Steroids 76 (2011) 365–375

Contents lists available at ScienceDirect

Steroids

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

# Disturbance in sex-steroid serum profiles of cattle in response to exogenous estradiol: A screening approach to detect forbidden treatments

Patricia Regal*, Carolina Nebot, Mónica Díaz-Bao, Rocio Barreiro, Alberto Cepeda, Cristina Fente

*Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Veterinary Medicine, University of Santiago de Compostela, Lugo, Spain*

ARTICLE INFO

*Article history:*
Received 29 July 2010
Received in revised form
10 November 2010
Accepted 11 December 2010
Available online 21 December 2010

*Keywords:*
HPLC–MS/MS
Bovine
Serum
Estradiol benzoate
Sex hormone

ABSTRACT

Estradiol benzoate (EB) has been one of the most widely used estrogenic agents in animal husbandry, as a way of exogenously introducing the natural hormone estradiol-17β into the animal organism. Estradiol was previously employed to induce anabolic effects or reproductive improvements in cattle. However, the employment of EB in European countries has been permanently forbidden by Directive 2008/97/EC to guarantee consumers' health. Despite this prohibition, the control of estradiol-17β and its esters continues to be a difficult task for residue-monitoring plans in European Communities because official analyses of natural thresholds for hormones in cattle have not yet been established, leading to a lack of confirmation for any exogenous administration of natural hormones. Several researchers have worked on excretion profiles of metabolites, variation in specific hormonal ratios and metabolomic fingerprints after hormonal treatments. This research focuses on the possible existence of disturbances in the serum profile of animals treated with EB in terms of steroid sex hormones (androgens, oestrogens and progestogens), by investigating the serum levels of several of these hormones. The serum samples were collected from three groups of cows: one treated with an intramuscular injection of EB, one treated with a combination of intravaginal EB and progesterone and a control (non-treated) group. The samples have been analysed by a validated high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method, and 17 natural hormones were identified and quantified. Subsequently, data from the serum profiles were submitted for statistic and multivariate analysis, and it was possible to observe a manifest variation between animal groups. The obtained results can help in the development of a viable screening tool for monitoring purposes in cattle.



Throughout history, humans have bred animals to accommodate increasing human requirements in terms of food and other products, such as leather and wool, the demand for which is particularly important in developed countries. A large number of substances, both natural and synthetic, have been applied in stock farming to speed up and improve animal growth, and to decrease feed costs [1,2]. Among these veterinary drugs, there are some natural hormones and substances with hormonal effects that have been applied to animals for different purposes, especially as growth promoters and as fertility regulators in cattle. The steroid hormones, which are steroids acting as hormones, contain the sex hormones oestrogens, gestagens and androgens (EGAs), and the corticosteroids. Although these hormones have a wide variety of applications within the veterinary field, they have been used in animal fattening due to the anabolic effect that increases weight gain in treated animals, and induces changes that are generally characterised by lower fat content and more lean mass [3–5]. The zootechnical use of some sex hormones, such as estradiol or its esters (i.e., estradiol benzoate (EB)), which successfully regulate oestrus in cattle, has also led to important improvements and financial gain in stock farming [6].

There have been several European regulations regarding the use of EGAs as animal growth promoters because of their possible toxic effect on public health. In the Council Directive 96/22/EC [7], the European Union prohibited the administration of substances having thyreostatic, oestrogenic, androgenic or gestagenic effects and of beta agonists in animal husbandry, while certain therapeutic applications of these drugs were still allowed. These anabolic steroids are included in group A substances according to Annex I of Directive 96/23/EC [8], which pertains to growth-promoting agents abused in animal fattening and unauthorised substances with no maximum residue limit (MRL). A zero-tolerance policy has been adopted, and especial analytical requirements have been stated in regard to these hormones [9]. In particular, estradiol-

* Corresponding author at: Laboratorio de Higiene e Inspección de Alimentos, Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Veterinaria, Universidad de Santiago de Compostela, 27002 Lugo, Spain.
Tel.: +34 982285900; fax: +34 982254592.
*E-mail addresses:* patricia.regal@rai.usc.es, patricia_regalopez@hotmail.com (P. Regal).

0039-128X/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2010.12.005

17β, used with the aim of promoting animal growth, was deemed as a complete carcinogen by the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) [10]. Estradiol-17β exerts both tumour-initiating and tumour-promoting effects, and the data currently available do not make it possible to obtain a quantitative estimate of the risk. Although Directive 2003/74/EC, amending Directive 96/22/EC, permanently prohibited the use of estradiol-17β and its ester-like derivatives as growth promoters, a temporary exemption was given until 14 October 2006 for their use as an oestrous-induction tool in cows, horses, sheep or goats. As alternative effective products exist and are implemented in the market [11], the European Parliament banned estradiol-17β and its ester-like derivatives, including those with a therapeutic purpose in farm animals, in 2008, to ensure human health protection within the European Community [12]. However, the possibility of widespread abuse of hormonal substances by unscrupulous farmers and veterinary professionals in some parts of Europe still exists, mainly due to the economic benefits these substances provide in animal husbandry [13].

The control of growth promoters in meat-producing animals is probably one of the most challenging tasks in the field of European residue-monitoring plans, as it involves a wide number of target substances, the variability of their chemical structures and their concentration levels and biological matrices used in residue surveillance [1,4]. With regard to the confirmation of use of xenobiotic analogues of natural sex steroids and non-steroidal compounds such as stilbenes and zeranol, there is an extensive range of successful methods that has been performed on different analytic matrices that have made the confirmation of illicit administrations of anabolics in cattle feasible [2,14–17]. However, hormones of natural origin, such as estradiol-17β, testosterone (T) or progesterone, are still a weak area in residue-monitoring plans due to their endogenous origin, as the target compound is always present. In such a case, the confirmation of an exogenous administration involves logical difficulties associated with distinguishing an exogenous origin from an endogenous (naturally occurring) presence of these hormones. In fact, it has been found that treatments with T or estradiol in bovines lead to equal or lower plasma concentrations of these compounds [18,19]. On the other hand, exogenous natural hormones are usually administered as simple semi-synthetic esters (i.e., 17β-estradiol benzoate and T decanoate), and a subsequent rapid hydrolysis of these compounds takes place as soon as they reach the bloodstream, where they generate non-esterified forms that are indistinguishable from naturally occurring forms [20]. These exogenous compounds follow the same pathways as the natural compounds biosynthesised by the animal, making the detection and confirmation of their exogenous administration difficult, if not impossible. These circumstances have led to the lack of success in detecting EB in serum or plasma, which has only been confirmed in hair from animals treated with this ester [14,21]. In addition, the demonstration of an exogenous administration of natural steroids, for instance, T, estradiol or cortisol, remains problematic, as no official threshold has been stated for natural hormone concentrations, mainly due to the fact that concentrations of naturally occurring hormones depend on the type of animal product, breed, gender, age, disease, medication and physiological condition [22]. Furthermore, no list of discriminative marker metabolites has ever been stated, accepted or published by the community reference laboratories (CRLs) or by the European Commission, regarding the misuse of natural hormones in stock farming.

The development of methods to provide unequivocal discrimination between the natural presence of an endogenous hormone and its presence as a consequence of an illegal exogenous administration remains a challenge. Some promising analytical approaches have been published in the past few years regarding this critical point of controlling residues in food of animal origin. The measurement of $^{13}C/^{12}C$ ratios by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) can be a powerful tool to trace the true origin of steroids, and is one of the most promising approaches for the control of exogenous administration of natural hormones, as it has already been applied for anti-doping in sports [23,24]. In recent years, the potential of '-omic' technologies (metabolomics, proteomics with transcriptomics) coupled with bioinformatics has been investigated for the development of reliable molecular biomarkers, and to obtain discrimination based on targeted profiling of metabolites [25–27]. Other research has focussed on the variation of the excretion profile of phase II metabolites as a consequence of exogenously administered steroids [28,29], and in variation of some urinary or plasmatic metabolites from the biosynthetic pathway of sex hormones [18,30], or, even in blood chemistry [31].

Summing up, more information concerning steroid levels in animals treated with natural hormones seems necessary to establish acceptable thresholds of natural hormones or for use as a screening approach in residue-monitoring plans. In the present study, an analytical evaluation of serum profiles of several natural hormones from the biosynthetic pathway of sex hormones has been performed to prove the existence of any disturbance in the serum profile in response to exogenous estradiol administration in cattle. Bovine serum samples were analysed using a method based on liquid chromatography–tandem mass spectrometry (high-performance liquid chromatography tandem mass spectrometry, HPLC–MS/MS) [32], previously validated according to Decision 657/2002/EC criteria [9]. The samples were obtained from cows treated intramuscularly with the main ester of natural estradiol, 17β-estradiol benzoate, and animals treated with a common intravaginal combination of 17β-estradiol benzoate and progesterone. Serum from non-treated animals, which were used as control animals, was also collected. Free plasma concentrations of 17 natural steroids belonging to the three existing groups of sex hormones (EGAs) were submitted for further statistical analysis. From the data analysis, useful and valuable descriptive information about the natural steroid levels in bovines was obtained. In addition, an overview of the disturbance in plasma profiles of cattle treated with the oestrogenic compound was gathered.

## 1. Experimental

### 1.1. Samples

Serum samples were obtained from 72 Holstein cows that were between 24 months and 5 years in age, all from the same intensive dairy farm. Twenty-four cows were treated with an intramuscular injection of EB, consisting of 5 ml of a veterinary drug (Neonida N from Pfizer S.A., Madrid, Spain) containing 1250 I.U. of chorionic gonadotropin and 5 mg of EB, while 13 other cows were treated with an intravaginal device composed of a progesterone-releasing spiral (1.55 g of progesterone dispersed in an elastomeric silicone matrix) and a capsule containing 10 mg of EB (PRID® from CEVA Salud Animal, Barcelona, Spain). The experimental cows were fed a diet typically used in animal husbandry practices, and provided *ad libitum* access to water.

The administration of these hormonal preparations took place within a typical and real bovine reproductive control programme under the supervision of a veterinary surgeon. The estrogenic compounds were administrated before their total prohibition in October 2006. Blood samples from the animals treated with EB and EB combined with progesterone were collected on days 3 and 6, respectively, after a single-dose treatment. Thirty-five untreated animals were used as a control group (so-called non-treated or C).

**Table 1**
HPLC–MS/MS transition used for the measurement of the assayed natural hormones, obtained *CC*α and *CC*β when relevant [29]. (IS: internal standard; RT: retention time; *CC*α: decision limit; *CC*β: detection capability).

| Steroid | RT (min) | MRM (*Q*1/*Q*3) | *CC*α (ng dL$^{-1}$) | *CC*β (ng dL$^{-1}$) |
|---|---|---|---|---|
| $P_4$ | 24.5 | 345.093/124.100 | 18.68 | 31.83 |
| $P_4$-$d_9$ (IS) | 24.3 | 354.124/128.200 | | |
| 17OH$P_4$ | 18.9 | 361.068/124.200 | 13.06 | 22.25 |
| $P_5$ | 23.4 | 332.065/86.200 | 10.92 | 18.60 |
| $P_5$-$d_4$ (IS) | 23.3 | 336.121/90.100 | | |
| 17OH$P_5$ | 18.5 | 348.081/330.300 | 7.33 | 12.49 |
| 17OH$P_5$-$d_3$ (IS) | 18.3 | 350.97/333.200 | | |
| $E_1$ | 18.3 | 286.064/253.000 | 7.27 | 12.39 |
| $E_1$-$d_2$ (IS) | 18.3 | 287.965/255.100 | | |
| 2OH$E_1$ | 16.6 | 302.041/269.100 | 6.95 | 11.84 |
| 2OH$E_1$-$d_4$ (IS) | 18.3 | 305.951/273.100 | | |
| 4OH$E_1$ | 17.2 | 302.041/269.100 | 6.00 | 12.10 |
| 2Me$E_1$ | 16.4 | 316.035/283.100 | 12.98 | 22.12 |
| 2Me$E_1$-$d_4$ (IS) | 18.1 | 319.95/287.000 | | |
| 4Me$E_1$ | 17.1 | 316.044/283.200 | 14.31 | 24.21 |
| 16OH$E_1$ | 17.5 | 301.912/251.100 | 8.99 | 15.33 |
| DHEA | 19.4 | 304.105/253.200 | 14.99 | 25.54 |
| DHEA-$d_2$ (IS) | 19.2 | 306.005/213.200 | | |
| T | 19.8 | 304.084/124.100 | 19.46 | 33.16 |
| T-$d_3$ (IS) | 19.7 | 306.994/124.100 | | |
| 7OHT | 17.4 | 320.086/112.000 | 19.38 | 33.02 |
| 19OHT | 17.2 | 320.071/143.100 | 7.48 | 12.75 |
| A | 18.6 | 317.086/112.200 | 9.16 | 15.60 |
| 7OHA | 18.1 | 333.05/112.100 | 13.33 | 22.71 |
| 19OHA | 17.8 | 333.034/124.100 | 10.65 | 18.14 |

In all cases, the samples were collected from the tail by a veterinary surgeon using vacuum-extraction tubes. After blood clotting into the extraction tubes and 10 min of centrifugation at 814 × *g*, the serum was removed from all samples and stored at −20 °C, until further analysis in 2009.

### 1.2. Reagents and chemicals

Experimental materials included the following deuterated analogues of steroids: deuterium-labelled progesterone ($P_4$-d9), pregnenolone ($P_5$-d4), 17-hydroxy pregnenolone (17OH$P_5$-d3), estrone ($E_1$-d2), 2-hydroxy oestrone (2OH$E_1$-d4), 2-methoxy estrone (2Me$OE_1$-d4), dehydroepiandrosterone (DHEA-d2) and T-d3, purchased from CDN Isotopes (Quebec, Canada). The formic acid was obtained from AcrosOrganics (Geel, Belgium). The compounds methyl tert-butylether (MTBE), hexane, HPLC-grade methanol and acetonitrile were supplied by Merck (Darmstadt, Germany). The hydroxylamine solution (99.999%, 50 wt. % in $H_2O$) and ascorbic acid were obtained from Sigma–Aldrich (St Louis, MO, USA). Milli-Q organic-free water from Millipore (Bedford, MA, USA) was used. All reagents were of analytical grade.

The blood extraction materials consisted of single-use blood collection tubes, needles and needle holders from Vacutainer (Plymouth, UK).

### 1.3. Materials and apparatus

The HPLC system consisted of a quaternary pump, degasser and autosampler (Agilent Technologies model 1100; Minnesota, USA). A Q-Trap 2000 mass spectrometer with ion source turbo spray from Applied Biosystems MSD Sciex (Toronto, Canada) was used. Nitrogen was produced by a high-purity nitrogen generator (PEAK Scientific Instruments Ltd, Chicago, IL, USA). Nitrogen was used for the curtain, nebuliser and collision gases.

Aliquots of sample extracts were separated by HPLC using a Synergi 2.5 μm Fusion-RP 100A (100 mm × 2 mm) column from Phenomenex (Torrance, CA, USA) with a guard column of the same filling material. The mobile phase was water and methanol, both with 0.1% formic acid, mixed in a binary gradient from a previously validated method [32].

### 1.4. Serum analysis by LC–MS/MS

The serum samples (0.5 ml) were processed following an analytical method previously validated according to the European Commission Decision 2002/657/EC criteria [9] and described by Regal et al. [32]. This method permitted reliable detection and quantification of hormones from the three groups of natural sex hormones (EGAs): pregnenolone ($P_5$) [5-pregnen-3ß-ol-20-one], progesterone ($P_4$) [4-pregnen-3,20-dione], 17-hydroxy-progesterone (17OH$P_4$) [4-pregnen-17-ol-3,20-dione], 17-hydroxy-pregnenolone (17OH$P_5$) [5-pregnen-3ß,17-diol-20-one], DHEA [5-androsten-3β-ol-17-one], androstenedione (A) [4-androsten-3,17-dione], 7α-hydroxyandrostenedione (7OHA) [4-androsten-7α-ol-3,17-dione], 7α-hydroxytestosterone (7OHT) [4-androsten-7α,17β-diol-3-one], T [4-androsten-17β-ol-3-one], 19-hydroxyandrostendione (19OHA) [4-androsten-19-ol-3, 17-dione], 19-hydroxytestosterone (19OHT) [4-androsten-17β,19-diol-3-one], estrone ($E_1$) [1,3,5(10)-estratien-3-ol-17-one], 2-methoxyestrone (2Me$OE_1$) [1,3,5(10)-estratien-2,3-diol-17-one 2-methyl ether], 4-methoxyestrone (4Me$OE_1$) [1,3,5(10)-estratrien-3, 4-diol-17-one 4-methyl ether], 2-hydroxyestrone (2OH$E_1$) [1,3,5(10)-estratrien-2, 3-diol-17-one], 16α-hydroxyestrone (16OH$E_1$) [1,3,5(10)-estratrien-3,16α-diol-17-one] and 4-hydroxyestrone (4OH$E_1$) [1,3,5(10)-estratrien-3,4-diol-17-one].

The serum samples (500 μl) were placed in a 2-ml Eppendorf tube containing 1000 μl of the internal standard mixture in acetonitrile (100 ng dL$^{-1}$) and 250 μl of hexane. The tubes were capped, vortexed for 1 min, sonicated for 30 min and centrifuged at 16 100 × *g* for 15 min using an Eppendorf Centrifuge 5415D (Hamburg, Germany). The assembly was cooled for 15 min at −20 °C. The hexane supernatant and the precipitate were discarded, and the acetonitrile layer was evaporated under a nitrogen gas stream at 37 °C. For steroid derivatisation, the residue was re-dissolved in 300 μl of a 1.5 M hydroxylamine solution (pH 10) for 30 min at 90 °C. After this derivatisation procedure, 700 μl of water was

**Table 2**
Median, mean, minimum (Min) and maximum (Max) values of hormonal levels in bovine serum ($ng\,dL^{-1}$) and the percentage of samples below the *CC*α level in each group of animals; statistically significant differences are also shown (SD: standard deviation).

| Natural hormones | Statistics | Animal group | | |
|---|---|---|---|---|
| | | Estradiol benzoate (EB) | EB and progesterone (EB + $P_4$) | Control |
| DHEA*,† | Median | 25.6 | <25.5 | <25.5 |
| | Mean ($ng\,dL^{-1}$) | 34.0 | <25.5 | <25.5 |
| | SD | 13.1 | 5.0 | 5.9 |
| | Min | <25.5 | <25.5 | <14.9 |
| | Max | 61.3 | 32.2 | 40.8 |
| | %<CCα | 0.0 | 0.0 | 11.4 |
| T | Median | <19.5 | 41.9 | <19.5 |
| | Mean ($ng\,dL^{-1}$) | <33.2 | 49.3 | 48.1 |
| | SD | 23.9 | 34.6 | 60.6 |
| | Min | <33.2 | <33.2 | <19.5 |
| | Max | 120.1 | 144.4 | 214.6 |
| | %<CCα | 0.0 | 0.0 | 17.1 |
| A*,† | Median | <15.6 | <9.2 | <9.2 |
| | Mean ($ng\,dL^{-1}$) | 20.5 | <15.6 | <15.6 |
| | SD | 15.5 | 1.8 | 2.8 |
| | Min | <15.6 | <9.2 | <9.2 |
| | Max | 70.0 | <15.6 | 21.1 |
| | %<CCα | 0.0 | 92.3 | 8.6 |
| 7OHT | Median | <33.0 | <33.0 | <19.4 |
| | Mean ($ng\,dL^{-1}$) | 33.4 | 35.4 | 28.9 |
| | SD | 13.6 | 7.8 | 12.7 |
| | Min | <19.4 | <33.0 | <33.0 |
| | Max | 65.2 | 51.1 | 71.5 |
| | %<CCα | 0.0 | 0.0 | 0.0 |
| 7OHA*,‡ | Median | <22.7 | 135.5 | 42.2 |
| | Mean ($ng\,dL^{-1}$) | 37.1 | 161.5 | 45.4 |
| | SD | 40.8 | 136.0 | 28.3 |
| | Min | <22.7 | 36.9 | <22.7 |
| | Max | 188.1 | 475.2 | 109.7 |
| | %<CCα | 0.0 | 0.0 | 0.0 |
| 19OHT | Median | 319.1 | 582.5 | 374.5 |
| | Mean ($ng\,dL^{-1}$) | 450.6 | 624.8 | 423.0 |
| | SD | 347.2 | 292.4 | 321.2 |
| | Min | 51.4 | 243.2 | 32.7 |
| | Max | 1198.6 | 1152.1 | 1264.6 |
| | %<CCα | 0.0 | 0.0 | 0.0 |
| 19OHA*,‡ | Median | 47.1 | 455.5 | 141.4 |
| | Mean ($ng\,dL^{-1}$) | 108.7 | 561.7 | 161.1 |
| | SD | 158.2 | 538.4 | 142.3 |
| | Min | 18.2 | 61.8 | <18.1 |
| | Max | 676.5 | 1804.0 | 755.8 |
| | %<CCα | 0.0 | 0.0 | 0.0 |
| $E_1$*,† | Median | 185.5 | 52.0 | 40.2 |
| | Mean ($ng\,dL^{-1}$) | 191.1 | 69.9 | 48.6 |
| | SD | 100.5 | 47.0 | 33.5 |
| | Min | 55.9 | 28.5 | <12.4 |
| | Max | 378.4 | 194.3 | 204.5 |
| | %<CCα | 0.0 | 0.0 | 0.0 |
| $2OHE_1$*,† | Median | 703.8 | 483.4 | 377.9 |
| | Mean ($ng\,dL^{-1}$) | 892.3 | 506.3 | 396.3 |
| | SD | 532.8 | 125.8 | 163.9 |
| | Min | 416.8 | 305.6 | 85.5 |
| | Max | 2718.6 | 676.0 | 913.1 |
| | %<CCα | 0.0 | 0.0 | 0.0 |
| $2MeOE_1$*,† | Median | 162.9 | 42.8 | 22.4 |
| | Mean ($ng\,dL^{-1}$) | 163.2 | 48.8 | 50.3 |
| | SD | 94.7 | 23.5 | 46.4 |
| | Min | 34.1 | <22.1 | <22.1 |
| | Max | 401.2 | 98.1 | 162.9 |
| | %<CCα | 0.0 | 0.0 | 0.0 |
| $4OHE_1$*,† | Median | 735.3 | 492.7 | 378.8 |
| | Mean ($ng\,dL^{-1}$) | 1123.9 | 530.7 | 396.3 |
| | SD | 938.9 | 149.3 | 163.9 |
| | Min | 416.8 | 305.6 | 85.5 |
| | Max | 3940.8 | 824.2 | 913.1 |
| | %<CCα | 0.0 | 0.0 | 0.0 |
| $4MeOE_1$*,†,‡ | Median | 96.7 | <14.3 | <14.3 |
| | Mean ($ng\,dL^{-1}$) | 114.1 | 23.1 | <22.1 |
| | SD | 94.7 | 19.2 | 2.4 |
| | Min | <14.3 | <14.3 | <14.3 |
| | Max | 354.6 | 81.9 | <24.2 |
| | %<CCα | 16.7 | 61.5 | 94.3 |

Table 2 (*Continued*)

| Natural hormones | Statistics | Animal group | | |
|---|---|---|---|---|
| | | Estradiol benzoate (EB) | EB and progesterone (EB + $P_4$) | Control |
| $16OHE_1$ *,† | Median | 107.1 | 35.0 | 33.9 |
| | Mean (ng $dL^{-1}$) | 140.8 | 38.8 | 43.1 |
| | SD | 114.7 | 30.0 | 42.8 |
| | Min | 22.7 | <8.9 | <8.9 |
| | Max | 450.2 | 109.9 | 218.6 |
| | %<*CC*α | 0.0 | 15.4 | 17.1 |
| $P_5$ | Median | 252.4 | 221.8 | 196.7 |
| | Mean (ng $dL^{-1}$) | 287.1 | 230.1 | 243.2 |
| | SD | 123.9 | 79.2 | 139.8 |
| | Min | 110.0 | 128.3 | 58.8 |
| | Max | 586.3 | 382.4 | 553.4 |
| | %<*CC*α | 0.0 | 0.0 | 0.0 |
| $P_4$ *,† | Median | 113.8 | 1302.0 | 2556.1 |
| | Mean (ng $dL^{-1}$) | 262.5 | 1460.4 | 2899.4 |
| | SD | 354.8 | 779.6 | 2774.3 |
| | Min | <18.6 | 26.0 | <18.6 |
| | Max | 1531.0 | 2669.6 | 12,364.3 |
| | %<*CC*α | 8.3 | 0.0 | 17.1 |
| $17OHP_5$ *,† | Median | 112.4 | 42.4 | 65.4 |
| | Mean (ng $dL^{-1}$) | 127.2 | 56.9 | 67.8 |
| | SD | 44.7 | 36.7 | 22.8 |
| | Min | 71.1 | <12.5 | 33.3 |
| | Max | 249.4 | 112.0 | 157.6 |
| | %<*CC*α | 0.0 | 0.0 | 0.0 |
| $17OHP_4$ *,† | Median | 22.3 | <13.1 | <13.1 |
| | Mean (ng $dL^{-1}$) | 33.0 | <22.3 | <22.3 |
| | SD | 26.9 | 2.5 | 21.0 |
| | Min | <13.1 | <13.1 | <13.1 |
| | Max | 126.4 | 22.3 | 127.8 |
| | %<*CC*α | 4.2 | 53.8 | 65.7 |
| Ratio $T/P_4$ *,† | Median | 0.179 | 0.027 | 0.013 |
| | Mean | 0.464 | 0.135 | 0.217 |
| | SD | 0.532 | 0.356 | 0.396 |
| | Min | 0.013 | 0.009 | 0.004 |
| | Max | 1.826 | 1.316 | 1.042 |
| Ratio $P_5/P_4$ *,† | Median | 2.599 | 0.139 | 0.095 |
| | Mean | 4.751 | 0.478 | 2.050 |
| | SD | 6.796 | 1.073 | 4.535 |
| | Min | 0.166 | 0.083 | 0.024 |
| | Max | 13.690 | 4.031 | 20.675 |

†Mann–Whitney *U* test for EB and C $p < 0.01$.
‡Mann–Whitney *U* test for CEB + P4 and C $p < 0.01$.
*KRUSKAL–WALLIS test between 3 groups $p < 0.01$.

added, and the oxime steroid derivatives were extracted twice with 2 ml of MTBE. After evaporation under a nitrogen gas stream, the residue in 100 μl of methanol:water (10:90 (v/v)) was used for the chromatographic procedure.

### 1.5. Identification and quantification of steroids

The transitions monitored for each of the assayed hormones and for their internal standards and main validation parameters, *CC*α and *CC*β (in ng $dL^{-1}$) [32], are shown in Table 1. The automatic quantification tool of Analyst 1.4.1 software (MDS SCIEX) was used to integrate the area corresponding to the chromatographic peaks of each hormone and internal standard. The serum levels of each hormone in their unconjugated forms were interpolated from calibration curves constructed by calculating the area ratios of the analyte peak area/internal standard (IS) peak area versus the analyte concentration (calibration with IS).

The data obtained on the level of free, unconjugated steroid hormones in animal serum samples were subsequently submitted to statistical analysis, to obtain descriptive results related to animal group and to observe discriminative and multivariate features. The results obtained from the serum samples were organised and evaluated in three groups: 25 animals treated with EB (EB, intramuscular), 13 treated with EB plus progesterone (EB + $P_4$, intravaginal) and 35 non-treated animals as group C.

Adequate measures were taken to minimise pain or discomfort to the animals, and the experiments were conducted in accordance with international standards on animal welfare, and were compliant with local and national regulations.

### 1.6. Data analysis

Univariate statistics were performed using PASW Statistics 18.0 (SPSS Ibérica, Madrid, Spain). Breakdown analyses are represented by the mean (ng $dL^{-1}$), median, standard deviation, ranges (maximum and minimum quantified level in ng $dL^{-1}$) and percentage of samples that were below the decision limit (*CC*α) in each group of animals. When an analyte was not detected (below the *CC*α), its value was expressed as the *CC*α level; when an analyte was detected but not accurately quantifiable (below *CC*β), it was expressed as the *CC*β level. The values of these performance limits, *CC*α and *CC*β, are shown in Table 1 for each hormone. In addition, discrimination tests were applied to search for any possible statistically significant differences in hormonal levels, according to animal group. Differences between groups were evaluated by means of the Mann–Whitney *U* test and Kruskal–Wallis test, as the distribution of variables was proven to be non-Gaussian.

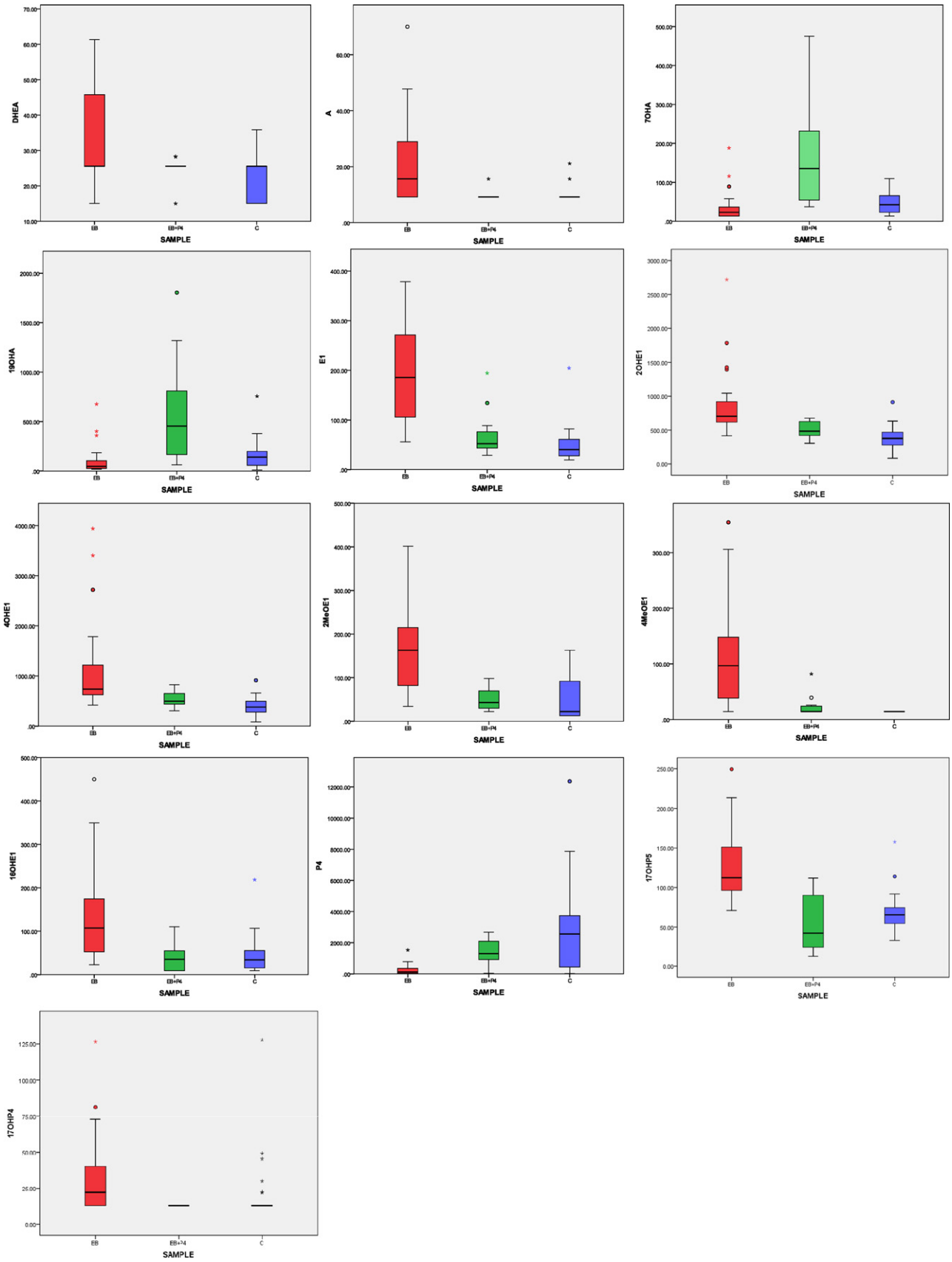


**Fig. 1.** Box plots showing serum levels of the steroid hormones identified by HPLC–MS/MS that were found to be significantly different ($p < 0.01$) between cows treated with estradiol benzoate (intramuscular, EB), treated with estradiol benzoate plus progesterone (intravaginal, EB + $P_4$) and the control group (C). The box limits are in the 25th and 75th percentile, and the band in the middle of the box is the median; the whiskers are in the 1.5 interquartile range. Data not included between the whiskers are plotted as an outlier with a small circle (mild) or star (extreme outlier).

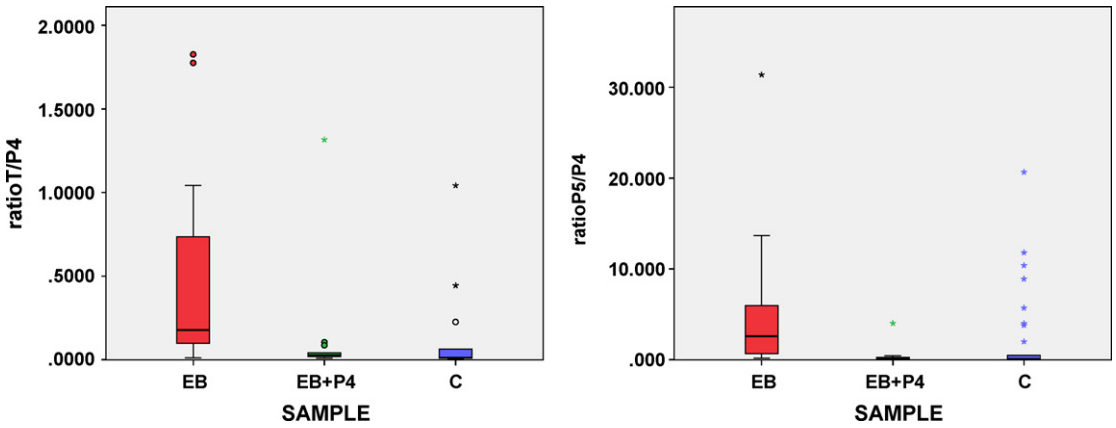


**Fig. 2.** Box plots showing the hormonal ratios that were found to be significantly different ($P_5/P_4$ and $T/P_4$) between cows treated with estradiol benzoate (intramuscular, EB), treated with estradiol benzoate plus progesterone (intravaginal, EB + $P_4$) and the control group (C), when $p < 0.01$. The box limits are in the 25th and 75th percentile, and the band in the middle of the box is the median; the whiskers are in the 1.5 interquartile range. Data not included between the whiskers are plotted as an outlier with a small circle (mild) or star (extreme outlier).

Multivariate analysis was performed by means of SIMCA-P+12.0 (Umetrics AB, Sweden) software. As the name indicates, multivariate analysis groups a set of techniques dedicated to the analysis of data sets with more than one variable. To perform multiple sample comparison by multivariate modelling, all samples must be described by a common set of variables, which, in this case, is the concentration of free natural hormones present in bovine serum. All variables were log transformed to become uniform and suitable for being submitted to multivariate analysis.

Unsupervised principal components analysis (PCA) is a method that reduces data dimensionality through a covariance analysis between factors without referring to class labels, which, in this case, are animal groups [33]. PCA was run to obtain a general overview of the variance in hormones throughout samples. Multivariate regression analysis in terms of orthogonal partial least squares discriminant analysis (OPSLS-DA) [34,35] was applied to extract the systematic variation in the quantified serum profiles related to the animal groups. OPLS-DA is a supervised method that uses a multiple linear regression technique to find the maximum covariance between a data set and the sample class. By analysing the S-plot, hormones that provide a greater ability to discriminate between animals treated with EB and the control animals were identified. The robustness of the OPLS-DA model was verified by a predictive model, in which a random selection of two-thirds of the samples (of known class) was used to predict the rest of the samples (of unknown class). The samples used as predictors were randomly selected with respect to a two-third-rate in each animal group.

## 2. Results and discussion

### 2.1. Selected drugs and sampling

Veterinary commercial products were selected based on their widespread employment in husbandry before the prohibition of 17β-estradiol and its esters by the European Union. The selected veterinary drugs were employed in the past for zootechnical purposes, such as controlling oestrus and synchronising pregnancy in cows. Due to the high effectiveness of estradiol in controlling and inducing oestrus in cattle [6], it can be illegally used nowadays, even though a permanent prohibition has been stated for it. It should be noted that these hormonal treatments were real, and the samples were collected from non-experimental dairy cows at a Spanish dairy farm, before the permanent prohibition of estradiol in October 2006, aiming to induce oestrus and, subsequently, inseminate treated cows. The sampling times of day 3 for EB and day 6 for EB + $P_4$ were established by the veterinary surgeon as the time fixed for artificial insemination when using each treatment, based on the purpose of these drugs.

The possible occurrence of problems in the hormonal stability due to long-term freezing storage has also been taken into account; therefore, control samples from the same farm were also collected at the same time. It is expected that possible variations in hormonal levels would affect both treated and non-treated samples, and differences in hormonal levels between groups would be maintained despite storage.

### 2.2. Analytical methodology

Oximation with hydroxylamine provided a method for the simultaneous analysis of 17 steroid compounds, including progestagens, androgens and estrogens, all of which have reactive keto groups. Monitored hormones were selected with the intention of including precursors and metabolites from the three existing groups of sex hormones (PGAs), and accounting for the possibility of oximation with hydroxylamine. Despite inhibiting the analysis of estradiol ($E_2$), hydroxylamine was selected based on previous research that was focussed on detecting the parent compound [36] without obtaining promising results in terms of discrimination of animals treated with EB. Moreover, deproteinisation and extraction with acetonitrile, without later steps, permitted the HPLC–MS/MS analysis of the obtained steroid–oxime derivatives. As the existing validation demonstrates [32], this LC–MS/MS methodology enables an efficient measurement of steroids from bovine serum with minimal background interferences. Good efficiency and peak shape were achieved with the proposed gradient conditions, and it was possible to separate closely related steroids when monitoring the same transitions (e.g., $2OHE_1$ and $4OHE_1$). The obtained derivatives were easily ionisable with an electrospray source in positive mode. Two multiple reaction monitoring (MRM) transitions (Table 1) were monitored for each analyte, and the most intense transition was monitored for quantification. Adequate chromatographic separation and evaluation of the two transitions enabled compound confirmation.

The serum concentrations of some analysed hormones were too low to be confirmed (below *CC*α) and/or quantified (below *CC*β), especially in the case of some androgens and, particularly, in animals treated with EB combined with P4 and in group C. To avoid eliminating these samples and some experimental animals with very low hormonal levels from the research, a fixed value was given

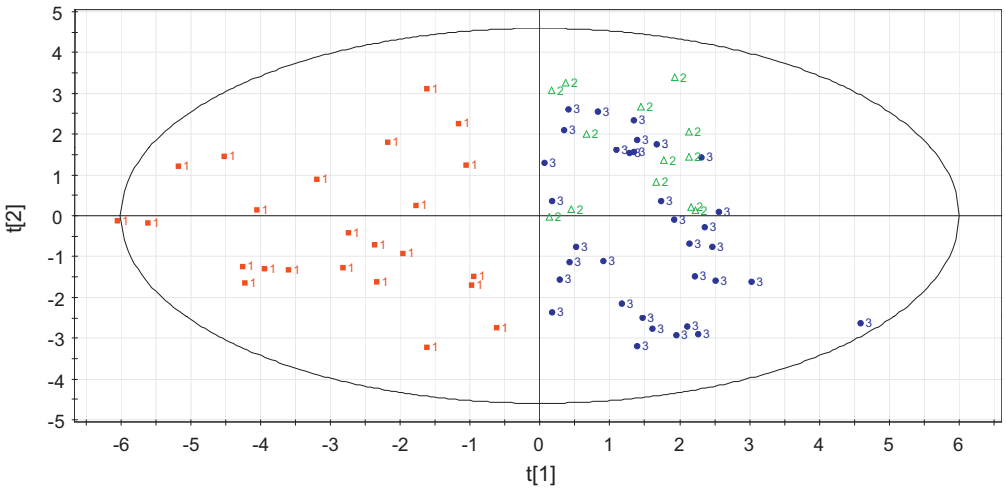


**Fig. 3.** Principal components analysis (PCA) score plots derived from the HPLC–MS/MS data set consisting of hormonal profiles from animals treated with estradiol benzoate (1, red box), estradiol benzoate and progesterone (2, green open triangle) and control animals (3, blue dot), showing that the profile is different between groups 1 and 3, but not between 2 and 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to the hormonal levels when they could not be quantified, as mentioned in Materials and Methods section, and this step must be taken into account for consideration throughout our research. Furthermore, as the relation between variables depends on the size of the sample as well, the results of discrimination regarding animals treated with EB + P4 ($n = 13$) should not be considered to be as reliable as the results from the EB-treated animals ($n = 24$), from a statistical point of view.

When analysing the obtained results, the mode of application of EB should be considered, as it is known that the rate of absorption of a steroid will differ significantly depending on the means of administration and formula [29,30]. When EB is injected intramuscularly, the absorption will be higher than for an intravaginal pill because the latter was previously a veterinary reproductive tool, which was not aimed at reaching high rates in blood, but only in reproductive organs. The above statement is in agreement with the results obtained for animals treated with the EB + P4 intravaginal device, as few differences were found after comparison with the control cows.

### 2.3. Univariate analysis

As for distribution of variables (hormonal levels), precise information was obtained by performing one of the tests of normality to determine the probability that the sample originated from a normally distributed population. The Kolmogorov–Smirnov test was performed on available quantitative variables corresponding to the hormonal level of each hormone in serum samples, and it was concluded that the hormonal levels did not follow a normal distribution ($p > 0.05$). In addition, corresponding histograms of variables were built to visualise the distribution of the data, confirming the results of this test. Subsequent discrimination tests were applied according to these results.

Non-parametric methods were chosen to evaluate the existence of statistically significant variations between animal groups in terms of serum profiles. When multiple groups were compared, then the Kruskall–Wallis analysis, which is the non-parametric equivalent of analysis of variance/multivariate analysis of variance (ANOVA/MANOVA) analysis, was used. If there are two independent groups of samples which could be compared by their mean values for some variable of interest, the *t*-test for independent samples is usually used; the Mann–Whitney *U* test is the non-parametric alternative for this test. In this study, the Mann–Whitney *U* test was performed to compare EB samples with C samples and EB + $P_4$ samples with C samples. Differences between the groups were considered statistically significant when $p < 0.01$. Both descriptive and discriminative results obtained from univariate statistical analyses are shown in Table 2.

When differences between the three groups were checked statistically by means of the Kruskall–Wallis test (see Table 2), it was concluded that the variations were significant in almost every assayed hormone, except for pregnenolone, T and its hydroxides (7OHT and 19OHT), as $p > 0.01$. When performing the Mann–Whitney *U* test for comparison of the hormonal profiles of the EB and C groups, steroids that were significantly different in the Kruskall–Wallis test were also selected ($p < 0.01$), except for the hydroxides of androstenedione (7OHA and 19OHA), which appeared to be non-altered. Interestingly, for animals treated with EB and $P_4$, variations in serum profiles were not evident in any hormone ($p > 0.01$), except for the hydroxides of androstenedione and $4MeOHE_1$. These statistically proven differences can be clearly seen in Fig. 1, which shows box plots for each hormone that was found significantly modified relative to group C ($p < 0.01$).

Hormonal ratios were also evaluated, but only $T/P_4$ and $P_5/P_4$ were found to be different among the three groups. These hypothetically significant ratios are also described in Table 2. Fig. 1 summarises the results of the concentrations of hormones in the free form of the three different groups: the C group, the EB-treated group and the EB + $P_4$-treated group. Here, statistically significant difference among the groups could be visualised when comparing the analysed hormones of animals and the significant ratios. Fig. 2 shows a comparison among the selected ratios through all the samples; it should be noted that an obvious variation of these ratios, $T/P_4$ and $P_5/P_4$, could be observed for the EB group ($p < 0.01$), but not for cows treated with EB + $P_4$, and the variation was particularly evident in the $P_5/P_4$ ratio. This finding shows that a single hormone could appear non-disturbed, as in the case of pregnenolone and T, although a significant change is observed in terms of the ratio of this hormone to related substances.

### 2.4. Multivariate analysis

Statistical analyses were performed on all continuous variables, representing the levels of several unconjugated EGAs, using SIMCA-

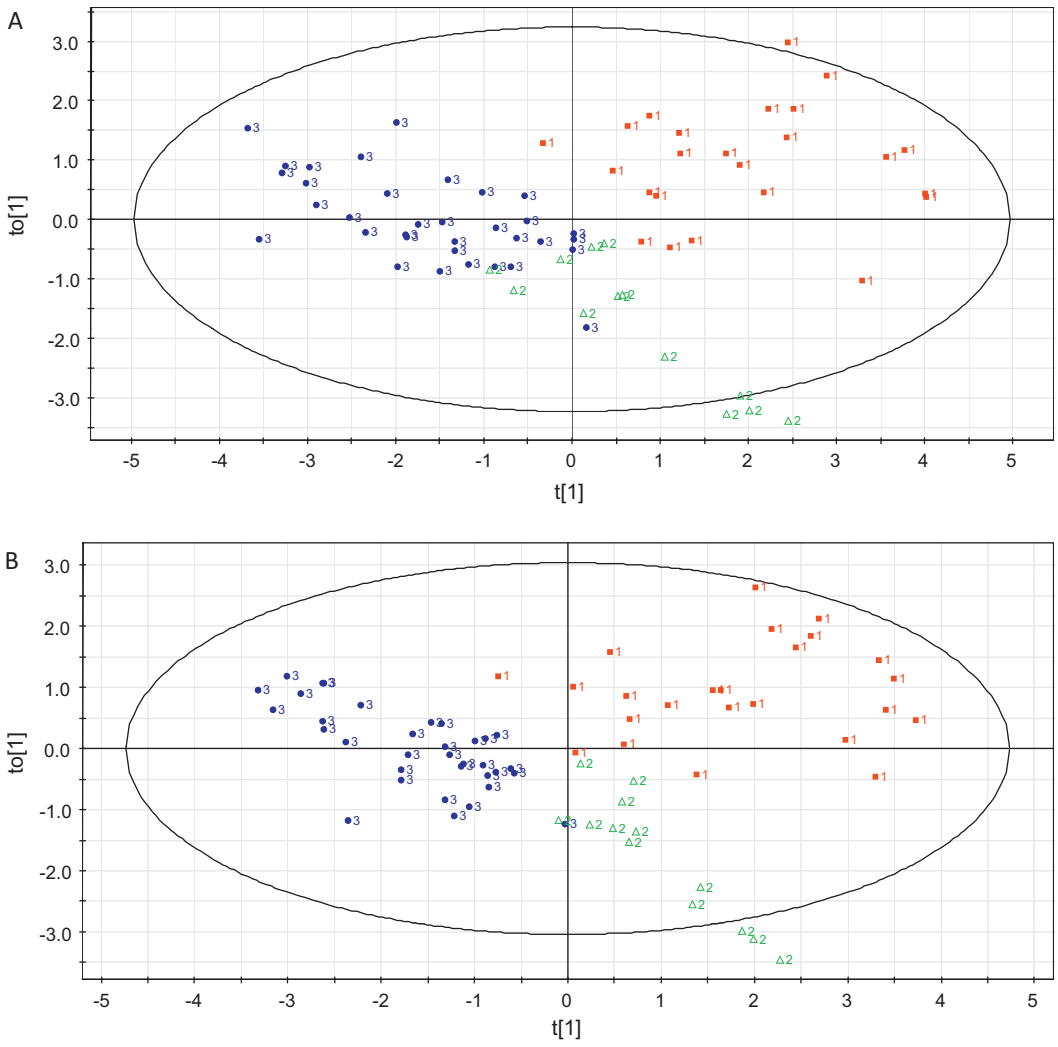


**Fig. 4.** OPLS-DA score plot derived from the HPLC–MS/MS data set consisting of hormonal profiles from animals treated with estradiol benzoate (1, red box), estradiol benzoate and progesterone (2, green open triangle) and control animals (3, blue dot). The first score plot (A) shows OPLS-DA derived from the complete data set, and the second (B) is derived from the data set without hormones that were not statistically different between groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

P + version 12.0 (Umetrics AB, Sweden). Unsupervised principal component analysis (PCA) was run to obtain a general overview of the variance in serum profiles, and supervised OPLS-DA was performed to obtain information on the differences in the hormonal profiles of samples.

### 2.5. PCA and OPLS-DA

As shown in the PCA score plot (Fig. 3), significant differences were detected between animals treated intramuscularly with EB and the control animals, but no difference was detected in the animals treated with progesterone combined with intravaginal EB, which were grouped with the control samples in the PCA score plot. The OPLS-DA model showed a good discrimination between the control and EB-treated animals, but the EB + P4-treated animals were not clearly distinguished from the other two groups ($R^2(Y) = 0.63$; $Q^2(Y) = 0.50$), as can be seen in Fig. 4. When eliminating these 13 samples from the data set, the characteristics the OPLS-DA model improved significantly ($R^2(Y) = 0.76$; $Q^2(Y) = 0.75$), resulting in a better discrimination between the EB group and the C group. A clear improvement could also be observed when the hormones that were not statistically different between samples (T and its hydroxides, 7OHT and 19OHT, and pregnenolone) were eliminated from the data set of OPLS-DA ($R^2(Y) = 0.70$; $Q^2(Y) = 0.61$), and the three groups can clearly be distinguished (Fig. 4). The S-plot from these two OPLS-DA models showed a high sharing of oestrogens in discriminatory power, androgens and especially T and its hydroxides being the less powerful components in terms of discrimination. The conclusions extracted from the S-plot were in agreement with the results obtained from the statistical tests from the previous univariate analysis.

### 2.6. Predictive model

The prediction ability of the previous OPLS model was assessed by a new OPLS built with two-thirds of the previous data set. Forty-eight samples (two-thirds of the data) were used to build the predictor set, and the other 24 (one-third) were used as the independent validation set to be predicted. The first model was built using the three groups of animals, and a second model included only the animals treated intramuscularly and group C. The resulting characteristics of the second model were better than in the first, in which animals treated intravaginally appeared slightly grouped but

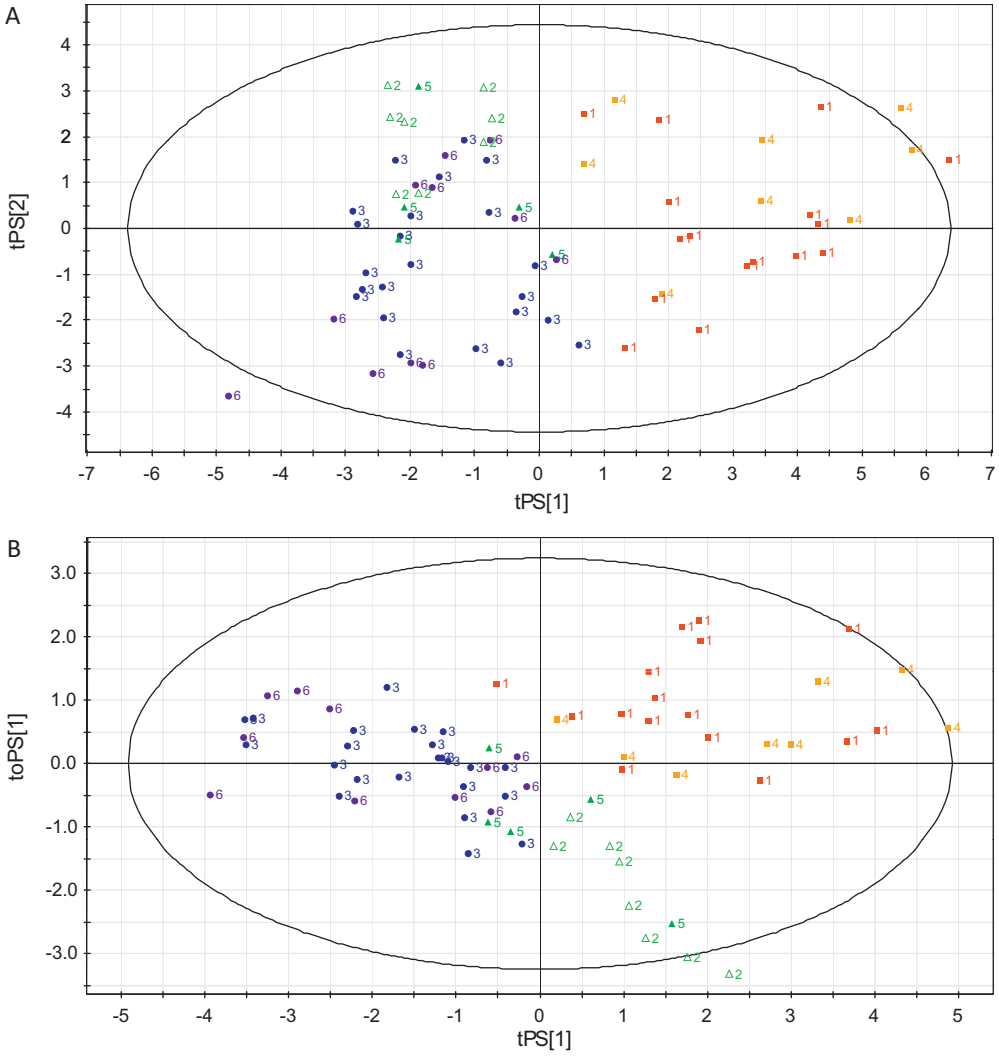


**Fig. 5.** Assessment of the predictive ability of the OPLS models built in Fig. 4, derived from the HPLC–MS/MS data set consisting of hormonal profiles from animals treated with estradiol benzoate (1, red box), estradiol benzoate and progesterone (2, green open triangle) and the control animals (3, blue dot). Here, two-thirds of the data were used for building the predictive model, and one-third of the data was used for the prediction, represented in the figure as follows: estradiol benzoate (4, orange box), estradiol benzoate and progesterone (5, green triangle) and control (6, purple dot). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were mixed with group C (Fig. 5). The independent set was successfully predicted in the second predictive OPLS model ($R^2(Y) = 0.77$; $Q^2(Y) = 0.73$).

## 3. Conclusions

In general, the obtained results showed that the main ester of estradiol-17β, EB, leads to changes in serum profiles of cattle with regard to sex steroid hormones. Variations in the serum profile after the administration of EB were not as evident in animals treated intravaginally as they were in animals treated intramuscularly with EB. The hormones that were not found to be evidently modified according to a first evaluation produced significant hormonal ratios. Multivariate analysis has hinted at the possibility of developing a future analysis using steroid serum profiles with screening purposes, as a contribution to the fight of the European Union against the prevalent use of illegal treatments with hormones in stock farming. However, further research in terms of age, breed, sex, nutritional status and/or animal location must be developed as well, as it is highly necessary to assess long-term administrations of EB and the possible existence or the absence of long-term serum disturbances associated with these treatments.

## Acknowledgements

We express our thanks for financial support received from Xunta de Galicia Programa Sectorial de Tecnoloxías da Alimentación, PGIDIT07TAL025261PR. Ms. Regal acknowledges a fellowship from Ministerio de Educación, Formación de Profesorado Universitario (FPU) del Programa Nacional de Formación de Recursos Humanos de Investigación, Plan Nacional de Investigación Científica, Desarrollo e Innovación Tecnológica 2008–2011 (Spain).

## References

[1] Kinsella B, O'Mahony J, Malone E, Moloney M, Cantwell H, Furey A, et al. Current trends in sample preparation for growth promoter and veterinary drug residue analysis. J Chromatogr A 2009;1216:7977–8015.
[2] De Brabander HF, Le Bizec B, Pinel G, Antignac J-P, Verheyden K, Mortier V, et al. Past, present and future of mass spectrometry in the analysis of residues of banned substances in meat-producing animals. J Mass Spectrom 2007;42(8):983–98.
[3] Hancock DL, Wagner JF, Anderson DB. Effects of estrogens and androgens on animal growth. In: Pearson AM, Dutson TR, editors. Growth regulation in farm animals: advances in meat research. London: Elsevier Applied Science; 1991. p. 255–97.

[4] Courtheyn D, Le Bizec B, Brambilla G, De Brabander HF, Cobbaert E, Van De Wiele M, et al. Recent developments in the use and abuse of growth promoters. Anal Chim Acta 2002;473(1–2):71–82.
[5] Lone KP. Natural sex steroids and their xenobiotic analogs in animal production: growth, carcass quality, pharmacokinetics, metabolism, mode of action, residues, methods, and epidemiology. Crit Rev Food Sci Nutr 1997;37(2):93–209.
[6] Cavalieri J, Rabiee AR, Hepworth G, Macmillan KL. Effect of artificial insemination on submission rates of lactating dairy cows synchronised and resynchronised with intravaginal progesterone releasing devices and oestradiol benzoate. Anim Reprod Sci 2005;90(1–2):39–55.
[7] Council Directive 96/22/EC. Off J Eur Comm 1996;L125:3–9.
[8] Council Directive 96/23/EC. Off J Eur Comm 1996;L125:10–32.
[9] Commission Decision 2002/657/EC. Off J Eur Comm 2002;L221:8–36.
[10] Directive 2003/74/EC. Off J Eur Comm L262:17–21.
[11] Lane EA, Austin EJ, Crowe MA. Oestrous synchronisation in cattle-current options following the EU regulations restricting use of oestrogenic compounds in food-producing animals: a review. Anim Reprod Sci 2008;109(1–4):1–16.
[12] Directive 2008/97/EC. Off J European Union 2008; L318:9–11.
[13] Stephany R. Hormones in meat: different approaches in the EU and in the USA. APMIS 2001;109:S357–63.
[14] Duffy E, Rambaud L, Le Bizec B, O'Keeffe M. Determination of hormonal growth promoters in bovine hair: comparison of liquid chromatography-mass spectrometry and gas chromatography–mass spectrometry methods for estradiol benzoate and nortestosterone decanoate. Anal Chim Acta 2009;637(1–2):165–72.
[15] Kaklamanos G, Theodoridis G, Papadoyannis IN, Dabalis T. Determination of anabolic steroids in muscle tissue by liquid chromatography–tandem mass spectrometry. J Agric Food Chem 2007;55(21):8325–30.
[16] Noppe H, Le Bizec B, Verheyden K, De Brabander HF. Novel analytical methods for the determination of steroid hormones in edible matrices. Anal Chim Acta 2008;611(1):1–16.
[17] Regal P, Nebot C, Vázquez BI, Cepeda A, Fente CA. Determination of 17α-methyltestosterone in bovine serum using liquid chromatography tandem mass spectrometry. J Food Drug Anal 2010;18(1):51–7.
[18] Simontacchi C, Perez De Altamirano T, Marinelli L, Angeletti R, Gabai G. Plasma steroid variations in bull calves repeatedly treated with testosterone, nortestosterone and oestradiol administered alone or in combination. Vet Res Commun 2004;28(6):467–77.
[19] Scippo M-, Degand G, Duyckaerts A, Maghuin-Rogister G, Delahaut P. Control of the illegal administration of natural steroid hormones in the plasma of bulls and heifers. Analyst 1994;119(12):2639–44.
[20] Stolker A, Groot M, Lasaroms J, Nijrolder A, Blokland M, Riedmaier I. Detectability of testosterone esters and estradiol benzoate in bovine hair and plasma following pour-on treatment. Anal Bioanal Chem 2009;395(4):1075–87.
[21] Regal P, Vázquez BI, Franco CM, Cepeda A, Fente CA. Development of a rapid and confirmatory procedure to detect 17β-estradiol 3-benzoate treatments in bovine hair. J Agric Food Chem 2008;56(24):11607–11.
[22] Hartmann S, Lacorn M, Steinhart H. Natural occurrence of steroid hormones in food. Food Chem 1998;62(1):7–20.
[23] Bichon E, Kieken F, Cesbron N, Monteau F, Prévost S, André F, et al. Development and application of stable carbon isotope analysis to the detection of cortisol administration in cattle. Rapid Commun Mass Spectrom 2007;21:2613–20.
[24] Cawley AT, Trout GJ, Kazlauskas R, Howe CJ, George AV. Carbon isotope ratio (δ13C) values of urinary steroids for doping control in sport. Steroids 2009;74:379–92.
[25] Gardini G, Del Boccio P, Colombatto S, Testore G, Corpillo D, Di Ilio C, et al. Proteomic investigation in the detection of the illicit treatment of calves with growth-promoting agents. Proteomics 2006;6(9):2813–22.
[26] Mooney MH, Situ C, Cacciatore G, Hutchinson T, Elliott C, Bergwerff AA. Plasma biomarker profiling in the detection of growth promoter use in calves. Biomarkers 2008;13(3):246–56.
[27] Della Donna L, Ronci M, Sacchetta P, Di Ilio C, Biolatti B, Federici G, et al. A food safety control low mass-range proteomics platform for the detection of illicit treatments in veal calves by MALDI-TOF-MS serum profiling. Biotechnol J 2009;4:1596–609.
[28] Antignac J-P, Brosseaud A, Gaudin-Hirret I, Andreĭ F, Bizec BL. Analytical strategies for the direct mass spectrometric analysis of steroid and corticosteroid phase II metabolites. Steroids 2005;70(3):205–16.
[29] Maume D, Le Bizec B, Pouponneau K, Deceuninck Y, Solere V, Paris A, et al. Modification of 17β-estradiol metabolite profile in steer edible tissues after estradiol implant administration. Anal Chim Acta 2003;(483):289–97.
[30] Becue I, Van Poucke C, Rijk JCW, Bovee TFH, Nielen M, Van Peteghem C. Investigation of urinary steroid metabolites in calf urine after oral and intramuscular administration of DHEA. Anal Bioanal Chem 2010;396:799–808.
[31] Cunningham RT, Mooney MH, Xia X-, Crooks S, Matthews D, O'Keeffe M, et al. Feasibility of a clinical chemical analysis approach to predict misuse of growth promoting hormones in cattle. Anal Chem 2009;81(3):977–83.
[32] Regal P, Vázquez BI, Franco CM, Cepeda A, Fente C. Quantitative LC–MS/MS method for the sensitive and simultaneous determination of natural hormones in bovine serum. J Chromatogr B 2009;877(24):2457–64, 8/15.
[33] Eriksson L, Antti H, Gottfries J, Holmes H, Johansson E, Lindgren F, et al. Using chemometrics for navigating in the large data sets of genomics, proteomics and metabolomics (gpm). Anal Bioanal Chem 2004;380:419–29.
[34] Trygg J, Wold S. Orthogonal projections to latent structures (O-PLS). J Chemom 2002;16:119–28.
[35] Bylesjö M, Rantalainen M, Cloarec O, Nicholson JK, Holmes E, Trygg J. OPLS discriminant analysis: combining the strenghts of PLS-DA and SIMCA classification. J Chemometr 2006;20:341–51.
[36] Fritsche S, Rumsey T, Meyer H, Schmidt G, Steinhart H. Profiles of steroid hormones in beef from steers implanted with synovex-S (estradiol benzoate and progesterone) in comparison to control steers. Z Lebensm Unters Forsch A 1999;208(5–6):328–31.