Research in Veterinary Science 136 (2021) 343–350

Contents lists available at ScienceDirect

Research in Veterinary Science

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

Development and validation of a liquid chromatography coupled to mass spectrometer (LC-MS) method for the simultaneous quantification of estrone-3-sulfate, progesterone, estrone and estradiol in serum of mares and American bisons

Patrice Dufour [a,*,1], Justine Courtois [a], Ysaline Seynaeve [a], Stéphanie Peeters [a], Caroline Le Goff [a], Etienne Cavalier [a], Jérôme Ponthier [b]

[a] Department of Clinical Chemistry, University of Liège (ULiège), CHU (B35), 4000 Liège, Belgium
[b] Equine Reproduction, Equine Clinic Department, University of Liège (ULiège), 5D, AV de Cureghem, B41, Quartier Vallée 2, 4000 Liège, Belgium

ARTICLE INFO

Keywords:
LC-MS
Immunoassays
Estrogens
Progesterone
Mammals

ABSTRACT

Steroid concentrations in serum are fluctuating during pregnancy of many mammal species. The current knowledge about endocrinology of gestation is mainly based on immunoassays. However, the lack of specificity of these assays hampers the reliability of the results. In the present work, we developed and validated a methodology associating liquid chromatography (LC) and mass spectrometry (MS) to simultaneously quantify, with high specificity and accuracy, estrone-3-sulfate (E3S), progesterone (PRO), estrone (E1) and estradiol (E2) in serum of two different mammal species.

The sample preparation procedure is based on a simple protein precipitation and a derivatization with dansyl chloride. After the chromatographical separation, compounds were analyzed with a triple-quadrupole mass spectrometer operating in multiple reaction monitoring. Mare and American bison serum samples were analyzed with the validated method and results were compared with concentrations measured with commercial radioimmunoassay (RIA), enzyme linked immunosorbent assay (ELISA) and chemiluminescent microparticle immunoassay (CMIA).

Following these criterions: relative standard deviation <15% and relative bias <15%, lower limits of quantification of 0.5 ng/mL (E3S), 0.1 ng/mL (PRO) and 2 pg/mL (E1 and E2) were achieved. Most of the comparison between immunoassays and LC-MS showed poor correlation and proportional differences.

Our LC-MS method is able to simultaneously quantify several steroid hormones with high specificity, accuracy and sensitivity in serum of two different mammal species. Our method constitutes a useful and performant tool for veterinary clinicians and LC-MS should thus be used to update and refine the current knowledge about the endocrinology of pregnancy in mammals.

1. Introduction

Estrogens are a complex class of steroid hormones, characterized by an aromatic A ring and an alcohol group on the 3-carbon (Senger and Phillip, 1997). Mammals use 5 alpha-aromatase to transform androgens in estrogens: e.g. estrone (E1) is directly derived from androstenedione (Senger and Phillip, 1997) (1). In numerous mammals' species, the placenta produces different estrogens that can be conjugated with sulfate or glucuronate chains and that can be assayed for pregnancy diagnosis. Among mammals, human and equine are remarkable for their high estrogen production during pregnancy (Raeside, 2017) (2). In women, there is a cooperation between placenta, fetal adrenal glands and gonads to produce high levels of estrogens during the last trimester of pregnancy (Raeside, 2017). The equine allanto-chorion uses fetal androgens to produce conjugated estrogens and unusual estrogens with an unsatured B-ring: equilin and equilinin (Senger and Phillip, 1997; Raeside, 2017; Raeside et al., 2009). Placenta also produces estrogens in cows (Henricks et al., 1972), goat (Refsal et al., 1991), ewe (Esteva et al.,

* Corresponding author.
E-mail address: pdufour@chuliege.be (P. Dufour).
[1] Full postal address: CHU (B35) University of Liege, 1, avenue de l'Hôpital, 4000 Liège, Belgium

https://doi.org/10.1016/j.rvsc.2021.03.014
Received 17 October 2020; Received in revised form 9 March 2021; Accepted 16 March 2021
Available online 18 March 2021

1988) and sow (Almond and Dial, 1987). Estrogens are also known to rise at the end of bison (*Bison bison)* pregnancy (Vervaecke and Schwarzenberger, 2006; Kirkpatrick et al., 1992).

Several immunological tests as radioimmunoassay (RIA), chemiluminescent microparticle immunoassay (CMIA) or enzyme linked immunosorbent assay (ELISA) have been developed for different species to assay the different estrogens secreted by the feto-placental unit in serum, urine and feces. They aimed to assess gestation, to decrease false positive pregnancy diagnosis caused by pseudo-gestation in goat (Refsal et al., 1991) or to detect pregnancy pathologies in the mare (Canisso et al., 2017). Nevertheless, some authors used immunoassays not specifically dedicated to the studied species and not properly validated for the determination of hormones in this species' biological matrix (Satué et al., 2011; Shikichi et al., 2017). Consequently, concentrations assayed may vary according to the method used. As an example, estrone-3-sulfate (E3S) concentration assayed by RIA was around 40 ng/mL in the serum of 9-month pregnant mare (Satué et al., 2011), when an ELISA used by another team gave values between 600 and 800 ng/mL for the same period of gestation (Canisso et al., 2017; Esteller-Vico et al., 2017). Concentrations measured with immunoassays, especially when the kit used was not specifically dedicated to the studied animal species, could thus be unreliable. Moreover, ELISA and RIA require different kits for each hormone and each species, thus increasing costs and consuming time in the commercial laboratories.

On the other hand, liquid chromatography coupled to mass spectrometry (LC-MS) is able to measure several steroids concentration. The LC-MS is an expensive instrument, but it uses rather inexpensive consumables (mainly solvents) and can perform simultaneously different steroids assays for many species. This multi-hormones method could help to understand endocrinology of estrogens and other steroids during pregnancy in several mammals. It may also offers opportunities for late gestational pathologies diagnosis.

The first objective of this study was to develop and validate a LC-MS method able to quantify simultaneously several steroid hormones (i.e. E3S, progesterone (PRO), E1 and estradiol (E2)) in sera of different mammal species. We tested the analytical methodology on bison and equine serum samples. The second objective was to compare the steroid concentrations measured with our LC-MS method in bison and mare samples and those determined with some commercial RIA, CMIA or ELISA assays in order to highlight potential bias induced by the use of immunoassays. This method agreement analysis was performed following the recommendation of Watson and Petrie (Watson and Petrie, 2010).

2. Materials and methods

2.1. Animals

In February 2019 and 2020, blood was sampled in 36 mares from two different stud-farms, one breeding Spanish pure breed horses and the other saddle breed horses. Insemination or breeding days, subsequent foaling day and observed adverse events like prematurity, dysmaturity, or septicemia were recorded. Sampled mares were thought to be over 7 months of pregnancy, except for 3 of them that were non-pregnant and used as negative control. Blood was sampled in jugular vein and directly centrifuged (1000 ×*g*, 10 min), then serum was stored frozen (−80 °C) until assays.

Between January and February 2019, and between February and March 2020, two ranches extensively breeding imported American Bison (*Bison bison*) in Belgium were gathering and immobilizing their herds in a mobile stock for mandatory diseases screening. An increased volume of blood was collected in dry-tubes in the vein under the tail. Blood samples were centrifuged (1000 x*g*) and stored frozen (−80 °C) until assays and subsequent calving day was recorded.

2.2. Chemicals and reagents

Powder of E3S sodium salt (Sigma-Aldrich, St. Louis, MO, USA) dissolved in methanol, solutions of 17β-estradiol (LGC Standards, Luckenwalde, Germany), estrone and progesterone (Cerilliant, Round Rock, TX, USA) were used as reference standard. D_4-estrone-3-sulfate sodium salt (Sigma-Aldrich, St. Louis, MO, USA) dissolved in methanol (stock solution), solutions of $^{13}C_3$-estrone, d_5-17β-estradiol and d_9-progesterone (Cerilliant, Round Rock, TX, USA) were used as an internal standard. Water, acetonitrile and methanol (LC-MS grade) were purchased from Biosolve (Biosolve, Dieuze, France). Ammonia solution (25% in water) (Merck KGaA, Darmstadt, Germany) was Suprapur® grade and was purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA), dansyl chloride and carbonate-bicarbonate buffer (0.05 M, pH = 10.5) used for the derivatization were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA).

2.3. Radioimmunoassays (RIA) for E3S and E1 in mares

The commercial RIA method RIA-2957 (DRG Instruments GmbH, Marburg, Germany) was used to quantify E3S in mares' samples. This kit follows the principle of competitive binding assay: the E3S present in the sample competes with a fixed amount of ^{125}I-labeled E3S for a fixed number of antibody binding sites. The mixture of 100 μL of sample, calibrator or quality control, 500 μL of tracer and 100 μL of antiserum was incubated during 3 h at room temperature on a shaker (180 rpm). Precipitating reagent was then added, tubes were centrifugated for 15 min at 1500 ×g and then washed. The amount of E3S was determined by measuring the radioactivity remaining in the tube. According to the manufacturer's information, the lower limit of quantification (LLOQ) of the kit was set at 0.01 ng/mL. Note that this kit is not specifically dedicated to equine samples.

The commercial RIA method Estrone-RIA-CT KIPI9100 (DIAsource ImmunoAssays, Louvain-la-Neuve, Belgium) was used to quantify E1 in mares' samples. This kit also follows the principle of competitive binding assay. One hundred μL of sample were transferred to tube coated with anti-E1 polyclonal antibodies and were shaken during 2 h at ambient temperature after the addition of 400 μL of a solution of ^{125}I-labeled E1 tracer. After incubation, supernatant was aspirated and the tube was washed twice. Finally, radioactivity was measured to determine the amount of E1 in the sample. The LLOQ was set by the manufacturer at 15 pg/mL. Once again, this kit is not specifically dedicated to equine serum.

2.4. Enzyme-linked immunosorbent assay (ELISA) for E3S in mares

The commercial ELISA method EIA-5223 (DRG Instruments Gmbh, Marburg, Germany) is specifically dedicated to equine serum and was used for mare samples. The kit is based on the principle of competition taking place on a microplate coated with a polyclonal antibody specific for E3S. Mixture of sample (20 μL) and biotin-labeled E3S was incubated during one hour on the microplate. Then, horseradish peroxidase-labeled streptavidine was added and the binding between the streptavidine and the biotinylated E3S took place during a 30 min incubation. Afterwards, the chromogenic substrate (3,3′,5,5'-Tetra-Methyl-Benzidine) was added and the enzymatic reaction was stopped after 30 min by the addition of hydrochloric acid. The optical density of the color solution was measured at 450 nm. The LLOQ was set by the manufacturer at 0.14 ng/mL.

2.5. Chemiluminescent microparticle immunoassay (CMIA) for PRO in bison

PRO was determined in serum samples collected in bison by using the Alinity I Progesterone CMIA kit from Abbott (Alinity I Progesterone

Reagent Kit 08P36). Briefly, PRO present in the sample competes with the anti-fluorescein antibody/fluorescein progesterone complex coated on paramagnetic microparticles for binding to anti-progesterone acridinium-labeled antibody. After washing and chemiluminescent reaction, the intensity of the light measured is inversely proportional to progesterone concentration in the sample. According to the manufacturer's information, the LLOQ of the kit was set at 0.5 ng/mL. This assay is not specifically dedicated to bison serum.

2.6. Sample preparation before LC-MS analysis

One hundred µL of calibrator, animal's serum sample or quality control were spiked with 10 µL of internal standard mixture (d_4-E3S at 500 ng/mL, d_9-PRO at 100 ng/mL. $^{13}C_3$-E1 and d_5-E2 at 2 ng/mL). Sample was then mixed with 390 µL of acetonitrile and vortexed for 20s. The mixture was centrifuged at 16100 ×*g* during 10 min at 4 °C. A volume of 300 µL of supernatant was then collected, transferred to a 2 mL Eppendorf vial and evaporated to dryness under vacuum at 40 °C. Then, 20 µL of bicarbonate/carbonate buffer and 30 µL of a solution of dansyl chloride 1 mg/mL in acetonitrile were added to the dried extracts to derivatize E1 and E2. Sample was incubated in a ThermoMixer (Eppendorf) at 60 °C and 1000 rpm during 10 min. The derivatization reaction was stopped by the addition of 50 µL of water. The mixture was then transferred to LC vials and injected (20 µL) into the LC-MS system.

2.7. Liquid chromatography and electrospray tandem mass spectrometry analysis

The chromatographical separation took place on a Shimadzu Nexera X2 LC-30 CE (Shimadzu Co., Kyoto, Japan) equipped with a BEH C18 column (2.1 mm × 100 mm, 1.7 µm particle size) (Acquity UPLC, Waters). Mobile phase A was 0.02% NH_4OH in water and mobile phase B was acetonitrile. The flow rate was 0.4 mL/min and the separation was achieved by using a gradient mode. The initial mobile phase composition was 20% of B and was held for 0.1 min. Between 0.1 min and 7 min the percentage of B linearly grew to 95%, proportion held for 3 min. Between each sample, column was allowed to equilibrate with the initial condition of gradient for 1 min. The HPLC system was connected to a linear combination of triple quadrupole and OrbiTrap mass analyzer, QTrap 6500 (ABSciex, Framingham, Massachussetts, USA) operating in triple-quadrupole mode. The resolution of quadrupoles Q1 and Q3 was set to unit. An ESI source operating in negative ionization mode during 3 min and then positive mode during 6 min was employed, the ion spray voltage was -4500 V (negative mode) or 5500 V (positive mode), gases 1, 2 and curtain gas were 30, 70 and 30, respectively and the ion source temperature was 650 °C. Table 1 gathered MRM transitions, declustering potentials, entrance potentials, collision energies and exit potentials for each analyte (quantifiers and qualifiers). Analyst 1.6.2 was used for data acquisition and processing.

2.8. LC-MS method validation and comparison with CMIA, RIA and ELISA

During the validation, we followed the guidelines provided by the United States Food and Drug Administration (FDA) (Food and Drug Administration, 2001). Calibration curves consisted in BSA solution (0.1% in phosphate buffer pH = 7.2) spiked with standard reference solutions to obtain eight calibration points (ranges: 0.25–500 ng/mL for E3S; 0.05–100 ng/mL for PRO; 1–2000 pg/mL for E1 and E2). Integrated peak area ratio between native hormones and marked internal standard was used to determine response. Validation standards were prepared in BSA solution, five levels were prepared with solutions of standards different from those used for the preparation of the calibration standards. Validation standards were analyzed in quintuplicate to assess intra-day precision and accuracy and then in triplicate during 3 days to assess inter-day precision and accuracy. Precision refers to the relative standard deviation (RSD = Standard deviation × 100/Mean value) and accuracy to relative bias (RB = (Mean of observed concentrations – Spiked concentration) × 100/Spiked concentration). LLOQs of the method were defined as the lowest concentrations in the validation standards that reported RSD and RB lower than 15% while the upper limits of quantification (ULOQ) were defined as the highest concentrations in the validation standards that reported RSD and RB lower than 15%.

Recoveries were estimated by analyzing three serum samples of bison and two serum samples of mare (samples were selected in order to have endogenous concentration of hormones near to or below the LLOQ) spiked with low and high levels of hormones before extraction (concentrations were reported in Table 3). Recoveries were calculated as the ratio between the response measured with spiked sample after extraction and the response obtained with the direct injection of the same amount of analytes into the LC-MS analyzer.

Finally, 36 mare's serum and 92 bison's samples were analyzed by LC-MS. Thirty-four mare's samples were also analyzed by ELISA and RIA to quantify E3S, and by RIA to quantify E1. Thirty bison's serum samples were analyzed by CMIA to determine PRO concentrations. Concentrations measured with the different methods were compared.

2.9. Statistical analyses

Statistical analyses were performed using RStudio (version 3.4.1; R Project for Statistical Computing). Passing-Bablok regressions and difference plots were computing using the package *mcr* while the concordance correlation coefficients (CCC) were computed using the package *DescTool*. For the interpretation of the CCC results, the following thresholds were used to characterize the quality of the correlation: poor (≤ 0.90), moderate (0.90–0.95), substantial (≤ 0.95–0.99) and almost perfect (> 0.99) (Cavalier et al., 2017; Watson and Petrie, 2010).

Table 1
MRM transitions, declustering potentials, entrance potentials, collision energies, exit potentials for analytes and internal standards (quantifiers and qualifiers).

Compounds	Q1 mass (*m*/z)	Q3 mass (m/z)	Declustering potentials (volts)	Entrance potentials (volts)	Collision energies (volts)	Exit potentials (volts)
Estrone-3-sulfate (quantifier)	349.2	269.25	-90	−7	−40	−13
Estrone-3-sulfate (qualifier)	349.2	145.1	−75	−5	−70	−10
d_4-Estrone-3-sulfate (IS)	353.2	273.2	−70	−7	−40	−13
Progesterone (quantifier)	315.2	97	80	10	27	6
Progesterone (qualifier)	315.2	109.1	80	10	29	18
d_9-Progesterone (IS)	324.2	100.2	80	10	31	44
Estrone (quantifier)	504.1	171	166	10	43	10
Estrone (qualifier)	504.1	115	166	10	115	10
$^{13}C_3$-Estrone (IS)	507	171	166	10	43	14
Estradiol (quantifier)	506.1	171	151	10	43	12
Estradiol (qualifier)	506.1	115	151	10	115	12
d_5-Estradiol (IS)	511.1	171	151	10	43	14

3. Results

3.1. LC-MS method validation

Selectivity of the current method was confirmed during the analysis of the routine samples. Calibration curves were calculated using 1/x-weighted quadratic regression and during all the validation process, the correlation coefficients were $r^2 \geq 0.99$. We prepared calibration standard in BSA solution because of the impossibility to obtain adequate volume of free-steroid mare's or bison's sera. Nevertheless, we prepared calibration curves in residual volume of serum from a mare and from a bison with hormones levels near or below the LLOQ, in both matrix: the correlation coefficients were $r^2 \geq 0.99$ and the curve were well correlated with curves prepared in BSA solution.

Relative standard deviation and RB are gathered in Table 2. For E3S, intra- and inter-day precision and accuracy ranged from 1.5% to 10.6% and from −9.8% to 9.6%, respectively, for PRO, from 1.2% to 5.8% (precision) and from −6.0% to 3.4% (accuracy), for E1 from 2.2% to 9.3% (precision) and from −11.7% to 6.0% (accuracy), for E2 from 2.6% to 14.2% (precision) and from −5.4% to 0.1% (accuracy). Consequently, the LLOQ were established at 0.5 ng/mL, 0.1 ng/mL, 2 pg/mL and 2 pg/mL for E3S, PRO, E1 and E2, respectively.

Extraction yields were reported in Table 3. Recovery ranged from 46.2% to 71.7% for E3S, from 50.8% to 92.0% for PRO, from 27.2% to 72.3% for E1 and from 21.7% to 71.5% for E2. The extraction yields observed for E1 and E2 were more variable probably because of an additional variability in the derivatization yield.

3.2. Application on real animal samples and comparison between LC-MS, ELISA, CMIA and RIA methods

Concentrations of steroid hormones measured in mare and bison samples were gathered in Table 4. Two abortions were observed in pregnant mares: no foaling was observed in the subsequent year, despite high E3S, E1, E2 concentrations. Samples of bisons used in this study were coming from a larger scale upcoming study and some of the sera were coming from bison cows with an unknown pregnancy status.

Passing-Bablok correlation plot between LC-MS and RIA methods and the Bland-Altman difference plot for the determination of E3S are presented in Fig. 1. The relation equation computed by Passing-Bablok regression was $Y_{RIA} = 21.3$ (95% CI: −0.51–54.1) + 0.96 (95% CI: 0.68–1.42) $X_{LC\text{-}MS}$. The mean difference between measurements obtained by LC-MS and RIA was not significant: −25.1 ng/mL (95% CI: −166.2–216.4). Nevertheless, relation between RIA and LC-MS showed important random errors and consequently, a CCC lowered ($\rho = 0.89$).

On the other hand, the relation between LC-MS and ELISA methods for the determination of E3S was marked by an important proportional difference, while the systematic bias was not significantly different from zero. The relation computed by Passing-Bablok regression is presented in Fig. 1 and the equation was $Y_{ELISA} = 1.93$ (95% CI: −52.4–29.3) + 2.44 (95% CI: 1.86–3.32) $X_{LC\text{-}MS}$. Consequently, the Bland-Altman difference plot (Fig. 2) presented a more important (but not significant) mean relative difference: 173.1 ng/mL (95% CI: −152.7–498.9). Despite the ELISA kit was specifically dedicated to equine samples, the relation between LC-MS and ELISA showed a poor correlation ($\rho = 0.68$). To explore the origins of this proportional difference, we measured the E3S concentration in the calibrators of the ELISA kit by using the LC-MS. The level measured in each ELISA calibrator was roughly 60% the expected concentration.

As illustrated by the Passing Bablok plot (Fig. 2), the correlation between PRO concentrations measured by LC-MS and those obtained with CMIA was far better ($\rho = 0.97$) and was considered as substantial. Nevertheless, the relation between LC-MS and CMIA showed a slight but significant proportional difference: $Y_{CMIA} = 0.089$ (95% CI: 0.049–0.32) + 1.18 (95% CI: 1.09–1.23) $X_{LC\text{-}MS}$. On the other hand, difference plot (Fig. 2) shows no systematic bias (mean relative difference: 0.9 ng/mL (95% CI: −1.16–2.97)).

Finally, we compared LC-MS and RIA for the quantification of E1. We observed (Fig. 3) an important proportional difference with E1 concentrations measured by RIA, twice higher than those determined by LC-MS: $Y_{RIA} = -38.4$ (95% CI: −140.6–20.3) + 2.28 (95% CI: 1.83–2.95) $X_{LC\text{-}MS}$. No systematic bias was highlighted in the difference plot (Fig. 3), mean relative difference: 156.9 pg/mL (95% CI: −109.2–423.1). The CCC computed ($\rho = 0.59$) also illustrated a poor correlation.

4. Discussion

Among mammals, the endocrinology of the pregnancy in horse is one of the best studied and probably one of the most complex (Senger and Phillip, 1997; Conley and Ball, 2019). However, steroid concentrations have historically been measured mostly through immunoassays. Moreover, end of pregnancy pathologies have been associated with changes in the placental steroids production: Shikichi et al. (Shikichi et al., 2017) and Canisso et al. (Canisso et al., 2017) both tried to diagnose placentitis or other late gestational pathologies on the basis of steroids assayed by immunoassays. A major drawback of immunoassays is the potential interferences from cross-reacting steroids: steroids all share the common core of four-membered hydrocarbon rings and consequently present a high similarity in chemical structure (Senger and Phillip, 1997; Conley and Ball, 2019). This lack of specificity is potentially linked to a reduction of the accuracy and consequently to a reduction of the reliability of the concentrations measured by immunoassay. This situation

Table 2
Intra-day and inter-day accuracy and precision of estrone 3-sulfate, progesterone, estrone and estradiol. These parameters were determined with validation standards prepared in BSA solution. Validation standards were analyzed in quintuplicate at day 1 (intra-day variation) and then in triplicate during 3 days (inter-day variation). Precision refers to the relative standard deviation (RSD = Standard deviation × 100/Mean value) and accuracy to relative bias (RB = (Mean of observed concentrations – Spiked concentration) × 100/Spiked concentration).

	E3S			PRO			E1			E2		
	Target (ng/mL)	RSD	RB	Target (ng/mL)	RSD	RB	Target (pg/mL)	RSD	RB	Target (pg/mL)	RSD	RB
Intra-day												
Level 1	0.5	10.6%	−9.8%	0.1	5.8%	−0.7%	2	5.5%	6.0%	2	6.4%	−2.4%
Level 2	1.25	2.7%	−6.4%	0.25	3.1%	−6.0%	5	3.1%	−11.7%	5	7.1%	0.1%
Level 3	2.5	4.3%	8.3%	0.5	2.2%	3.4%	10	2.2%	1.5%	10	4.3%	−1.2%
Level 4	375	1.5%	−1.7%	75	4.7%	1.6%	1500	3.8%	−2.6%	1500	7.7%	−0.4%
Level 5	500	1.6%	−1.9%	100	1.2%	−0.3%	2000	2.4%	−8.1%	2000	2.6%	−5.4%
Inter-day												
Level 1	0.5	7.4%	−6.8%	0.1	3.9%	1.3%	2	9.3%	5.6%	2	14.2%	−5.0%
Level 2	1.25	6.6%	1.5%	0.25	4.0%	−1.1%	5	8.4%	−3.1%	5	6.8%	−1.1%
Level 3	2.5	3.3%	9.6%	0.5	2.8%	2.3%	10	3.9%	−1.7%	10	10.2%	−5.2%
Level 4	375	3.8%	−1.7%	75	3.4%	0.3%	1500	6.2%	−2.9%	1500	10.9%	−1.9%
Level 5	500	3.3%	−0.3%	100	2.8%	−2.2%	2000	4.4%	−7.0%	2000	9.2%	−3.6%

Table 3
Extraction yields of estrone 3-sulfate, progesterone, estrone and estradiol, calculated as the ratio between the response measured with spiked sample after extraction and the response obtained with the direct injection of the same amount of analytes into the LC-MS analyzer. Each sample was spiked with low and high levels of hormones before extraction.

	E3S		PRO		E1		E2	
Sample	Low level (2.5 ng/mL)	High level (375 ng/mL)	Low level (0.5 ng/mL)	High level (75 ng/mL)	Low level (10 ng/mL)	High level (1500 ng/mL)	Low level (10 ng/mL)	High level (1500 ng/mL)
Bison 1	68.6%	56.4%	65.4%	67.7%	27.2%	69.0%	49.9%	70.0%
Bison 2	57.2%	51.9%	98.0%	62.2%	36.4%	63.1%	24.3%	61.5%
Bison 3	65.9%	56.0%	ND	64.3%	61.6%	72.3%	44.7%	71.5%
Jument 1	71.7%	50.2%	ND	59.3%	39.6%	27.6%	69.4%	25.5%
Jument 2	55.8%	46.2%	92.0%	50.8%	37.3%	47.5%	21.7%	45.7%

Table 4
E3S, E1 and E2 levels in serum of pregnant and not pregnant mares. E3S, PRO and E1 levels in serum of male bisons, pregnant female bisons, not pregnant female bisons and female bisons with unknown status (pregnant, not pregnant, cycling or in anoestrus).

		E3S (ng/mL)		PRO (ng/mL)		E1 (pg/mL)		E2 (pg/mL)	
	n	Median	Range	Median	Range	Median	Range	Median	Range
Mares									
Not pregnant	5	<LOQ	<LOQ - 2.51	<LOQ	<LOQ – 7.1	<LOQ	<LOQ - < LOQ	<LOQ	<LOQ - < LOQ
Pregnant	31	108.9	29.0–683.7	0.11	<LOQ – 0.68	154.4	40.9–776.1	11.8	3.6–31.2
Bisons									
Male	6	<LOQ	<LOQ - < LOQ	1.3	0.2–2.5	<LOQ	<LOQ - < LOQ	<LOQ	<LOQ - < LOQ
Pregnant female	40	3.5	<LOQ - 13.6	8.1	0.59–21.9	22.5	<LOQ - 71.5	3.11	<LOQ – 147.8
Not pregnant female	22	<LOQ	<LOQ - 3.8	0.8	<LOQ - 15.2	<LOQ	<LOQ - 26.3	<LOQ	<LOQ – 19.3
Unknown status female	24	<LOQ	<LOQ - 18.5	1.6	<LOQ - 16.9	<LOQ	<LOQ - 110.5	<LOQ	<LOQ – 12.5

is particularly critical in animal samples: despite kit manufacturers assessing the percentage of cross-reactivity for many steroids (Krasowski et al., 2014; Wudy et al., 2018), few immunoassays are specifically dedicated to animal samples and these samples may contain known or even unknown steroids, that are not relevant for humans. E.g., only one of the four kits assessed in the present work was dedicated to equine samples, and the human kits used were not tested for cross-reactivity with equilin and equilinin that could potentially interfere in the immunoassay (Senger and Phillip, 1997). Moreover, the levels of interfering compounds may be very different in animal serum compared to those measured in human serum. Ideally, immunoassays dedicated to one species should be revalidated before being used for the determination of hormones in another animal species, which is not always the case. For instance, Shikichi et al. (Shikichi et al., 2017) used immunoassays with anti-progesterone and anti-17β-estradiol antibodies: given the lack of specificity of the antibodies, they could not exclude cross reactivity with other estrogens and progestins, whereas progesterone secretion by placenta is low at this period of pregnancy (Senger and Phillip, 1997). They consequently reported their results as progestins and estrogens concentrations. This example shows that the current knowledge about endocrinology of the pregnancy in horse is potentially biased by this lack of specificity and accuracy and it should be updated and refined by the use of a more specific and accurate technology (Senger and Phillip, 1997; Krasowski et al., 2014; Raeside, 2017; Wudy et al., 2018). As demonstrated by the analysis of our mare's samples, our LC-MS method allows the quantification of E3S, E1, E2 and PRO in mares' late pregnancy. Moreover, our methodology showed good accuracy and precision during the validation process and could thus highlight subtle changes in steroid concentration associated with some pathologic conditions at the end of the pregnancy. It could thus be used to explore more in depth the findings of Shikichi et al. (2017) and subsequently, could be routinely used to diagnose placentitis and other late gestational pathologies instead of immunoassays.

Some other authors developed methodologies associating chromatography and mass spectrometry to quantify simultaneously several steroid hormones in equine serum with high specificity and good accuracy. Legacki et al. proposed two LC-MS methods to quantify several hormones in mare's serum (Legacki et al., 2016; Legacki et al., 2019). The methods of Legacki et al. are interesting tools for the veterinary clinician/researcher, but our methodology provides additional and complementary perspectives. Legacki et al. (Legacki et al., 2016) proposed a method to quantify E1 with a LLOQ of 500 pg/mL. Due to the derivatization step, we were able to increase the sensitivity by a factor 250, and we also obtained a good sensitivity for E2. Consequently, we were able to quantify E1 and E2 in our pregnant mare's samples which would not have been possible with the method of Legacki et al. (Legacki et al., 2016; Legacki et al., 2019). Our method requires lower volume of serum than the LC-MS method of Legacki et al. (100 µL vs 500 µL or 1000 µL) which offers the perspective of increasing the sensitivity by analyzing higher volume of serum. Surprisingly, Legacki et al. measured in equine late pregnancy E3S levels as high as 60,000 ng/mL, while we observed in our mares (also in the late pregnancy) a maximum concentration of 683.7 ng/mL. This important discrepancy could have been due to limited sensibility of previous measuring methods, and should be further investigated in the future.

American bison's pregnancy endocrinology has been less studied than equine. To the best of our knowledge, only few studies assessed steroid concentration in serum, urine or feces. In these studies, authors followed the fluctuation of steroid concentrations during the pregnancy but had also difficulties to identify specific compounds and they reported their results as "progestins" or "estrone conjugates" levels (Goodrowe et al., 2007; Kirkpatrick et al., 1992). Our LC-MS methodology could help to identify the steroid hormones fluctuating during pregnancy in bison. Our preliminary results showed that our method is able to discriminate pregnant females from non-pregnant females and males according to the levels of E3S, PRO, E1 and E2. In the future, this LC-MS method should be used to follow the variations of steroid concentration during normal or pathologic pregnancies in order to update the current data.

We compared concentrations measured with LC-MS and those determined by immunoassays. Our results showed a poor correlation between LC-MS and immunoassays (CCC ρ ranging from 0.59 to 0.89 except for PRO quantified by CMIA: $\rho = 0.97$). Moreover, relations between E1 quantified by RIA and E3S determined with ELISA and the

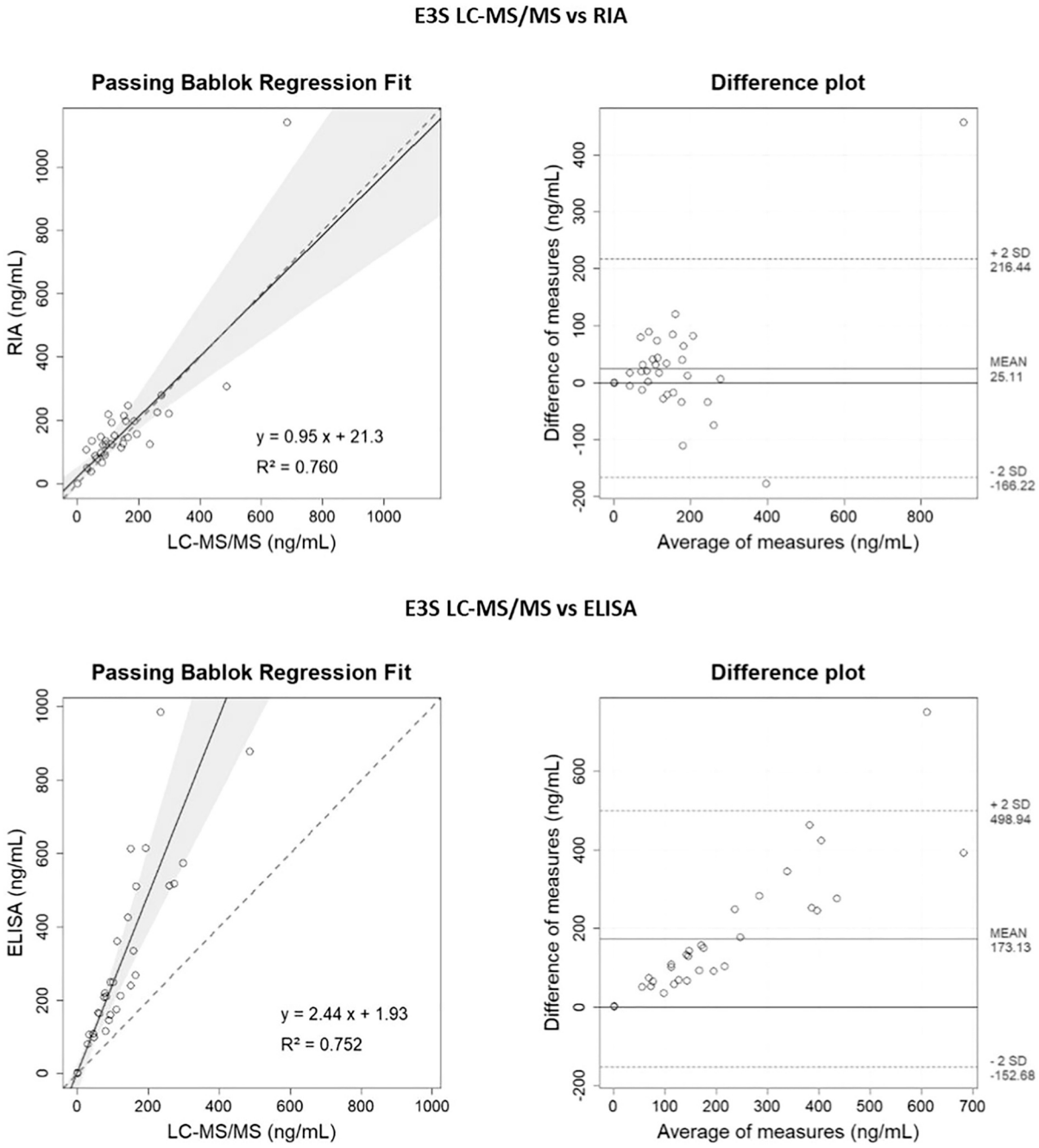

Fig. 1. Comparison (Passing Bablok regression and Different plots) of E3S concentrations measured by LC-MS/MS versus RIA or ELISA.

results obtained with LC-MS were marked by an important (more than a factor 2) proportional difference. We also observed a moderate (+20%) proportional difference in the relation between LC-MS and CMIA for the quantification of PRO. Our results highlight the potential bias induced by the use of immunoassays not specifically dedicated and validated for the studied species. The lack of specificity of immunoassays, due, as discussed above, to the presence of interfering compounds may explain the higher levels measured with immunoassays (for instance, according to the E3S kit manufacturers, cross reactivity with estrone is 7.8% and 4.9% for ELISA and RIA assays, respectively). This could also be worsened by the presence of additional steroids specific to the animal in the serum. Moreover, we showed that the calibrators of the E3S ELISA kit are biased which partly explains the proportional difference observed and casts doubt on the results obtained with kit specifically developed for the quantification of steroid hormones in equine serum. Finally, for the determination of E3S with RIA, most of the samples were above the ULOQ and we consequently had to dilute samples which added an additional source of variability. We cannot exclude the existence of isobaric coeluting steroid compounds in the sample which would interfere with the quantification of hormones by LC-MS, but the probability of presence of interfering compounds is drastically reduced by the sample preparation, the chromatographical separation and the analysis of two MRM transitions for each compound. Consequently, our results show that LC-MS is a more reliable method for measurement of steroid concentrations in American bison (Krasowski et al., 2014; Wudy et al., 2018).

Moreover, the sample preparation is simple and requires a low volume of serum. We applied our method on mares and American bisons samples, but, we assume that the method could be easily transferred to others mammals species and some other steroid compounds could be

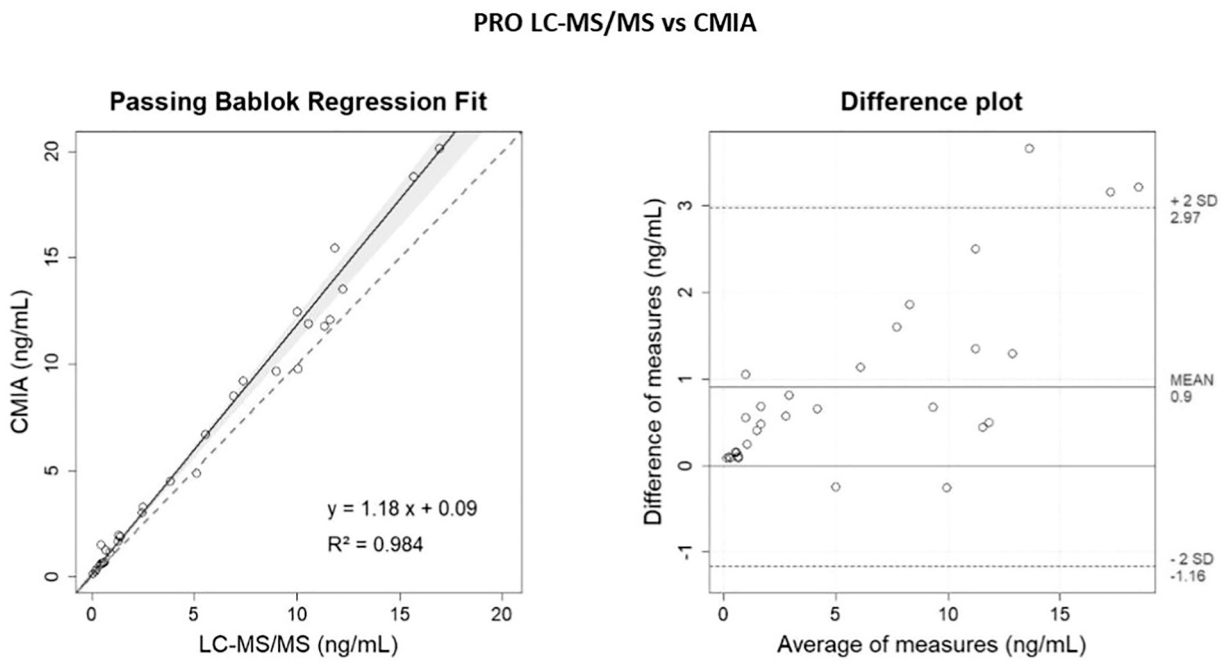

Fig. 2. Comparison (Passing Bablok regression and Different plots) of PRO concentrations measured by LC-MS/MS versus CMIA.

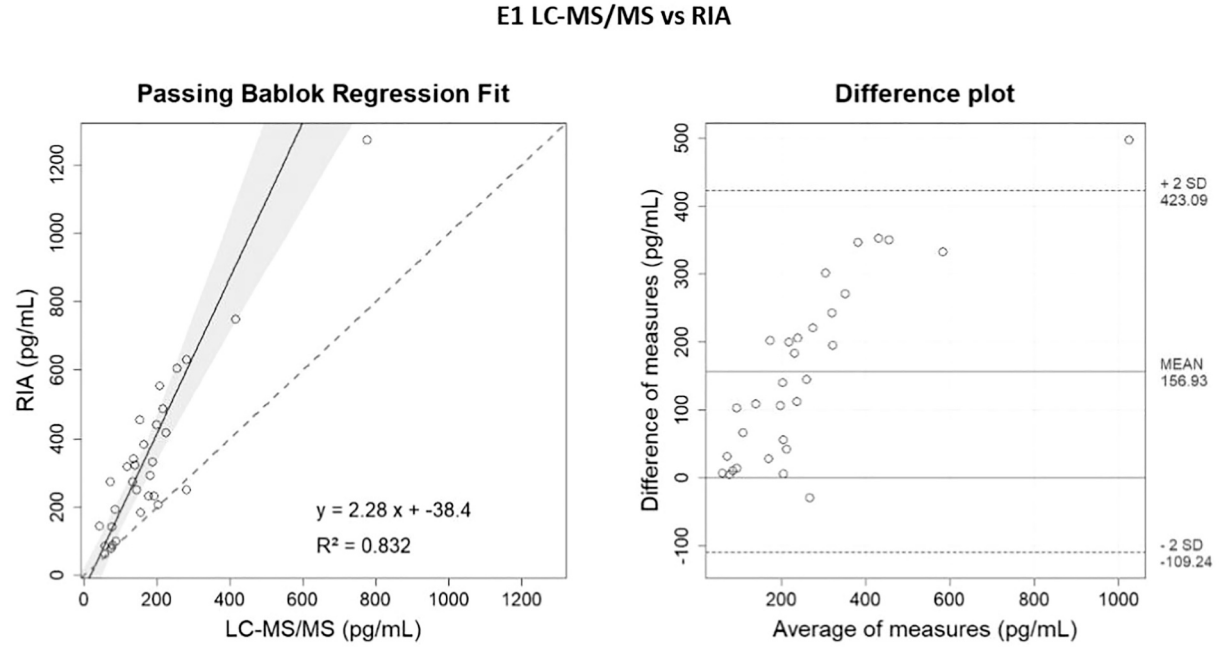

Fig. 3. Comparison (Passing Bablok regression and Different plots) of E1 concentrations measured by LC-MS/MS versus RIA.

added to the present method if necessary. However additional investigations are necessary to confirm these hypothesis.

5. Conclusion

We developed and validated a LC-MS method to quantify multiple steroid hormones (e.i. E3S, PRO, E1 and E2) in animals' serum. We demonstrated the good accuracy, precision and specificity of our method during the validation process and by analyzing mare and bison samples. Although requiring low volume of serum, good sensitivity was achieved. LC-MS apparatus allows to quantify simultaneously several compounds which reduce the time and the materials required for the assay of multiple hormones concentrations. LC-MS could also be easily transferred to other animals' species. LC-MS is associated with high specificity which contributes to enhance the reliability of the concentrations measured. Consequently, this LC-MS method is a valid tool for veterinary clinicians and researchers to explore pregnancy or endocrine pathologies in mammals.

Declaration of Competing Interest

None.

References

Almond, G.W., Dial, G.D., 1987. Pregnancy diagnosis in swine: principles, applications, and accuracy of available techniques. J. Am. Vet. Med. Assoc. 191, 858–870.

Canisso, I.F., Ball, B.A., Esteller-Vico, A., Williams, N.M., Squires, E.L., Troedsson, M.H., 2017. Changes in maternal androgens and oestrogens in mares with experimentally-induced ascending placentitis. Equine Vet. J. 49, 244–249. https://doi.org/10.1111/evj.12556.

Cavalier, E., Lukas, P., Bekaert, A.-C., Carlisi, A., Le Goff, C., Delanaye, P., Souberbielle, J.-C., 2017. Analytical and clinical validation of the new abbot architect 25(OH)D assay: fit for purpose? Clin. Chem. Lab. Med. 55, 378–384. https://doi.org/10.1515/cclm-2016-0566.

Conley, A.J., Ball, B.A., 2019. Steroids in the establishment and maintenance of pregnancy and at parturition in the mare. Reproduction 158, R197–R208. https://doi.org/10.1530/REP-19-0179.

Esteller-Vico, A., Ball, B.A., Troedsson, M.H.T., Squires, E.L., 2017. Endocrine changes, fetal growth, and uterine artery hemodynamics after chronic estrogen suppression during the last trimester of equine pregnancy. Biol. Reprod. 96, 414–423. https://doi.org/10.1095/biolreprod.116.140533.

Esteva, M., Mañes, S., del Palacio, M.A., López-Sebastián, A., 1988. Effect of ovariectomy on plasma levels of progesterone, free and conjugated estrone in pregnant sheep. Rev. Esp. Fisiol. 44, 197–203.

Goodrowe, K.L., Mastromonaco, G.F., Othen, L.S., 2007. CHAPTER 137 - reproductive patterns in female Bison (*Bison bison sp.*). In: Youngquist, R.S., Threlfall, W.R. (Eds.), Current Therapy in Large Animal Theriogenology (Second Edition). W.B. Saunders, Saint Louis, pp. 1000–1004. https://doi.org/10.1016/B978-072169323-1.50140-9.

Henricks, D.M., Dickey, J.F., Hill, J.R., Johnston, W.E., 1972. Plasma estrogen and progesterone levels after mating, and during late pregnancy and postpartum in cows. Endocrinology 90, 1336–1342. https://doi.org/10.1210/endo-90-5-1336.

Kirkpatrick, J.F., Bancroft, K., Kincy, V., 1992. Pregnancy and ovulation detection in bison (Bison bison) assessed by means of urinary and fecal steroids. J. Wildl. Dis. 28, 590–597. https://doi.org/10.7589/0090-3558-28.4.590.

Krasowski, M.D., Drees, D., Morris, C.S., Maakestad, J., Blau, J.L., Ekins, S., 2014. Cross-reactivity of steroid hormone immunoassays: clinical significance and two-dimensional molecular similarity prediction. BMC Clin. Pathol. 14, 33. https://doi.org/10.1186/1472-6890-14-33.

Legacki, E.L., Scholtz, E.L., Ball, B.A., Stanley, S.D., Berger, T., Conley, A.J., 2016. The dynamic steroid landscape of equine pregnancy mapped by mass spectrometry. Reproduction 151, 421–430. https://doi.org/10.1530/REP-15-0547.

Legacki, E.L., Scholtz, E.L., Ball, B.A., Esteller-Vico, A., Stanley, S.D., Conley, A.J., 2019. Concentrations of sulphated estrone, estradiol and dehydroepiandrosterone measured by mass spectrometry in pregnant mares. Equine Vet. J. 51, 802–808. https://doi.org/10.1111/evj.13109.

Raeside, J.I., 2017. A brief account of the discovery of the fetal/placental unit for estrogen production in equine and human pregnancies: relation to human medicine. Yale J Biol Med 90, 449–461.

Raeside, J.I., Christie, H.L., Waelchli, R.O., Betteridge, K.J., 2009. Estrogen metabolism by the equine embryo proper during the fourth week of pregnancy. Reproduction 138, 953–960. https://doi.org/10.1530/REP-09-0235.

Refsal, K.R., Marteniuk, J.V., Williams, C.S.F., Nachreiner, R.F., 1991. Concentrations of estrone sulfate in peripheral serum of pregnant goats: relationships with gestation length, fetal number and the occurrence of fetal death in utero. Theriogenology 36, 449–461. https://doi.org/10.1016/0093-691X(91)90474-R.

Satué, K., Domingo, R., Redondo, J.I., 2011. Relationship between progesterone, oestrone sulphate and cortisol and the components of renin angiotensin aldosterone system in Spanish purebred broodmares during pregnancy. Theriogenology 76, 1404–1415. https://doi.org/10.1016/j.theriogenology.2011.06.009.

Senger, P.L., Phillip, L., 1997. Pathways to pregnancy and parturition. In: Pathways to pregnancy and parturition, 3rd editionCurrent conceptions. Redmond.

Shikichi, M., Iwata, K., Ito, K., Miyakoshi, D., Murase, H., Sato, F., Korosue, K., Nagata, S., Nambo, Y., 2017. Abnormal pregnancies associated with deviation in progestin and estrogen profiles in late pregnant mares: a diagnostic aid. Theriogenology 98, 75–81. https://doi.org/10.1016/j.theriogenology.2017.04.024.

Vervaecke, H., Schwarzenberger, F., 2006. Endocrine and behavioral observations during transition of non-breeding into breeding season in female American bison (Bison bison). Theriogenology 66, 1107–1114. https://doi.org/10.1016/j.theriogenology.2006.03.005.

Watson, P.F., Petrie, A., 2010. Method agreement analysis: a review of correct methodology. Theriogenology 73, 1167–1179. https://doi.org/10.1016/j.theriogenology.2010.01.003.

Wudy, S.A., Schuler, G., Sánchez-Guijo, A., Hartmann, M.F., 2018. The art of measuring steroids: principles and practice of current hormonal steroid analysis. J. Steroid Biochem. Mol. Biol. 179, 88–103. https://doi.org/10.1016/j.jsbmb.2017.09.003.