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First insights into serum metabolomics of trenbolone/estradiol implanted bovines; screening model to predict hormone-treated and control animals' status

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Abstract The use of anabolic agents in livestock production is a subject of much concern. Although prohibited for more than 20 years within the EU, growth promoting practices are still widely suspected. To meet the current challenges for detecting illicit practices, 'omics' strategies have recently been demonstrated as important new investigative tools. These investigations, based on the observation of physiological disturbances, mainly in urine, demonstrated the possibility to monitor biomarkers enabling high throughput determination of animal status in terms of hormonal treatment. In this context, serum was investigated for the first time as an alternative and potential complementary sample type. A metabolomic approach based on liquid chromatography coupled to high resolution mass spectrometry, was exploited in order to, highlight metabolic perturbations in serum of Revalor-XS[®] (trenbolone acetate/estradiol) implanted bovines. Univariate and multivariate statistical analyses were carried out to establish descriptive and predictive models. These models

enabled the discrimination of anabolised from control animals, and highlighted a number of metabolites which contributed the most in the observed discrimination. Further, a screening model combining a set of selected markers intensities was generated and it successfully classified animals according to their status, up to 4 weeks post Revalor-XS[®] implant. This research indicates, for the first time, that serum metabolomics has an important role in screening to detect for anabolic misuse in bovines.

Keywords LC-HRMS metabolomics · Screening · Steroids implant · Bovines · Serum

1 Introduction

The use of hormonal growth promoters ('anabolic') in livestock production is still a current topic and a subject of much debate. Very recently, in its newsletter published on the 4th April 2014, Global Meat News reported that Russia banned imports of both chilled and frozen beef from Australia as Trenbolone (steroid) was detected during inspections.

The main classes of growth promoters used in animal production include: steroid hormones (steroids), β 2-adrenergic substances (β -adrenergic agonists), and growth hormones (somatotropins). In meat production, growth promoters are used to increase productivity and to reduce costs by improving weight gain and feed efficiency (Moran et al. 1991; Lange et al. 2001). The purpose is to obtain more edible muscle meat while not increasing production costs i.e. to improve feed conversion rates and producing preferably more tender (e.g. beef) and/or leaner (e.g. pork). Thus economics is the main driver for growth promoter abuse (Stephany 2010). International disputes on the potential

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detrimental effects to human health of growth promoter residues carryover in foods still persist, leading to differing legislation around the world. Their use was banned in animal production in Europe (European Council Directive 88/146/EC 1988) principally as a precautionary safety measure (Serratos et al. 2006). The history behind the ban can be traced to a loss of European consumer confidence in meat production due to a scandal involving illegal use of a steroid (diethylstilbestrol) in Italy, which resulted in multiple cases of breast development in young children aged 3–13 years (Fara et al. 1979). This caused consumer organizations to lobby for a blanket Europe-wide ban on all hormones. Despite strong protests to the World Trade Organization from other regions of the world, most notably from the United States of America, arguing that it is a trade barrier restricting the importation of their products from hormone-treated animals to European Union (EU) markets (Mooney et al. 2009; Stephany 2010).

At the time of the initial imposition of the ban, there were only five licensed hormonal growth promoters: the naturally occurring 17β -estradiol, testosterone and progesterone, and two synthetic hormones, trenbolone and zeranol. Over time, people involved in these practices turned to alternative compounds, alternative formulations and alternative methods of administration to evade detection (Galbraith 2002).

Council Directive 96/23/EC (1996) and Commission Decision 2002/65/EC lay down the requirements for residue testing and performance of analytical methods in order to ensure compliance with EU prohibition. Although prohibited for more than 20 years within the EU, growth promoting practices are still suspected (Courtheyn et al. 2002). The confirmation of anabolic compounds administration is generally not an issue, though the screening stage constitutes a limiting factor. To meet the current challenges for screening illicit practices, ‘omics’ strategies, in particular metabolomics seems promising new tools (Riedmaier et al. 2009, Pinel et al. 2010, Nebbia et al. 2011, Graham et al. 2012). These approaches, based on the observation of physiological perturbations, may offer a reliable solution to tackle steroids abuse in livestock. Several metabolomics studies reporting mass spectrometry based urinary fingerprinting to discriminate anabolised from control animals, have shown this proof of concept for various screening applications such as growth hormone (Kieken et al. 2009; Boyard-Kieken et al. 2011), steroids (Rijk et al. 2009; Dervilly-Pinel et al. 2011, 2012; Jacob et al. 2013) as well as β -agonists (Courant et al. 2009; Dervilly-Pinel et al. 2014). Urine has primarily been the matrix of choice, due to the non invasive nature of the sample collection.

In the scope of the present research, serum was investigated as an alternative biological fluid and its potential to complement urine analysis was evaluated. Revalor-XS[®] is

a new steroid combination implant which has been approved in the United States for use in steers fed in confinement for slaughter. Revalor-XS[®] implant contains 200 mg trenbolone acetate and 40 mg estradiol as a slow release delivery system which increases rate of weight gain and improves feed efficiency for up to 200 days. The delayed release of trenbolone acetate and estradiol eliminates the need to re-implant cattle (FDA 2007). In beef steers, the use of a combination of implants containing trenbolone acetate and 17β -estradiol resulted in a 10–30 % increase in average daily gain (ADG) and 5–15 % improvement in body weight (BW) gain efficiency (Duckett et al. 1997; Preston 1999).

Recently, Jacob et al. (2014) reported a metabolomics workflow to compare cattle urinary metabolic profiles from Revalor-XS[®] implanted and non-implanted bovines. To complement the urine metabolomics models, the present study reports results obtained from an untargeted serum metabolomics strategy to reveal Revalor-XS[®] administration in bovines. Serum was assessed as the target matrix for screening of anabolic practices. The final objective of these studies is to reveal biomarkers of steroids administration that will be further assessed for their potential to predict a large range of steroid-based anabolic practices and that will also allow extended detection time windows compared to current strategies.

Liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) analytical platforms were employed for sera profiling, in polarity switching mode HESI+/-; data generated were processed, univariate and multivariate statistical analysis were performed, and models were evaluated for their descriptive and prediction capabilities. Further, a weighted equation combining a set of selected markers intensities was determined, to predict sample coordinates on the model and in the view of implementing the tool as screening model. To the best of our knowledge, this is the first time HRMS based metabolomics and serum have been combined to investigate steroid abuse in cattle.

2 Materials and methods

The recommendations of the metabolomic standards initiative (MSI) (Sumner et al. 2007) were considered during the present investigation, i.e., sample preparation, chromatography, MS, data processing and metabolite characterization steps.

2.1 Animal experimentation and biological samples collection

The experimentation was approved by the Animal Experimentation Ethical Committee of the University of Bologna

and carried out in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The animal management also meets the criteria of Directive 86/609/EEC regarding the protection of animals used for experimental or other scientific purposes, enforced by the Italian D Lgs n° 116 of 27th January 1992 and Directive 63/2010.

Thirty-two 10–14 months male beef cattle (Charolaise breed) were randomly divided into control ($n = 16$ animals) and treatment groups ($n = 16$ animals). Animals were allowed a sixteen day acclimatization period, following which, each animal from the treatment group received at day 0, a subcutaneous implant of Revalor-XS® (Merck Animal Health, NJ, USA), containing trenbolone acetate (200 mg, i.e., 20 mg/pellet) and estradiol (40 mg, i.e., 4 mg/pellet). Each implant contains ten pellets: four uncoated and six coated. Animals were implanted once at the start of the treatment, with the implant placed behind the ear by means of special implanter (Revalor Gun®) for subcutaneous administration of the steroids. During the experimentation, animals were normally fed; hay and water were available ad libitum.

Blood samples were collected at regular time points, for both treated (T) and control (C) animals, once a week over a 10 week period following implant administration. The blood was allowed to clot and serum was obtained by centrifugation, frozen in liquid nitrogen and then stored at -80°C . Data presented in the current study correspond to sera collected on day 8, day 22 and day 29 post implant.

2.2 Chemical and reagents

All solvents and reagents used in this study were of analytical or HPLC grade quality. Acetonitrile (ACN), methanol (MeOH), chloroform (CHCl_3) were purchased from Carlo Erba Reactifs (SDS, Peypin, France), and formic acid was sourced from Merck (Briarele-Canal, France). Isotope labeled standards, namely, Leucine- d_3 , L-tryptophan- d_3 , Indole-acetic acid d_5 , 1,4-tetradecanedioic acid- d_{24} were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France) and CDN Isotopes (Québec, Canada). MSCAL6 ProteoMass LTQ/FT-Hybrid, standard mixtures used for external calibration of the MS instrument (positive and negative ionization mode) were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). The calibration solution for the positive ionization mode, consisting of caffeine, L-methionyl-arginyl-phenylalanyl-alanine acetate, and Ultramark 1621, whilst for the negative ionization mode, it consisted of sodium dodecyl stearate, taurocholic acid sodium salt hydrate and Ultramark 1621. Water was deionized and purified using an ultra pure water system (Milli-Q, Millipore Corporation, Billerica, Massachusetts, USA). For metabolite identification, commercially

available chemical reference standard of Dopamine was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

2.3 Sample preparation

Prior to the extraction, serum samples were allowed to thaw on ice. The extraction procedure firstly reported by Bligh and Dyer (1959) and further re-adapted by Bird et al. (2011) was employed. The first step in the sample preparation scheme was a deproteinization step, which consisted of removing high molecular weight species including proteins. It was also employed as mean for preserving the LC–MS system integrity and to reduce matrix effects. The deproteinization was achieved by organic solvent precipitation using ice-cold methanol. The procedure consisted of a triphasic liquid liquid extraction with $\text{MeOH}:\text{CHCl}_3:\text{H}_2\text{O}$. Briefly, 190 μl of MeOH was added to 30 μl serum sample and the mixture was vortex-mixed for 20 s. Next, 380 μl of CHCl_3 was added, the mixture was again vortexed for 20 s, and finally, 120 μl of H_2O to induce phase separation. The sample was further mixed for 10 s and allowed to equilibrate at room temperature for 10 min before centrifugation ($8,000\times g$, 10 min, 4°C). The upper MeOH phase was then collected. An aliquot of the isotope labeled standards ($1.25\text{ ng }\mu\text{l}^{-1}$ each) was added to the MeOH phase in a 1:3 ratio; the mixture was evaporated to dryness at 35°C under gentle nitrogen stream, and subsequently reconstituted with H_2O .

A pooled quality control (QC) sample, deriving from all the study' subject population was prepared, to ensure that no or minimal metabolic information was lost (Want et al. 2010). QC samples were extracted along with each samples batch and analysed throughout the analytical run, in order to provide robust quality assurance for each metabolic feature detected.

2.4 Liquid chromatography (LC)-high resolution mass spectrometry (HRMS) instrumentation

A 1200 Infinity Series high performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, California, USA) coupled to an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray (H-ESI II) source was used for sample analysis.

The chromatographic separation was achieved with a XSelect CSH C_{18} (150 mm $\times$ 2.1 mm; 3.5 μm particle size, Waters, Dublin, Ireland) together with the corresponding guard column, maintained at 35°C . Gradient conditions were adapted from Dunn et al. (2011). The mobile phase consisted of H_2O in channel A, and MeOH in channel B, both containing 0.1 % formic acid. The system

was programmed to hold initial condition (97 % A) for 1 min, and then to perform gradient elution from 97 to 3 % A over a 15 min period, hold at 3 % A for 4 min, return to initial conditions over 2 min and then hold these conditions for a further 5 min. The flow rate was 300 $\mu\text{L min}^{-1}$ and the injection volume was 5 μL .

The HPLC column was connected to the H-ESI interface operating in polarity switch mode, i.e., both positive and negative ionization mode.

High resolution MS fingerprints were acquired using the following parameters: capillary voltage 30/–45 V, capillary temperature 350 °C, spray voltage 3/–3.5 kV, sheath gas flow rate 55, auxiliary gas flow rate 10 a.u. (arbitrary units), sweep gas flow rate 0, skimmer voltage 26/–26 V, tube lens 100 V, heater temperature 300 °C. Full scan mass spectra were acquired from m/z 65 to 1,000 at a mass resolving power of 50,000 Full Width at Half Maximum (FWHM) at m/z 200. The scan speed was 2 Hz in profile mode. Daily instrument calibration was performed using a MSCAL6 ProteoMassT LTQ/FT-Hybrid ESI Pos/Neg (Sigma–Aldrich). Xcalibur 2.2 (Thermo Fisher Scientific, San Jose, CA, USA) was used for data acquisition and analysis. Samples were analyzed by batch (day 8, day 22 and day 29) in randomized order.

Further, for the characterization of metabolite chemical standard, MS/MS spectra were acquired on a hybrid quadrupole-orbitrap (Q-ExactiveTM) mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).

2.5 Data processing and inter-batch normalization

LC-HESI (+/–) HRMS raw data files were initially pre-processed with Xcalibur 2.2 (Thermo Fisher Scientific, San Jose, CA, USA). The raw (*.raw) files were subsequently converted to a more exchangeable format (*.mzXML) and polarity split using MSConvert (Kessner et al. 2008) and XCMS (Smith et al. 2006) software implemented with the R language. Further data processing was performed using the same software; multiple steps including: peak alignment, peak picking, retention time alignment followed by a peak grouping and integration step. The following parameters were applied: CentWave algorithm (Tautenhahn et al. 2008); peakwidth = 5–25; $mzdiff$ = 0.001, $mzwid$ = 0.008, and $minfrac$ = 0.85. The resulting XCMS matrix containing MS features for each sample, was subsequently exported to Excel[®] for univariate analysis, inter-batch normalization and later to SIMCA-P+[®] (Version 13, Umetrics AB, Sweden) software for multivariate statistical analysis.

Inter-batch normalization was applied to the collected data from day 8, day 22 and day 29 for both controls and Revalor-XS[®] implanted animals; each sampling day corresponding to one analytical batch (sequence). The normalization followed the procedure first described by van

der Kloet et al. (2009), assuming that the measurement errors in a single batch are randomly distributed, and that different batches can be compared and corrected using the average or median value of the QC samples in a batch.

2.6 Data analysis

A combination of univariate and multivariate methods were used to unveil relevant information. To examine the MS features responsible for differences observed between treated and control animals, a two-level data analysis was performed. Sequences data from day 8, day 22 and day 29, were first analyzed individually, and then MetaXCMS (Tautenhahn et al. 2012a) was applied for second order sample class comparison and to identify shared metabolites across the study time points.

2.6.1 Univariate analysis

Univariate statistics were exploited to estimate instrumental variability. The coefficient of variation (CV) within QC samples, was calculated using the standard deviation divided by the mean intensities of each feature detected in the QCs; a histogram of the resulting CV distribution was plotted. Besides, retention time CVs and intensity CVs were calculated for each labeled isotope standard.

Following this, computation of the fold change (ratio of abundance observed between control and treated animals) and the p value (statistical significance calculated from a student t test) further served for metabolites selection. MS features selection was based on the following criteria: CV <30 %, p value <0.05 and fold change >1.5. It is worth noting that these filters were considered when analyzing individual sequences, i.e., samples from day 8, day 22 and day 29, respectively. Whilst, only the criterion CV <30 % was applied to the data resulting from MetaXCMS matrix. Extracted ion chromatograms (EICs) visualization was achieved in order to withdraw potential artifacts from the ion list.

2.6.2 Multivariate statistical analysis

MetaXCMS was employed to identify shared metabolites across the collection time points (day 8, day 22 and day 29). The data matrix was subsequently exported to SIMCA-P+[®] and subjected to multivariate statistical analysis. In order to reveal discriminating features and building descriptive and predictive models, first, a Principal Component Analysis (PCA) with logarithmically-transformed and Pareto scaling was undertaken followed by, a supervised (Orthogonal) Projection of Latent Structures (O)PLS. The PCA aims at providing an overview of the data structure while the OPLS models attempt to explain a Y variable (which in the present

study represents the status of the animal, i.e., control or Revalor-XS[®] treated animals) from the X matrix of all the ions constituting the fingerprint.

MS features (ions) were considered as independent variables while days of experiment were assigned to the dependent variables. The validation and robustness of each model were evaluated by R²(Y) and Q²(Y) parameters, cross validation-analysis of variance CV-ANOVA (Eriksson et al. 2008), and permutation test. Characteristics of the resulting statistical model were exploited to generate an equation based on combined individual abundances of a set of selected markers. The model was further tested on the data set for screening purposes.

2.7 Putative metabolites identification

First, metabolites of interest were identified by online database searches. Accurate mass of each putative metabolite was submitted to databases for identification of the corresponding metabolite. Metabolite hits were searched against two databases, i.e. METLIN (Zhu et al. 2013; Tautenhahn et al. 2012b, Smith et al. 2005) and HMDB (Wishart et al. 2007, 2009, 2013). Metabolite hits were searched for using a tolerance of ± 0.001 Da (HMDB), ± 5 ppm (METLIN), molecular species, charge state and adduct types. Additionally, results generated after database searches were assessed in relation to metabolites physicochemical properties, spectral similarities, matrix of occurrence and commercially available chemical reference standards; commercially available reference standards were purchased for metabolites with successful database hits.

3 Results and discussion

Regarding the efficiency of Revalor-XS[®] treatment in the current investigation, animal experiment outcomes have recently been reported (Jacob et al. 2014). Briefly, a body weight gain resulting in a conversion efficiency of 9.54 and 8.51 was noted, for controls and treated animals respectively, attesting to the increased performances gained upon such treatment. Release of trenbolone acetate and estradiol from Revalor-XS[®] implant was monitored in urine of treated animals and confirmed the sustained release of the steroids in the animals throughout the experiment (Bianotto et al. personal communication).

The urinary concentrations of drug residues (α -estradiol, β -estradiol, α -trenbolone and β -trenbolone), in animals treated with Revalor-XS are reported in Table 1. In the urine of animals belonging to the control group, only endogenous α -estradiol was detectable at the day 8, 22 and 29 at the concentration of $(0.97 \pm 0.22) \mu\text{g l}^{-1}$; $(1.43 \pm 0.24) \mu\text{g l}^{-1}$ and $(1.06 \pm 0.12) \mu\text{g l}^{-1}$, respectively.

3.1 Sample preparation

Blood sample preparation for LC-MS metabolomics is one of the most challenging; blood is a complex matrix which contains higher molecular weight species comparing to other biological matrices such as urine. Urine has primarily been the matrix of choice for metabolomic investigations related to anabolic practice detection in rearing animals (Gallart-Ayala et al. 2015). Serum LC-HRMS-metabolomics in this field has not been performed yet, though serum might represent an interesting matrix to investigate, in order to assess its complementarity to urine and its relevance for screening anabolic practices in bovines, highlighting the novelty of the current investigation.

Due to the wide chemical diversity of potential metabolites a complete holistic overview using metabolomics is not possible and decisions have to be made as to which part of the metabolome is to be investigated. The sample extraction procedure employed herein presents the advantage of obtaining both polar and non-polar fractions of the serum, with the lower CHCl₃ phase to further serve for lipidomics investigations, where appropriate. In the scope of the present study, we set out to investigate low-molecular-weight metabolites present in the polar fraction of implanted and non-implanted bovine serum. Bovine sera were investigated at day 8, 22 and 29 post-implantation, as two to 4 weeks growth of promoter treatment is usually the period used by those involved in such illegal practices.

3.2 Quality of LC-HRMS data

As a quality control procedure, an isotope labeled standard mixture consisting of Leucine-5,5,5-d₃, L-tryptophan 2,3,3-d₃, Indole-2,4,5,6,7,3-acetic acid d₅ and 1,4-tetradecanedioic acid d₂₄ ($1.25 \text{ ng } \mu\text{l}^{-1}$) was used. It was added to each serum sample before LC-HRMS and served to evaluate the retention time stability, the consistency of signal intensities, the mass accuracy within and between sequences, and eventual drifts in instrumental sensitivity. Deviation from standard accurate mass remained below 5 ppm and no significant drift in retention time was noticed within and between sequences. A pooled quality control (QC) sample, derived from the complete study population was also employed in order to check for consistency during analysis. QCs were injected at the beginning ($n = 4$), the end and also along the analytical run ($n = 6$ samples bracketed by QC).

These measures have been taken to ensure that differences in the fingerprints would not be caused by analytical variability but would reflect metabolome modifications upon Revalor XS[®] administration.

Table 1 Urinary concentrations of α -estradiol, β -estradiol, α -trenbolone and β -trenbolone determined in animals treated with Revalor-XS

Sampling day	α -estradiol	β -estradiol	α -trenbolone	β -trenbolone
8	2.69 $\pm$ 0.41	ND	2.06 $\pm$ 0.33	0.49 $\pm$ 0.04
22	3.86 $\pm$ 0.47	0.07 $\pm$ 0.02	2.02 $\pm$ 0.28	0.26 $\pm$ 0.03
29	3.47 $\pm$ 0.28	0.12 $\pm$ 0.02	1.42 $\pm$ 0.18	0.27 $\pm$ 0.02

The table reports mean concentration values of drug residues ($\mu\text{g l}^{-1}$) and the associated standard error

3.3 Serum metabolomics profiling in HESI polarity switch mode and selection of metabolites of interest

The chromatographic separation was achieved via a recent stationary phase called Charged Surface Hybrid (CSH) C18. In CSH technology, a well controlled surface charge is applied to the packing material. This low level charge greatly improves the peak shape, retention and loading behavior of ionized compounds in reverse phase separations. Other advantages include: improved batch-to-batch reproducibility, rapid column re-equilibration, excellent stability at low pH and a wide range of selectivity.

In the scope of the present study, reverse phase conditions combined to electrospray ionization in polarity switch mode (+/−) were implemented, as recognized to be the more open in terms of application range and suited for the detection of markers of anabolic practices (Dervilly-Pinel et al. 2011; Courant et al. 2009, 2012). In relation to the organic phase, methanol was preferred to acetonitrile. As reported by Dunn et al. (2011), acetonitrile can present problems with elevated background and reduced sensitivity at high acetonitrile concentrations. Elution with methanol also proved to be a good compromise in terms of price, background noise and chromatographic separation (supplementary Fig. 1). Peaks for the isotope labeled standards were already visible on the total ion chromatogram (TIC) when elution was performed with a methanol/water gradient instead of acetonitrile/water; this rapid and visual check up was deemed useful for our quality control procedure. For these reasons, metabolic profiling of serum samples was performed in the study using a methanol/water gradient. Figure 1 illustrates representative TICs obtained in LC-HRMS from control and treated cattle (Fig. 1a, b, respectively). The complex interplay between solvent, stationary phase and solute that enacts a separation in RPLC was recently addressed through a comparison of methanol/water and acetonitrile/water systems. The effects of acetonitrile/water and methanol/water as mobile phase in reversed-phase liquid chromatography was studied more in detail by molecular simulation and reported by Rafferty et al. (2011).

We examined 96 serum samples from Revalor-XS[®] treated and non-treated bovine (day 8, day 22 and day 29 post-implant), in the building of predictive and screening models. Sequences for each treatment day (day 8, day 22, day 29) were first analyzed separately as described in Sect. 2.6. Metabolic fingerprints generated in positive and negative ionization mode were combined to build ‘posneg’ models for each treatment day.

The OPLS fit criteria were found to be the following: $R^2(X) = 0.81$, $R^2(Y) = 0.99$, and $Q^2(Y) = 0.98$ at day 8 (27 animals), $R^2(X) = 0.84$, $R^2(Y) = 0.96$, and $Q^2(Y) = 0.90$ at day 22 (29 animals), $R^2(X) = 0.73$, $R^2(Y) = 0.96$, and $Q^2(Y) = 0.93$ at day 29 (32 animals). CV-ANOVA p values were equal to: 5.417×10^{-15} at day 8, 3.142×10^{-8} at day 22 and 2.770×10^{-13} at day 29. As expressed by these high values, the models demonstrated good descriptive and predictive performances.

Subsequently, the MetaXCMS application was implemented to highlight common features across the treatment period with the aim of building a global model. Venn diagrams revealed 1689 features were common between day 8, day 22 and day 29 samples in negative mode, compared to 1828 common features in positive mode (supplementary Fig. 2).

Ions displaying a CV below 30 % in QC samples were retained for further statistical analysis; application of this filter led to the selection of 1105 ions in negative mode and 1209 ions in positive mode. To remove between-batch offsets and drifts, data were normalized following the procedure described by van der Kloet et al. (2009), assuming that the measurement errors in a single batch are randomly distributed, and that different batches can be compared and corrected using the average or median value of the QC samples in a batch.

The normalized data matrix contained features characterized by their accurate m/z, retention time, fold change, and p value. A non-supervised PCA provided an overview of the data structure; QC samples from all three treatment periods were well-clustered, ensuring that further separation observed would reflect metabolome modifications upon Revalor XS[®] administration, and not analytical variability. The resulting OPLS model (Fig. 2) further allowed the selection of 26 discriminating ions, i.e., the most significant to the model when comparing C and T animals. These ions displayed mass-to-charge ratios ranging from 93 to 617 Da and retention time ranging from 82 s (1.37 min) to 1,034 s (17.23 min).

The model exhibited good descriptive and predictive abilities, as expressed by high values of $R^2(X) = 0.69$, $R^2(Y) = 0.891$ and $Q^2(Y) = 0.842$ parameters. The OPLS model shows that most of the serum samples from C animals have coordinates opposed to serum samples from T animals. Only one sample, control animal # 14 at day 29

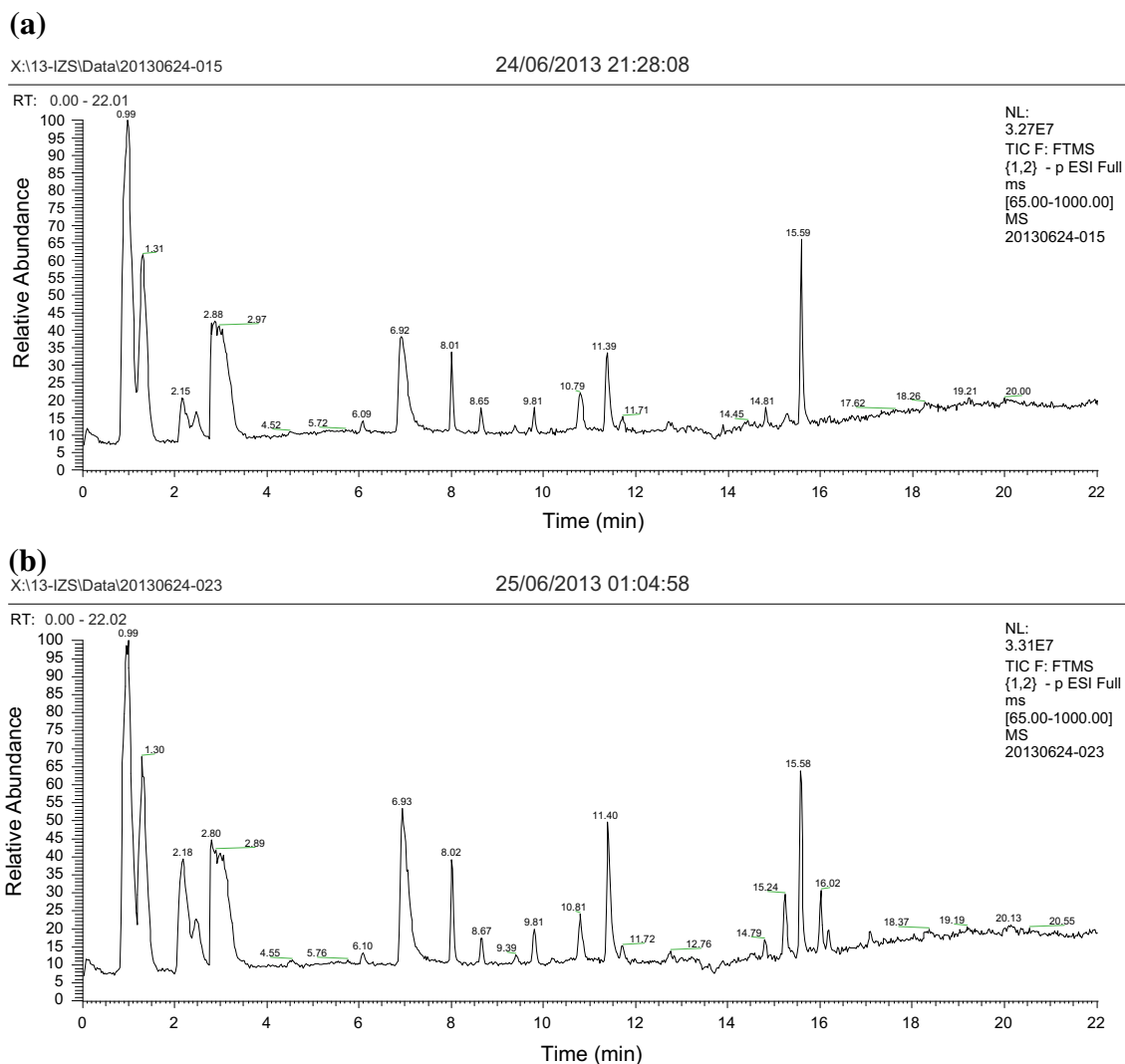


Fig. 1 Typical Total Ion Chromatograms obtained from serum of **a** control and **b** Revalor XS[®] implanted bovine under RPLC-HESI⁻

(four weeks post implant), could not be discriminated from the treated animals' population. In addition, looking at the OPLS plot, it seems that, within the T group, samples collected 8 days post implant formed a separate cluster from those of day 22 and day 29. This difference might be due to the growth of the animals or the result of intense metabolic perturbation a week following administration.

As reported by Saccenti et al. (2013), (O)PLS techniques can over fit the dataset and this has to be controlled by using appropriate cross-validation strategies. Thereby, the validation of the global model created was undertaken. The robustness of the model was assessed using CV-ANOVA, permutation test and cross validation (1/3 2/3 prediction) analysis. The p-value calculated from the CV-ANOVA was equal to 1.26×10^{-25} suggesting that the differences observed through the model are significant. As further evidenced in Fig. 3, permutation test and cross validation (1/3 2/3 prediction) achieved satisfactory results. The cross

validation consisted in eliminating one-third of randomly selected samples from the model and predicting their status (or class) on the newly established model. This validation step further confirmed the descriptive and predictive capabilities of our global model, as demonstrated by high values of $R^2(X) = 0.731$, $R^2(Y) = 0.892$, $Q^2(Y) = 0.842$ parameters. In relation to the permutation test, the rationale behind it was to ensure that the results were not due to a chance factor. The goodness of fit of the original model was compared to the goodness of fit of several models based on data where the order of the sample classes had been randomly permuted, while the data set was kept intact. It can be drawn from Fig. 3 that the multivariate model is valid, robust, and reflects a proper discrimination between control and steroid treated populations up to four weeks post steroid implant administration.

The next step was to generate a predictive model for screening purposes. Finally, metabolites of further interest

Fig. 2 Score plot for OPLS model based on 26 selected ions from the HESI+/HESI– dataset. Control samples are represented by *green triangles*; serum samples from treated animals collected on day 8, day 22 and 29 are represented by *red triangles* (Color figure online)

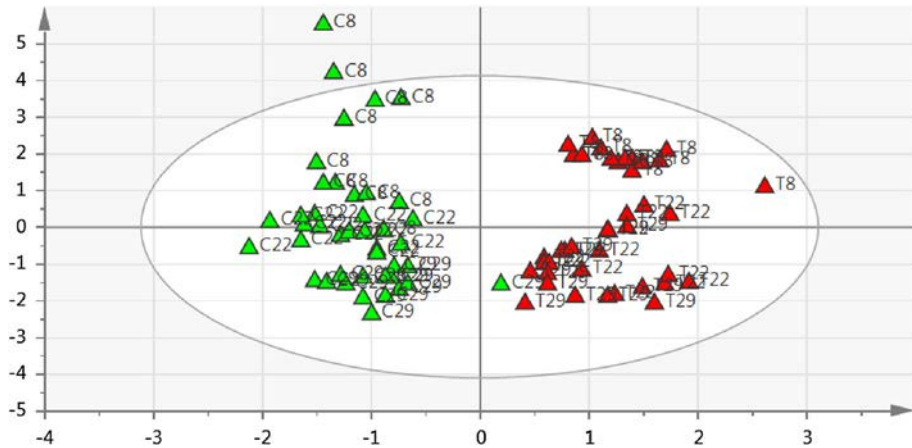


Fig. 3 Validation of dataset presented in Fig. 2 by permutation test

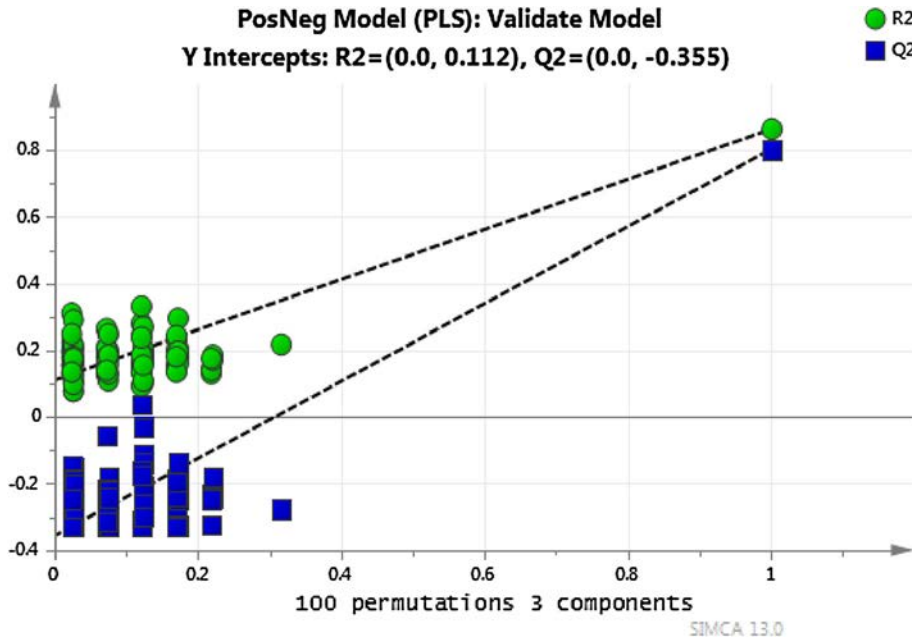


Fig. 4 Results from a weighted equation based on a suspicion threshold using the contribution of 9 discriminant metabolites highlighted from multivariate models. Control samples (C) are represented by *triangles* and Revalor XS® implanted (T) animals by *circles*

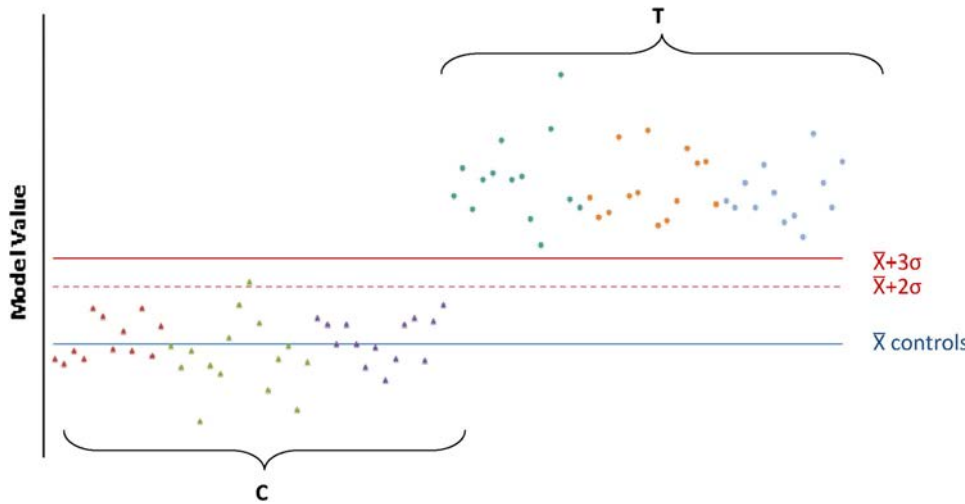


Table 2 Putative metabolites associated to the screening model

Metabolites	Mass/charge ratio	RT (s)	Adduct	Day 8		Day 22		Day 29		Up- or down-regulated	Putative metabolites
				Fold Change	P value	Fold Change	p value	Fold Change	p value		
M108T769	108.082126	768.524	[M-H-CO ₂]-	1.6	0.00854	2.5	0.00012	1.8	0.00046	Up	4 Metabolites returned from Metlin and HMDB search including Dopamine and <i>p</i> -Octopamine
M152T768	152.0719918	768.459	[M-H]-	1.6	0.01157	2.3	0.00023	1.8	0.00005	Up	4 metabolites returned from Metlin and HMDB search including Dopamine and <i>p</i> -octopamine
M93T748	93.0347159	747.86	[M-H-CO ₂]-	1.2	0.00851	1.9	0	1.3	0.0075	Down	16 Metabolites returned from Metlin and 10 from HMDB search including Dopaquinone
M194T576	194.046319	576.468	[M-H]-	1.2	0.17523	3.4	0	1.1	0.43775	Down	
M327T659	327.0884667	658.505		2.2	0.00002	2.6	0	1	0.81675	Up	
M297T894	297.17142	893.906		8.6	0	1.3	0.16684	1.1	0.35961	Down	
M587T1037	587.3609458	1,037.48		1.2	0.10503	3.5	0.00001	3.6	0	Down	
M617T1034	617.371632	1,033.89		2	0.00412	1.8	0.00982	4	0	Down	
M299T719	299.1094991	719.15		4.1	0	1	0.75621	1	0.68109	Down	

were selected based on their coefficient plot in both OPLS and PLS models, as evidenced in supplementary Fig. 3; the error bars indicate the confidence intervals of the coefficients. The coefficient is significantly ($p < 0.05$) above the noise, when the confidence interval does not include zero; the application of this principle led to the selection of 9 ions of interest (supplementary Fig. 4) amongst the 26 ions used in the previous multivariate model. Six of these ions exhibited higher intensities in the T group, while the 3 others showed an opposite trend.

The putative markers highlighted in this serum metabolomics study are different from the markers reported by Jacob et al. (2014) in the frame of urinary fingerprinting subsequent to the same steroid treatment. This proves that urine and serum can be considered as complementary matrices.

3.4 Predictive model for screening

The predictive model for screening purposes was based on a weighted equation including individual abundance of the 9 ions of interest as follows (Eq. 1).

$$\begin{aligned}
 Y = & -0.284511 \times [\text{M93T748}] + 0.25078 \times [\text{M108T769}] \\
 & + 0.246162 \times [\text{M152T768}] - 0.266365 \times [\text{M194T576}] \\
 & - 0.178207 \times [\text{M297T894}] - 0.351371 \times [\text{M299T719}] \\
 & + 0.257659 \times [\text{M327T659}] - 0.601153 \times [\text{M587T1037}] \\
 & - 0.32375 \times [\text{M617T1034}]
 \end{aligned}
 \quad (1)$$

The equation determines the coordinates of a given sample in the model, based on individual measured abundance of each biomarker. As illustrated on Fig. 4, the application of the equation to the 82 samples of the study (41 control and 41 treated animals) provides very good visual discrimination of treated and control sample groups.

Additionally, a screening criterion for Revalor XS[®] abuse has been established. It takes into account a threshold which was determined as the mean value of all control samples plus two or three times the standard deviation ($\bar{x}_{\text{control}} + 2\sigma$) or ($\bar{x}_{\text{control}} + 3\sigma$) which corresponds to 95 and 99 % level of confidence, respectively. Such an approach has already been successfully proposed for the implementation of screening criteria in the context of steroid abuse in horses (Kaabia et al. 2014) or β -agonists administration to bovines (Dervilly-Pinel et al. 2014).

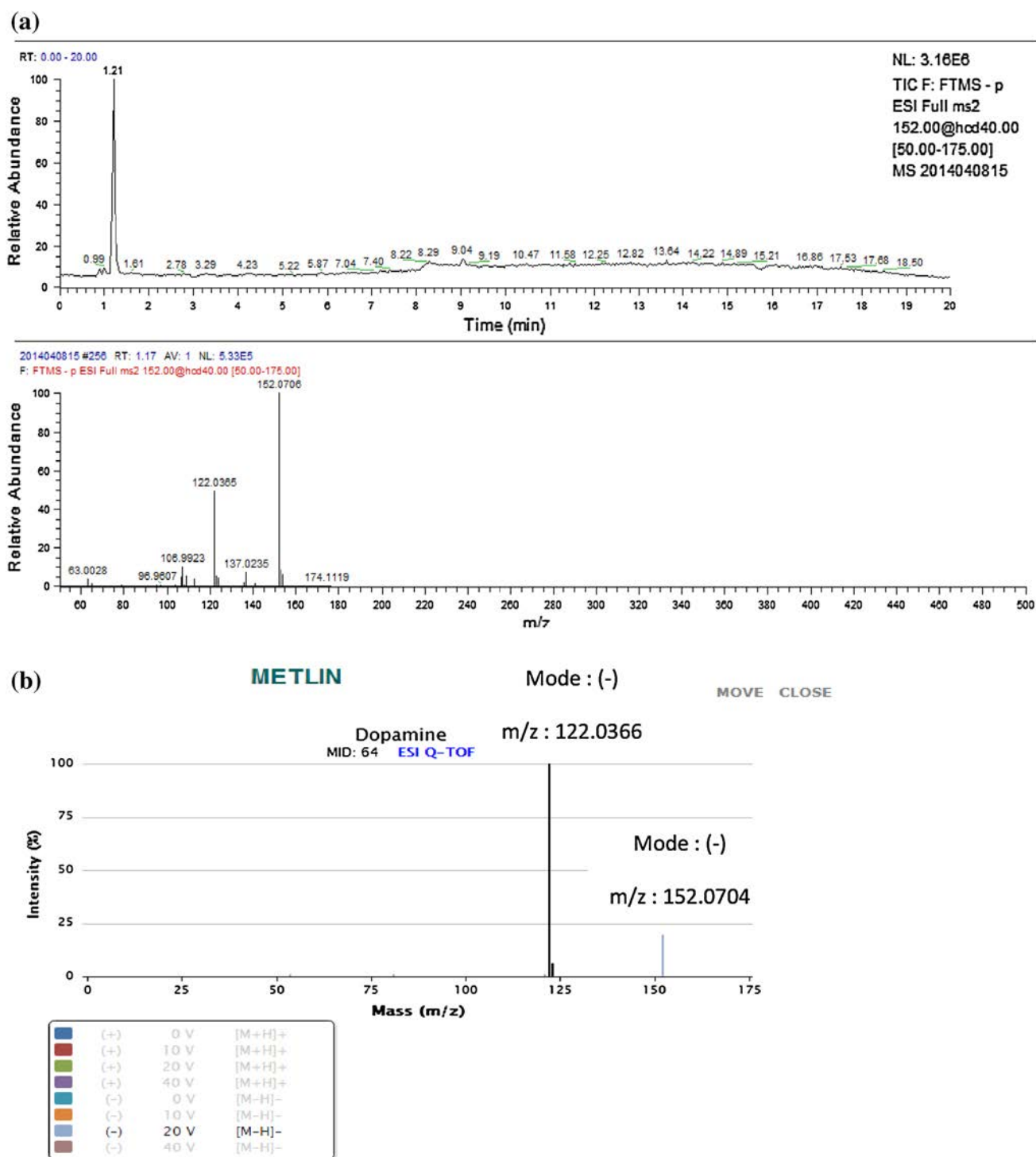


Fig. 5 **a** LC-HR MS/MS analysis of Dopamine chemical standard under identical experimental conditions, using a Q-Exactive mass spectrometer; **b** screenshot of the MS/MS spectrum of Dopamine in Metlin database

As illustrated in Fig. 4 and based on the 99th percentile level confidence, the model correctly predicts C and T samples, and lead to no false non-compliant (false positive) nor false compliant (false negative) animals. Whilst considering the 95th percentile level of confidence, the model

resulted in 2.38 % of false non-compliant rate; the false non-compliant animal was the same animal already highlighted in the multivariate model, i.e., control animal # 14 at day 29. However, no false compliant samples were observed. Although further validation steps including

additional samples (both control and treated) is required to assess the robustness and performances of the selected markers—which was not the purpose of the present research—the established model exhibit preliminary interesting performances, in good agreement with the Commission Decision 2002/657/EC (false compliant rate below 5 % required) implying that serum is also a matrix of interest for screening anabolic practices in bovine.

3.5 Tentative identification of candidate biomarkers

Whatever the objectives of a metabolomic study, one critical step consists in the structural identification of mass spectrometric features revealed by statistical analysis and this remains a real challenge. Indeed, this requires both an understanding of the studied biological system, the correct use of various analytical information (retention time, molecular weight experimentally measured, isotopic golden rules, MS/MS fragment pattern interpretation), or querying online databases (Courant et al. 2012).

First, the metabolites of interest were identified by searching freely available online databases using accurate masses obtained from HRMS experiments. Metabolite hits were taken into account only when they were found by both Metlin and HMDB databases. Among the 9 putative biomarkers, hits were found for 3 of them, i.e., M108T769, M152T768 and M194T576 (Table 2). Results generated were assessed in relation to metabolites physicochemical properties such as retention times, log P, and matrix of occurrence. M108T769 and M152T768 were found to have structural similarities with Dopamine, Octopamine and two other isomers, whilst M194T576 was found to have similarities with Dopaquinone and nine isomers.

However, in terms of physicochemical properties, these matches do not agree with the retention time of our putative biomarkers. Metabolite hits obtained from the database query tend to be hydrophilic molecules with a logP below zero, thus they would elute early under our experimental conditions. To confirm this, dopamine, a representative chemical standard of this cluster was purchased and analyzed under identical experimental conditions and proved to elute earlier (1.21 min $\approx$ 73 s) as suspected ($\approx$ 769 s) (Fig. 5a). Successful standard characterization of dopamine was achieved through MS/MS fragmentation using a Q-Exactive mass spectrometer and compared to dopamine MS/MS spectrum available in Metlin database (Fig. 5b).

It is important that database search results are followed up by comparison with their standard compound when available. In our case, database matches were not confirmed using chemical standards, which lead to the classification of our metabolites to levels 2 and 3 according to the current MSI reporting standards.

4 Concluding remarks

Our primary aim in this study was to investigate serum as an alternative to urine to establish a metabolomics model to screen for Revalor XS[®] misuse in animal breeding. In summary, the resulting multivariate models displayed good descriptive and prediction capabilities and could serve as future tools for the classification of Revalor XS[®] implanted and non-treated bovine. Furthermore, a weighted equation using individual abundance of 9 putative biomarkers succeeded in screening animal status up to four weeks post steroid implant administration, a period usually employed by those involved in illicit practices. The rate of false compliant samples using the equation was equal to 0 %, in accordance with Decision 2002/657/EC. The performance of the screening model was therefore considered fit for purpose.

Our data implies that serum should also be considered as a matrix of interest for screening anabolic practices in bovines. Further prospective studies including a greater number of control and treated animals might be interesting to reinforce the screening model presented here.

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Compliance with Animal Studies and Ethical standards All institutional and national guidelines for the care and use of laboratory animals were followed. Animals were managed according to Directive 86/609/EEC for the protection of animals used for experimental or other scientific purposes, enforced by the Italian D. Lgs n° 116 of January 27, 1992 and Directive 63/2010. The animal study was approved by the Animal Experimentation Ethical Committee of the University of Bologna and was therefore performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Conflict of interest The authors declare no conflicts of interest.

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