate issued by Vilnius University (concordant to FELASA C category) No. 194 (2012-02-06)) and competent personnel. Sample collection on the Isle of May was performed by personal license holders/designated and competent personnel under UK Home Office license PPL 60/4009. This work received ethical approval from the University of St Andrews Animal Welfare and Ethics Committee (AWEC). Sampling was performed in compliance with the Animal (Scientific Procedures) Act (ASPA) 1986 and the EU directive on the protection of animals used for scientific purposes (2010/63/EU).

Credit author statement

Shannen Thora Lea Sait: Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Visualization, Writing – original draft. Vaida Survilienė: Funding acquisition, Investigation, Resources, Software, Writing – review & editing. Mart Jüssi: Funding acquisition, Resources, Writing – review & editing. Susana V. Gonzalez: Methodology, Resources, Writing – review & editing. Tomasz Maciej Ciesielski: Funding acquisition, Resources, Investigation, Supervision, Writing – review & editing. Bjørn Munro Jenssen: Funding acquisition, Resources, Investigation, Supervision, Writing – review & editing. Alexandros G. Asimakopoulos: Funding acquisition, Methodology, Investigation, Resources, Project administration, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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