Nandrolone and estradiol biomarkers identification in bovine urine applying a liquid chromatography high-resolution mass spectrometry metabolomics approach

Sadia Ouzia, Anne-lise Royer, Marzia Pezzolato, Alessandro Benedetto, Elena Biasibetti, Yann Guitton, Bruno Le Bizec, Gaud Dervilly, Elena Bozetta

Received: 22 March 2021 | Revised: 14 June 2021 | Accepted: 7 July 2021

DOI: 10.1002/dta.3126

SPECIAL ISSUE - RESEARCH ARTICLE

WILEY

Nandrolone and estradiol biomarkers identification in bovine urine applying a liquid chromatography high-resolution mass spectrometry metabolomics approach

Saadia Ouzia[1] | Anne-Lise Royer[1] | Marzia Pezzolato[2] | Alessandro Benedetto[2] | Elena Biasibetti[2] | Yann Guitton[1] | Bruno Le Bizec[1] | Elena Bozetta[2] | Gaud Dervilly[1]

[1]Oniris, INRAE, LABERCA, Nantes, France

[2]Centro di Referenza Nazionale Indagini Biologiche Anabolizzanti Animali - CIBA, Experimental Zooprophylactic Institute of Piedmont, Liguria and Valle d'Aosta, Torino, Italy

Correspondence
Gaud Dervilly and Saadia Ouzia, Oniris, INRAE, LABERCA, Nantes, 44307 France.
Email: gaud.dervilly@oniris-nantes.fr; sadiaa.ouzia@oniris-nantes.fr

Abstract

With the aim of specifically investigating patterns associated with three steroid treatments (17β-nandrolone, 17β-estradiol, and 17β-nandrolone + 17β-estradiol) in bovine, an reversed phase liquid chromatography (RPLC)-electrospray ionization (ESI) (+/−)-high-resolution mass spectrometry (HRMS) study was conducted to characterize the urinary profiles of involved animals. Although specific fingerprints with strong differences could be highlighted between urinary metabolite profiles within urine samples collected on control and treated animals, it appeared further that significant discriminations could also be observed between steroid treatments, evidencing thus specific patterns and candidate biomarkers associated to each treatment. An MS-2 structural elucidation step enabled level-1 identification of two biomarkers mainly involved in energy pathways, in relation to skeletal muscle functioning. These results make it possible to envisage a global strategy for the detection of anabolic practices involving steroids, while at the same time providing clues as to the compounds used, which would facilitate the confirmation stage to follow.

KEYWORDS
biomarkers, bovine, pathways, screening, steroids

1 | INTRODUCTION

Although the use of anabolic compounds has been banned in livestock since the late '80s,[1] the new regulatory scheme induced by the application of the Official Control Regulation[2] and repealing Dir 96/22/EC[3] confirms this provision by defining "A substances" as "Prohibited or unauthorised pharmacologically active substances which may be used for illegal treatment in food producing animals" listing "A1 substances" as "Substances with hormonal and thyrostatic action and beta agonists the use of which is prohibited under Dir 96/22/EC." Steroids in particular are being listed as A1c substances under this new regulatory framework. Thus, Europe firmly reaffirms its position with regard to these substances in livestock and, with a public health perspective, its commitment to the performance of the associated controls. Although the analytical strategies developed by the control laboratories rely on robust targeted mass spectrometric approaches to identifying and confirming steroids abuse,[4–10] it appears that the preliminary screening stage is limiting in that it is confronted with certain critical points such as the use of natural steroid hormones, synthetic ones whose structure is not described yet or the use of low-dose cocktails. These shortcomings led over the last past 15 years to the development of innovative untargeted approaches, consisting in the investigation of the physiological effects induced as a consequence of illegal practices.

The objective is to reveal biomarkers of effect that may subsequently be monitored for screening purposes. Such so-called omics strategies mainly referring to the study of changes in mRNA-expression, protein or small molecule profiles, have already proven their relevance using respectively either transcriptomics,[11–14] proteomics,[15–18] or metabolomics.[19–33]

Compared with other omics strategies, metabolomics gathered more interest from the residue-control world because it involves analytical platforms similar to those already available in laboratories in this area. Furthermore, biomarkers evidenced upon metabolomics studies are considered as easier to subsequently monitor since involving targeted analytical strategies close to those already available in corresponding laboratories in charge of the control.

Although metabolomics research work performed up to now is a mandatory step in initial assessment of the strategy, steps toward official or commercial implementation of corresponding screening tools are still to be taken,[34,35] and so far only one metabolomics-based screening method is reported accredited and officially implemented in national control and monitoring plans, targeting the use of β-agonists compounds.[22] The reason this strategy could be brought to effective implementation is because the various stages of biomarkers validation have characterized them as sufficiently specific to β-agonists administration, but generic markers are enough to address the effect of the whole β-agonist family of compounds. Consequently, monitoring these biomarkers made it a suitable tool for detecting the use of known, new or even low dose β-agonists compounds. With regard to steroids, numerous metabolomics studies have also revealed disturbed profiles, and some biomarkers have been identified.[29,36] However, it appears that the knowledge generated is insufficiently robust at this stage to select specific biomarkers of steroid effects in farm animals. Indeed, the models proposed in the literature often appear overfitted; that is, they have the ability to properly describe the samples involved in the study in question but do not allow to predict samples collected outside this specific scheme, not allowing to generalize the approach.

In order to provide additional information and feed the subject, this study proposes to include three types of administration protocols involving the use of steroids in young cattle to determine whether it is possible to identify specific markers common to these three treatments or on the contrary whether the profiles are specific to each treatment. The first option would allow consideration of a nandrolone and/or estradiol screening tool in general, whereas the second option should lead to thinking about specific tools for each case.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Reagents and solvents were of LC–MS grade quality. Acetonitrile and acetic acid were purchased from Honeywell Chromasolv (Bucharest, Romania) and water from VWR (Fontenay-sous-Bois, France). Isotope-labeled internal standards, namely, L-leucine-5,5,5-d3, L-tryptophan-2,3,3-d3, indole-2,4,5,6,7-d5–3-acetic acid, and 1,14-tetradecanedioic-d24 acid were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France) and CDN Isotopes (Québec, Canada). Pure chemical standards for targeted MS2 confirmation were from Sigma-Aldrich and Acros Organic. MSCAL6 ProteoMass LTQ/FT-Hybrid, standard mixtures used for calibration of the MS instrument (positive and negative ionization mode) were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France).

2.2 | Samples

According to ethical agreement of the University of Turin (Italy) and to experimental authorization of the Italian Ministry of Health, 13 Friesian male veal calves aged between 15 and 35 days were randomly divided in groups and housed in box under the same controlled conditions for 6 months according to Council Directive 86/609/EEC. To prevent infections, the animals were vaccinated against IBR, Para influenza (PI3), BRSV, and BVDV (CATTLEMASTER_4 Pfizer Animal Health, New York, USA). Clinical evaluation was carried out daily by a veterinarian and included a daily observation and, if necessary, a physical assessment. Treatments for occurring infections were performed without using hormonal active substances. During 6-month veal, calves were treated by intramuscular injection (IM) in the neck with 17β-nandrolone decanoate ester (DECADURABOLIN®) or/and 17β-estradiol cypionate ester (ESTRADIOL DEPO®). Animals have been treated with steroids on 4 time points (D0, D7, D14, and D21). For each injection time point, five of them received 50-mg 17β-nandrolone decanoate (ANDR), the other five received 3-mg 17β-estradiol cypionate (ESTR), and the remaining three were treated with the mixture of the two steroid hormones consisting of 50-mg 17β-nandrolone decanoate + 3 mg 17β-estradiol cypionate (ANDR + ESTR). Control urine samples (CONTR, $n = 13$, collected at D0, before the first administration) were collected from each animal. In total, 78 urine samples were collected for about a month at regular time points, before and after the injections. All the samples were stored at −20°C prior analysis.

2.3 | Sample preparation

An aliquot of 500 μl of each urine sample was thawed on ice. After 10 sec of vortex and 5 min of centrifugation at 750*g* at 4°C. Afterwards, the different aliquots were normalized with LC–MS quality water through dilution according to their specific gravity (SG), measured by refractometry (Digital Urine Specific Gravity Refractometer, 4410 [PAS-10s], Cole-Parmer, USA), in order to obtain a final reference specific gravity (SGref) of 1.003 for each sample. Then, a quality control (QC) was created with an equal volume of all the urine samples. To eliminate the proteins, 350 μl of the recovered supernatant were centrifuged in a 10 kDa at 14,000*g* for 30 min at 6°C. Before the liquid chromatography high-resolution mass

spectrometry (LC-HRMS) analysis, 30 and 300 µl of samples and QC filtrate were deposited, respectively, in insert vials already containing 10 and 100 µl at 2 ng/µl of evaporated mix metabolomics (L-leucine-5,5,5-d3, L-tryptophan-2,3,3-d3, indole-2,4,5,6,7-d5–3-acetic acid, and 1,14-tetradecanedioic-d24 acid) used as internal standards.

2.4 | Liquid chromatography

The chromatographic separation was performed on a Hypersil Gold C18 column (2.1 × 100 mm, 1.9 µm particle size, Thermo Fisher Scientific) using a high-performance liquid chromatography (HPLC) system equipped with a pump (1260 QuAT, Agilent Technologies, Santa Clara, CA, USA) and an autosampler (CTC Analytics AG, Switzerland). Elution solvents were 0.1% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B). The elution gradient (A:B, v/v) was as follows: 95:5 from 0 to 2.40 min; 75:25 at 4.50 min; 30:70 at 11 min; 0:100 at 14–16.5 min; 95:5 at 19–25 min. The flow rate was set at 0.4 ml/min, the injection volume was 5 µl, and the column's temperature was 35°C.

2.5 | High-resolution mass spectrometry

Acquisition was performed on an Exactive-Orbitrap system (Thermo Fisher Scientific, Bremen, Germany) in both positive and negative (switch polarity) electrospray ionization mode (ESI+/−). The spectrometric parameters optimized similarly in ESI+/− are as follows: spray voltage 3.0 kV, capillary temperature 350°C, sheath gas flow rate 55 AU, gas flow rate 10 AU, and heater temperature 50°C. The spectrometric parameters optimized differently in ESI+/− are as follows: capillary voltage 3.0 (ESI+) to 2.5 kV (ESI−). The full scan mass spectra were acquired from 65 to 1,000 m/z with a mass resolution of 25,000 FWHM (at m/z 200) in centroid mode and a maximum time injection of 200 ms. Xcalibur (version 2.2 SP1 48 1.1-135305/1.1.4.1354) integrated software was used for data acquisition.

Regarding the structural identification part of the study, targeted MS2 was performed on a selection of metabolites. The chromatographic system was an UltiMate® 3000 Series HPLC system coupled to a hybrid quadrupole-orbitrap (Q-Exactive™) mass spectrometer (ThermoFisher Scientific, Bremen, Germany) equipped with a heated electrospray (H-ESI II) source. Two inclusion lists of selected ions were used for subsequent fragmentation in the positive or negative targeted-MS2 mode using higher energy collisional dissociation (HCD) after unit mass quadrupole filtering with parameters as follows: resolving power 17,500 FWHM (at 200 m/z); AGC Target, 2×10^5; maximum IT, 100 ms; isolation window 1.0 m/z, and normalized collision energy (NCE) at 35%.

2.6 | Quality controls

In order to guarantee and then verify the quality of the data, a set of measures were implemented within the framework of this study, as follows: (i) the samples were randomly distributed during the injection sequence; (ii) the mixture of four isotope-labeled internal standards had been added to each sample before HPLC-HRMS analysis in order to evaluate the retention time stability, the consistency of the signal intensities and mass accuracy within sequence; (iii) the same QC pool was injected at regular intervals throughout the sequence; (iv) the LC-HRMS instrument was cleaned and calibrated before the injection sequence; and v) the quality of the chromatograms (total ion chromatogram [TIC]) has been manually controlled before data processing.

2.7 | Data preprocessing and availability

After converting the raw data files (*.raw) to (*.mzML) files using MSConvert software (ProteoWizard version 3.0.11537), a data preprocessing step was performed on the platform Workflow4Metabolomics.org (version 3.3),[37] with essentially XCMS[38] (version 3.4.4)) and CAMERA (version 2.2.4) packages (parameters are available in Table S1). To account for the matching of data (same animals before and after treatment), a multilevel transformation was applied to all datasets studied.[39,40] In order to take into account of the particular nature of the mass spectrometry data, a log10 transformation and a "Pareto" scaling were applied. All data are available on the Metabolights repository (https://www.ebi.ac.uk/metabolights/study/MTBLS2474/). The whole data processing is available as reference history W4M00009 (https://workflow4metabolomics.usegalaxy.fr/histories/list_published).[41]

2.8 | Statistical analysis

Statistical analysis was carried out using both the Workflow4Metabolomics platform (version 3.3)[37] and SIMCA-P+® (Version 13.0.2, Umetrics AB, Sweden) software. After checking data quality with unsupervised principal component analysis (PCA) to assess QC grouping and potential outliers, partial least squares discriminant analysis (PLS-DA) supervised analyzes were performed to investigate further the data. The validity and the robustness of the models were evaluated by the diagnostic criteria of the model R2 (Y) and Q2 (Y), as well as by permutation tests (N permutations = 1,000). The p-value threshold considered was set < 0.05. Then, only the discriminant ions exhibiting variable influence in projection (VIP) > 1.5 were selected. Finally, a representative OPLS-DA loading S-plot was achieved to present the relative distribution patterns and the expression level of the discriminating selected ions (not shown).

2.9 | Structural identification of candidate biomarkers

The identification step was performed for a set of selected signals. As a first step, the signals were compared with an internal database

comprising >800 metabolites, including information as exact mass of the ions and retention time. The identification thus obtained corresponds to the level 2 of identification,[42] it is based on the m/z ratio (Delta = 10 ppm) and the retention time (Delta = 60 s). In a second step, a confirmatory analysis was carried out performing targeted MS2 analysis of the corresponding pure analytical standard.

3 | RESULTS AND DISCUSSION

3.1 | Data quality

Quality of the data has been assessed checking internal standards criteria (Delta RT < 1 s, Delta m/z < 10 ppm, signal intensity CV < 30%) in the analytical sequences. Further, QCs on the PCA (Figure 1a) were evaluated, justifying subsequent data normalization using the "Lowess" method available within the Workflow4Metabolomics normalization tool that successfully enabled grouping of the QC samples (Figure 1b).[43] Finally, ions exhibiting a signal variability above 30% (QC.CV > 30%) were discarded.

3.2 | Investigation of bovine urine metabolome-specific patterns upon steroid treatments

In the present study, a protocol whose relevance and robustness have already been proven within the laboratory to address similar topic has been implemented.[20] It allowed investigating the effect of three different steroid treatments in bovine urine.

3.2.1 | Characterization of steroid effect on urinary metabolome: Control versus treated animals

In a first step and to individually assess the effects associated to the three steroid-based treatments, urine samples datasets from control animals have been compared with those collected after steroid administrations, that is, CONTR versus ANDR, CONTR versus ANDR + ESTR, and CONTR versus ESTR conditions. The PLS-DA supervised multivariate analysis revealed the associated effect on cattle metabolism as detailed hereafter. Validated models enabled confirming for the three considered cases that most of the variance in animal status could be explained by the respective models that highlighted a strong effect of the various steroid treatments and the capacity of such a metabolomics approach to describe them (Supporting Information).

Control versus nandrolone

Investigating nandrolone effect on urinary metabolome was achieved comparing ANDR and CONTR datasets. Corresponding PLS-DA resulted in highlighting 279 (ESI+) and 268 (ESI−) ions as significantly different between both groups (S-plot, VIP > 1.5). Parameters of both models were similar as follows: R2Y = 0.914, Q2Y = 0.642, pR2Y = 2e-03, and pQ2Y = 2e-03 in ESI+ and R2Y = 0.92, Q2Y = 0.687, pR2Y = 1e-03, and pQ2Y = 1e-03 in ESI− (Figure 2a), attesting for significant impact of nandrolone on the urinary metabolome of treated animals.

Control versus estradiol

Investigating estradiol effect on urinary metabolome was achieved comparing ESTR and CONTR datasets. Corresponding PLS-DA analysis resulted in highlighting 251 (ESI+) and 258 (ESI−) ions as significantly different between both groups. Parameters of both models were similar as follows: R2Y = 0.943, Q2Y = 0.661, pR2Y = 3e-03, and pQ2Y = 1e-03 (ESI+) and R2Y = 0.948, Q2Y = 0733, pR2Y = 3e-03, and pQ2Y = 1e-03 (ESI−) (Figure 2b). As observed above with nandrolone, in the case of estradiol administration, also the urinary metabolome of the treated animals appeared as deeply modified.

Control versus nandrolone + estradiol

Finally, the effect of the mixed nandrolone and estradiol combined treatment was studied on the basis of CONTR and ANDR + ESTR datasets analysis. Generated PLS-DA enabled highlighting 287 and 256 ions in ESI+ and ESI− modes, resp., as significantly different between both groups. Performances of associated models were as follows: R2Y = 0.896, Q2Y = 0.609, pR2Y = 1e-03, and pQ2Y = 1e-03 (ESI+) and R2Y = 0.894, Q2Y = 0.619, pR2Y = 1e-03, and pQ2Y = 1e-03 (ESI−) (Figure 2c). Here again, the descriptive models generated make it possible to conclude as to the significant effect of the steroid treatment applied on the urinary metabolome of the animals involved in the study.

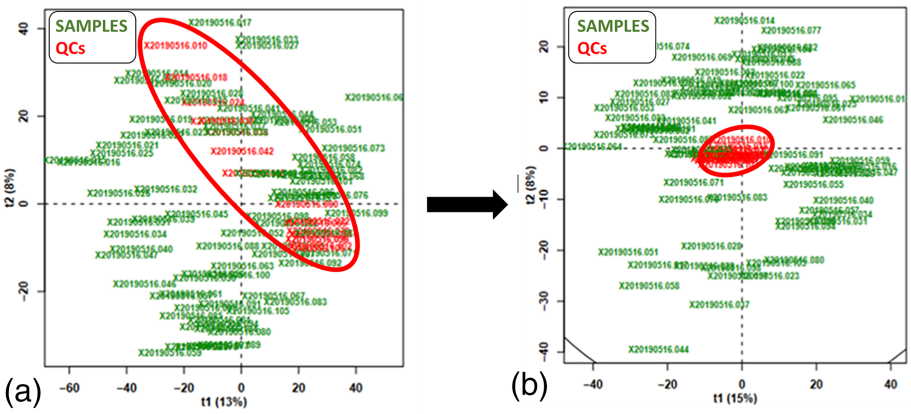

FIGURE 1 Principal component analysis (PCA) (score plot) performed on metabolomics dataset (RP, ESI+), before (a) and after (b) lowess standardization on quality control (QC) (pool)

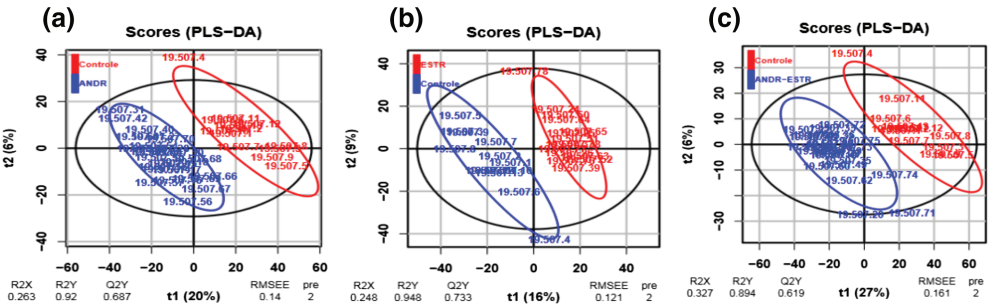

FIGURE 2 Partial least squares discriminant analysis (PLS-DA) score on ESI− datasets (a: ANDR vs. CONTR, b: ESTR vs. CONTR, and c: ANDR + ESTR vs. CONTR

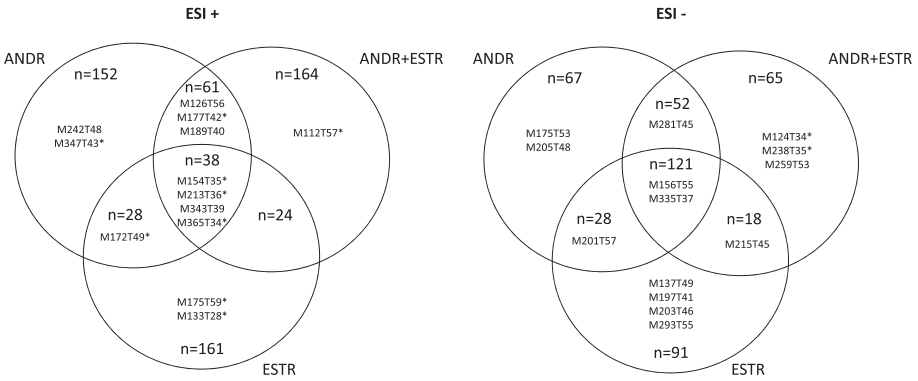

FIGURE 3 Venn diagram of the number of discriminant ions by conditions investigated or in common between treatments as well as the metabolites annotated in both ESI+/− datasets. (MxxTyy: M exact mass and T retention time in sec, * lower urinary concentration in considered group)

Conclusions on control versus treated animals study

The comparison of the ions highlighted as presenting significantly altered profiles between the groups studied also made it possible to observe similarities of effects between the three steroid treatments. In positive and negative ionization mode, resp., 38 and 121 discriminating ions were common for the three treatments. Further, 61 and 52 were observed as only common to ANDR and ANDR + ESTR, 28 and 28 to ANDR and ESTR, and finally 24 and 18 to ESTR and ANDR + ESTR groups (Figure 3). It should be noticed here that because of adducts and ionization modes, some metabolites may be monitored under several forms; therefore, this number of common ions does not necessarily correspond to the number of common metabolites.

3.2.2 | Comparison of the different steroid treatments and their effects

Although the preliminary study described above made it possible to highlight significant differences between the metabolomes of the control animals and those of the animals having been treated by three different steroid protocols, the remainder of the study was interested in the comparison of specific induced effects caused by these treatments, which are considered to be probable in breeding. Indeed, although some ionic signals appeared to be impacted in the same way by the three treatments (Figure 3, $n = 121$ ions), allowing to conclude as to probable similar mechanisms of action, these results also pointed to the fact that a certain number of ions were not shared between the conditions studied and could be related to specific effects. The exploration of this aspect then motivated the rest of the work, therefore focusing on ANDR, ESTR, and ANDR + ESTR datasets investigation for pair comparison purposes. Whatever the conditions compared, the PLS-DA models could explain most of the variance in animal status, as detailed hereafter.

Estradiol versus nandrolone effects

Nandrolone and estradiol respective effects on the bovine urinary metabolome were studied using ANDR and ESTR datasets. Corresponding PLS-DA resulted in highlighting 273 (ESI+) and 255 (ESI−) ions as significantly different between both groups. Parameters of both models were similar as follows: R2Y = 0.926, Q2Y = 0.638, pR2Y = 1e-03, and pQ2Y = 1e-03 (ESI+) and R2Y = 0.893, Q2Y = 0.574, pR2Y = 9e-03, and pQ2Y = 1e-03 (ESI−) (Figure 4a), confirming that both treatments also induce specific effects, which may be highlighted with the current approach.

Nandrolone versus nandrolone + estradiol effects

Comparing the specific effects associated to nandrolone alone or nandrolone in combination with estradiol was then achieved using ANDR and ANDR + ESTR datasets. Descriptive PLS-DA models with similar performances in both ionization modes could be obtained as follows: R2Y = 0.930, Q2Y = 0.457, pR2Y = 1e-03, and pQ2Y = 1e-03 (ESI+) and R2Y = 0.960, Q2Y = 0.482, pR2Y = 1e-03, and pQ2Y = 2e-03 (ESI−) (Figure 4e). Both groups could be differentiated by 280 ions in ESI+ and 226 ions in ESI−. In the present case, although the model exhibited very good descriptive ability (R2Y), confirming that the use of a cocktail of an androgen with an estrogen induces different effects than with the androgen alone. In the present model, the predictive performance (Q2Y) as determined by cross-validation however appeared as less satisfactory than in the other models generated within the present study.

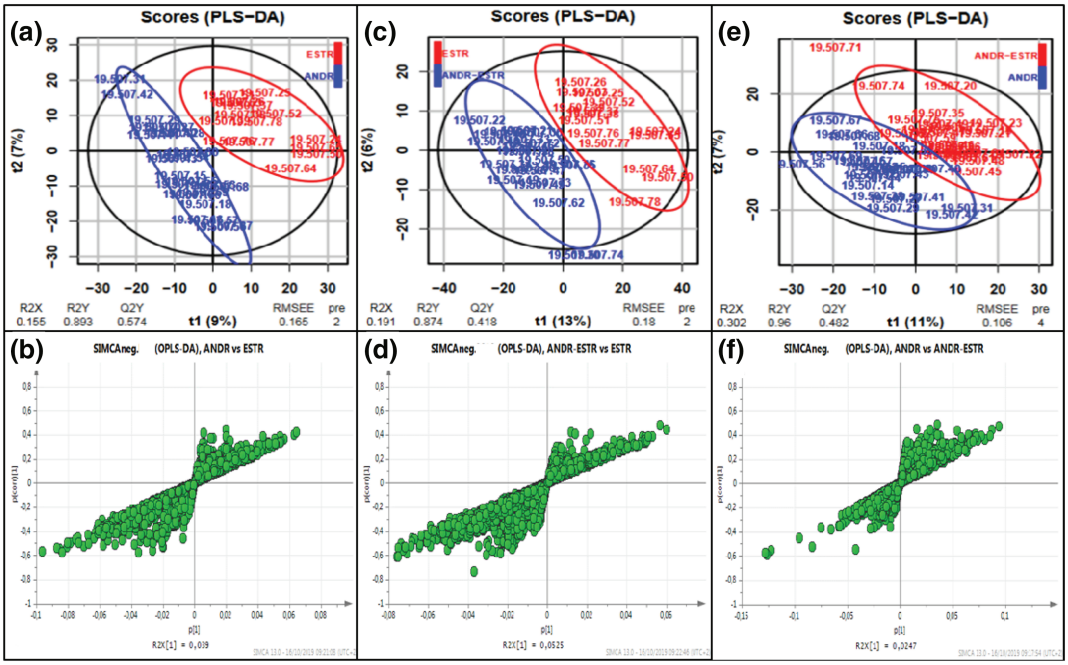

FIGURE 4 Partial least squares-discriminant analysis (PLS-DA) score and S-plots on ESI− datasets (a and b: ANDR [blue] vs. ESTR [red]; c and d: ESTR [red] vs. ANDR + ESTR [blue]; and e and f: ANDR [blue] vs. ANDR + ESTR [red])

Estradiol versus nandrolone + estradiol effects

Finally, the specific effects associated to estradiol were compared with those induced by estradiol in combination with nandrolone. The analysis of ESTR and ANDR + ESTR datasets enabled generating PLS-DA models as follows: R2Y = 0.893, Q2Y = 0.551, pR2Y = 1e-03, and pQ2Y = 1e-03 (ESI+) (273 ions) and R2Y = 0.874, Q2Y = 0.418, pR2Y = 2e-03, and pQ2Y = 1e-03 (ESI−) (239 ions) (Figure 4c). These robust performances again make it possible to conclude here as to the ability to distinguish the metabolic profiles of the urine of bovines treated with estradiol alone or in a cocktail with nandrolone, thereby indicating effects specific to these two conditions.

The ions significantly associated with the discrimination between the groups studied were selected using the S-plot of the PLS-DA (Figure 4b,d,f) and considering a VIP > 1.5 confirming the previous observations, thus listing a certain number of signals as specifically associated with one of the steroid treatments or else common to two of them or even all three (Supporting Information).

3.3 | Biomarkers investigation

Although a number of ions of interest could be specifically associated in bovine urine with nandrolone, estradiol, or a mix of nandrolone and estradiol treatment, the next step consisted in their tentative structural elucidation. Although such a step is recognized the main metabolomics bottleneck,[44] 28 "level-2" candidate biomarkers could be further investigated using targeted MS2 strategy to reach a "level-1" identification level for 2 of them. For level-1 identifications, detailed MS and MS/MS confirmation in the Supporting Information.

Two of the level-2 biomarkers were identified in the ESI+ generated fingerprints. In particular, M189T40 was assigned to $[M + H]^+$ of homoarginine, which was observed with higher concentration levels in androgen treated animals. The ion M133T28 was assigned to the $[M + H]^+$ of ornithine, presenting lower concentration in E2-treated animals. Finally, the only level-1 ion identified in ESI+ is M154T35 and was assigned to $[M + Na]^+$ of creatine, showing lower concentration level in the urine of cattle treated with any of the steroids considered.

In the ESI− fingerprints, the M124T34 could be identified (level-1) as $[M - H]^-$ of taurine. Taurine exhibited lower concentration levels in the urine of animals treated with 17β-estradiol.

Although the remaining ions listed in the Supporting Information could not be identified at level-1 despite MS2 efforts, these signals are worth being shared with the community because their concentration levels may provide robust evidence of steroid abuse in cattle.

The metabolites identified in the context of this study show that not only common but also specific metabolic pathways are activated by the various treatments studied. In particular, the pathways involving ornithine appear to be solicited only when estradiol alone is used, whereas creatine responds to treatment with estradiol whether used alone or as a cocktail. Taurine was selectively associated to the use of nandrolone combined with estradiol.

Creatine is a naturally occurring compound of which the primary metabolic role is to generate phosphocreatine, subsequently used to regenerate adenosine triphosphate (ATP), it is therefore essential for energy and muscle metabolism. This molecule is well known for its use by athletes, as it is indeed associated with muscle performance. It has further already been demonstrated as a biomarker of the effect of anabolic practices in livestock, in particular through a metabolomics study as well.[19,33,36] As in the previous study, creatine was observed

as less concentrated in the urine of treated animals, which corresponds to a decreased urinary excretion to the benefit of an increased use by the muscles upon anabolic solicitation.

Ornithine and homoarginine are metabolites involved in creatine biosynthesis, which is consequently not surprising having them also involved as candidate biomarkers within an anabolic framework.[45]

Taurine is involved in many biological roles, such as conjugation of bile acids, antioxidation, osmoregulation, membrane stabilization, and modulation of calcium signaling; it is therefore essential for a range of functions among them the development and function of skeletal muscle, which may explain in the present context its role as steroid biomarkers.[46] As creatine, it is observed in lower concentration in the urine of treated animals, enabling hypothesizing its increased use by the muscle.

4 | CONCLUSION

An reversed phrase liquid chromatography (RPLC)-HRMS metabolomics workflow has been applied to the characterization of urine samples collected on bovine calves treated with different steroid protocols with the aim of investigating both common and specific features. Although strong differences could be highlighted between urinary metabolite profiles within urine samples collected on control and treated animals, it appeared further that significant discriminations could also be observed between steroid treatments, evidencing thus specific patterns and candidate biomarkers associated to each treatment. Although robust models have been obtained, making it possible to plan for the development of tools for predicting the status of samples in the context of screening for such anabolic practices in breeding, two important steps must be taken: on the one hand, the validation of these models by carrying out challenge tests (prediction of independent samples), and on the other hand, markers identification. The lock of structural elucidation indeed remains because only two ions could be identified without ambiguity. The biological pathways associated with these few biomarker candidates are, however, relevant in that they involve energy pathways linked to the functioning of skeletal muscles. In the long term, it will be necessary to develop a targeted approach, typically by tandem mass spectrometry (QqQ), in order to reinforce the robustness of the measurement of these biomarkers and to increase the predictive potential of the model, as well as to make the strategy applicable in laboratories that do not have an HRMS system.

ACKNOWLEDGEMENT

The Corsaire metabolomics core facility through MELISA platform (Biogenouest) is acknowledged for its involvement in this research work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Gaud Dervilly https://orcid.org/0000-0002-1867-0008

REFERENCES

1. Council Directive 88/146/EEC. Prohibiting the use livestock farming of certain substances having a hormonal action. 1988 (16):16-18.
2. European Parliament and Council. REGULATION (EU) 2017/625 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products, amending Regulations (EC) No 999/2001, (EC) No 396/2005, (EC) No 1069/2009, (EC) No 1107/2009, (EU) No 1151/2012, (EU) No 652/2014, (EU) 2016/429 and (EU) 2016/2031 of the European Parliament and of the Council, Council Regulations (EC) No 1/2005 and (EC) No 1099/2009 and Council Directives 98/58/EC, 1999/74/EC, 2007/43/EC, 2008/119/EC and 2008/120/EC, and repealing Regulations (EC) No 854/2004 and (EC) No 882/2004 of the European Parliament and of the Council, Council Directives 89/608/EEC, 89/662/EEC, 90/425/EEC, 91/496/EEC, 96/23/EC, 96/93/EC and 97/78/EC and Council Decision 92/438/EEC (Official Controls Regulation). In. OJ L 95, 7, 4, 2017, pp. 1-142.
3. Council Directive 96/22/EC. Council Directive 96/22/EC of 29 April 1996 concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of beta-agonists, and repealing Directives 81/602/EEC, 88/146/EEC and 88/299/EEC. 1996.
4. De Brabander HF, Le Bizec B, Pinel G, 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):983-998.
5. Dervilly-Pinel G, Rambaud L, Sitthisack P, et al. 5alpha-Estrane-3beta,17beta-diol and 5beta-estrane-3alpha,17beta-diol: definitive screening biomarkers to sign nandrolone abuse in cattle? *J Steroid Biochem Mol Biol*. 2011;126(3–5):65-71.
6. Destrez B, Bichon E, Rambaud L, et al. Criteria to distinguish between natural situations and illegal use of boldenone, boldenone esters and boldione in cattle 2. Direct measurement of 17beta-boldenone sulpho-conjugate in calf urine by liquid chromatography–high resolution and tandem mass spectrometry. *Steroids*. 2009;74(10–11):803-808.
7. Doue M, Dervilly-Pinel G, Pouponneau K, Monteau F, Le Bizec B. Analysis of glucuronide and sulfate steroids in urine by ultra-high-performance supercritical-fluid chromatography hyphenated tandem mass spectrometry. *Anal Bioanal Chem*. 2015;407(15):4473-4484.
8. Le Bizec B, Pinel G, Antignac JP. Options for veterinary drug analysis using mass spectrometry. *J Chromatogr A*. 2009;1216(46):8016-8034.
9. Pinel G, Rambaud L, Cacciatore G, et al. Elimination kinetic of 17beta-estradiol 3-benzoate and 17beta-nandrolone laureate ester metabolites in calves' urine. *J Steroid Biochem Mol Biol*. 2008;110(1–2):30-38.
10. Pinel G, Rambaud L, Monteau F, Elliot C, Le Bizec B. Estranediols profiling in calves' urine after 17 beta-nandrolone laureate ester administration. *J Steroid Biochem*. 2010;121(3–5):626-632.
11. Riedmaier I, Becker C, Pfaffl MW, Meyer HH. The use of omic technologies for biomarker development to trace functions of anabolic agents. *J Chromatogr A*. 2009;1216(46):8192-8199.
12. Riedmaier I, Benes V, Blake J, et al. RNA-sequencing as useful screening tool in the combat against the misuse of anabolic agents. *Anal Chem*. 2012;84(15):6863-6868.
13. Riedmaier I, Tichopad A, Reiter M, Pfaffl MW, Meyer HH. Influence of testosterone and a novel SARM on gene expression in whole blood of Macaca fascicularis. *J Steroid Biochem Mol Biol*. 2009;114(3–5):167-173.
14. Reiter M, Walf VM, Christians A, Pfaffl MW, Meyer HH. Modification of mRNA expression after treatment with anabolic agents and the

usefulness for gene expression-biomarkers. *Anal Chim Acta*. 2007; 586(1–2):73-81.
15. Nebbia C, Urbani A, Carletti M, et al. Novel strategies for tracing the exposure of meat cattle to illegal growth-promoters. *Vet J*. 2011; 189(1):34-42.
16. Cacciatore G, Eisenberg SW, Situ C, et al. Effect of growth-promoting 17beta-estradiol, 19-nortestosterone and dexamethasone on circulating levels of nine potential biomarker candidates in veal calves. *Anal Chim Acta*. 2009;637(1–2):351-359.
17. Mooney MH, Bergwerff AA, van Meeuwen JA, Luppa PB, Elliott CT. Biosensor-based detection of reduced sex hormone-binding globulin binding capacities in response to growth-promoter administrations. *Anal Chim Acta*. 2009;637(1–2):235-240.
18. Mooney MH, Elliott CT, Le Bizec B. Combining biomarker screening and mass-spectrometric analysis to detect hormone abuse in cattle. *TrAC Trends Anal Chem*. 2009;28(6):665-675.
19. Courant F, Pinel G, Bichon E, Monteau F, Antignac JP, Le Bizec B. Development of a metabolomic approach based on liquid chromatography-high resolution mass spectrometry to screen for clenbuterol abuse in calves. *Analyst*. 2009;134(8):1637-1646.
20. Dervilly-Pinel G, Chereau S, Cesbron N, Monteau F, Le Bizec B. LC-HRMS based metabolomics screening model to detect various β-agonists treatments in bovines. *Metabolomics*. 2015;11(2):403-411.
21. Dervilly-Pinel G, Courant F, Chereau S, et al. Metabolomics in food analysis: application to the control of forbidden substances. *Drug Test Anal*. 2012;4:59-69.
22. Dervilly-Pinel G, Royer AL, Bozzetta E, et al. When LC-HRMS metabolomics gets ISO17025 accredited and ready for official controls—application to the screening of forbidden compounds in livestock. *Food Addit Contam Part A-Chem Anal Control Expos Risk Assess*. 2018;35(10):1948-1958.
23. Dervilly-Pinel G, Weigel S, Lommen A, et al. Assessment of two complementary liquid chromatography coupled to high resolution mass spectrometry metabolomics strategies for the screening of anabolic steroid treatment in calves. *Anal Chim Acta*. 2011;700(1–2):144-154.
24. Gallart Ayala H, Chéreau S, Dervilly-Pinel G, Le Bizec B. Potential of mass spectrometry metabolomics for chemical food safety. *Bioanal Rev*. 2015;7(1):133-146.
25. Guitton Y, Dervilly-Pinel G, Jandova R, Stead S, Takats Z, Le Bizec B. Rapid evaporative ionisation mass spectrometry and chemometrics for high-throughput screening of growth promoters in meat producing animals. *Food Addit Contam Part A-Chem Anal Control Expos Risk Assess*. 2018;35(5):900-910.
26. Jacob C, Dervilly-Pinel G, Biancotto G, Monteau F, Le Bizec B. Global urine fingerprinting by LC-ESI(+)-HRMS for better characterization of metabolic pathway disruption upon anabolic practices in bovine. *Metabolomics*. 2014;11:184-197.
27. Kieken F, Pinel G, Antignac J, et al. Generation and processing of urinary and plasmatic metabolomic fingerprints to reveal an illegal administration of recombinant equine growth hormone from LC-HRMS measurements. *Metabolomics*. 2011;7:84-93.
28. Kieken F, Pinel G, Antignac JP, et al. Development of a metabonomic approach based on LC-ESI-HRMS measurements for profiling of metabolic changes induced by recombinant equine growth hormone in horse urine. *Anal Bioanal Chem*. 2009;394(8):2119-2128.
29. Kouassi Nzoughet J, Dervilly-Pinel G, Chereau S, et al. First insights into serum metabolomics of trenbolone/estradiol implanted bovines: screening model to predict hormone-treated and control animals' status. *Metabolomics*. 2015;11(5):1184-1196.
30. Kouassi Nzoughet J, Gallart-Ayala H, Dervilly-Pinel G, Biancotto G, Le Bizec B. Original combination of hydrophilic interaction (HILIC) and reverse phase (RPLC) high resolution LC-MS for characterizing lipids profile disruption in serum of anabolic implanted bovines. *Metabolomics*. 2015;11(6):1884-1895.
31. Marchand J, Martineau E, Guitton Y, Le Bizec B, Dervilly-Pinel G, Giraudeau P. A multidimensional H-1 NMR lipidomics workflow to address chemical food safety issues. *Metabolomics*. 2018;14(5):60.
32. Peng T, Royer AL, Guitton Y, Le Bizec B, Dervilly-Pinel G. Serum-based metabolomics characterization of pigs treated with ractopamine. *Metabolomics*. 2017;13(6):1-15.
33. Pinel G, Weigel S, Antignac JP, et al. Targeted and untargeted profiling of biological fluids to screen for anabolic practices in cattle. *TrAC Trends Anal Chem*. 2010;29(11):1269-1280.
34. Esslinger S, Riedl J, Fauhl-Hassek C. Potential and limitations of non-targeted fingerprinting for authentication of food in official control. *Food Res Int*. 2014;60:189-204.
35. Riedl J, Esslinger S, Fauhl-Hassek C. Review of validation and reporting of non-targeted fingerprinting approaches for food authentication. *Anal Chim Acta*. 2015;885:17-32.
36. Pinel G, Weigel S, Lommen A, et al. Assessment of two complementary LC-HRMS metabolomics strategies for the screening of anabolic steroid treatment in calves. *Anal Chim Acta*. 2011;700(1–2):144-154.
37. Giacomoni F, Le Corguille G, Monsoor M, et al. Workflow4Metabolomics: a collaborative research infrastructure for computational metabolomics. *Bioinformatics*. 2015;31(9):1493-1495.
38. Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching and identification. *Anal Chem*. 2006;78(3):779.
39. Liquet B, Cao K, Hocini H, Thiébaut R. A novel approach for biomarker selection and the integration of repeated measures experiments from two assays. *BMC Bioinf*. 2012;13(325):1-14.
40. Westerhuis J, van Velzen E, Hoefsloot H, Smilde A. Multivariate paired data analysis: multilevel PLSDA versus OPLSDA. *Metabolomics*. 2010;6(1):119-128.
41. Guitton Y, Tremblay-Franco M, Le Corguille G, et al. Create, run, share, publish, and reference your LC-MS, FIA-MS, GC-MS, and NMR data analysis workflows with the Workflow4Metabolomics 3.0 Galaxy online infrastructure for metabolomics. *Int J Biochem Cell Biol*. 2017;93:89-101.
42. Schymansky E, Jeon J, Gulde R, et al. Identifying small molecules via high resolution mass spectrometry: communicating confidence. *Environ Sci Technol*. 2014;48(4):2097.
43. van der Kloet FM, Bobeldijk I, Verheij ER, Jellema RH. Analytical error reduction using single point calibration for accurate and precise metabolomic phenotyping. *J Proteome Res*. 2009;8(11):5132-5141.
44. Chaleckis R, Meister I, Zhang P, Wheelock C. Challenges, progress and promises of metabolite annotation for LC–MS-based metabolomics. *Curr Opin Biotechnol*. 2019;55:44-50.
45. (KEGG). KEoGaG. Arginine and Proline metabolism. Creatine pathway. https://www.genome.jp/pathway/map 00330+M00047. Accessed 11th June, 2021.
46. Spriet LL, Whitfield J. Taurine and skeletal muscle function. *Curr Opin Clin Nutr Metab Care*. 2015;18(1):96-101.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Ouzia S, Royer A-L, Pezzolato M, et al. Nandrolone and estradiol biomarkers identification in bovine urine applying a liquid chromatography high-resolution mass spectrometry metabolomics approach. *Drug Test Anal*. 2022;14(5):879-886. https://doi.org/10.1002/dta.3126