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Development, validation and application to real samples of a multiresidue LC-MS/MS method for determination of β_2 -agonists and anabolic steroids in bovine hair

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KEYWORDS: β_2 -agonists; anabolic steroids; bovine hair; liquid chromatography-tandem mass spectrometry; multiresidue.

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Abstract

β_2 -agonists are often abused in cattle breeding because of their effects on animal growth and meat properties. The use of β_2 -agonists as growth promoters is forbidden in the European Union (Council Directive 96/23/EC classifies them into group A of Annex I), due to their toxicity and carcinogenic properties, as for anabolic steroids, which are often administered in combination with β_2 -agonists, to promote the storage of proteins and increase muscle size.

A unique confirmatory LC-MS/MS method for the quantitative detection of thirteen β_2 -agonists and anabolic steroids plus the qualitative identification of other three analytes in bovine hair was developed and validated, according to Decision 2002/657/CE. Hair samples were washed with dichloromethane, digested within a NaOH solution, and subjected to liquid–liquid extraction. The analysis was performed by HPLC coupled to a triple quadrupole mass spectrometer operating in the selected reaction monitoring mode. The absence of matrix interferences, together with good repeatability of both retention times and relative abundances of diagnostic transitions, allowed the correct identification of all analytes. The quantitative calibrations obtained from spiked blank hair samples proved linear in the range tested. $CC\alpha$ and $CC\beta$ ranged from 0.5 ng/g to 30 ng/g. Intralaboratory reproducibility (CV%) ranged between 5.0 and 17.7 and trueness between $96\% \pm 7\%$ and $105\% \pm 8\%$. The applicability of the method to real positive samples was demonstrated for both β_2 -agonists and anabolic steroids.

17α -boldenone was found in most (70%) hair samples obtained from untreated animals, supporting the hypothesis of endogenous production of this steroid.

Introduction

β_2 -agonists are synthetic phenethanolamine compounds used for therapeutic purposes as bronchodilator and tocolytic agents. They are structurally similar to the naturally occurring catecholamines, such as dopamine, norepinephrine and epinephrine^[1]. β_2 -agonists are usually divided into two main sub-classes, that is aniline-like (clenbuterol-like) and phenol-like (salbutamol-like) compounds^[2]. In the clenbuterol-like compounds, the presence of an amino-group accounts for their basic nature. In the salbutamol-like compounds the occurrence of a β -hydroxyl group produces relatively high polarity, which is amplified when further phenolic or alcoholic hydroxyl groups are present in the molecule^[3].

β_2 -agonists are often abused in cattle breeding^[4] because of their growth-promoting effect, produced by stimulation of β_2 -adrenergic receptors on the cell surface. Moreover, they promote protein synthesis and cell hypertrophy, by inhibition of proteolysis in the muscle tissue, and induce lipolysis in the adipose tissue. These effects may result in a reduction of carcass fat up to 40% and an increase of carcass protein content up to 40%, yielding a consistent advantage for the meat industry, by increasing the animal lean mass and reducing its fat content.

In order to obtain these growth-enhancing effects, the applied doses have to be 10-100 times higher than necessary for the therapeutic treatment^[5,6]. The health risks connected to β_2 -agonists abuse have been underscored by several human poisoning accidents, when the consumption of animal food products containing clenbuterol residues was implicated^[7]. At the beginning of the '90s, some cases of human poisoning after ingestion of contaminated liver were reported in Spain^[8], France^[9], and later in Italy^[10].

β_2 -agonists are occasionally abused in combination with anabolic steroids^[4] to enhance further the protein storage and increase the muscle size. Due to their toxic and carcinogenic properties^[11], the non-therapeutic use of both anabolic steroids and β_2 -agonists has been forbidden in the European

Union. Council Directive 96/23/EC classifies β_2 -agonists and anabolic steroids into group A of Annex I – substances having an anabolic effect and unauthorised substances^[12].

Highly sensitive and reliable analytical methods are necessary to provide evidence of illicit pharmaceutical treatments in cattle breeding, and to detect and quantify the presence of trace drug residues in biological matrices and food of animal origin. Beside traditional target matrices (urine, liver) other less conventional matrices, such as hair and retina, proved to be suitable to detect anomalous anabolic steroids or β_2 -agonists (typically clenbuterol) concentrations. In particular, hair analysis offers many advantages: it permits the surveillance of living animals, hair collection is very easy and preserves the full exposure history of the animals^[13]. Thanks to bioaccumulation, Nielen et al. showed that the hair matrix allows largely prolonged detectability of stanozolol, unlike urine^[14]. Fente et al. detected clenbuterol residues from both therapeutic and growth-promoting treatments in hair of a calf until 140 days after the end of the treatment.^[15] A few other studies were subsequently published, dealing with the determination of either β_2 -agonists or anabolic steroids in animal hair, but the two classes of drugs were never considered together^[16-18].

Purpose of the present study was to develop and validate a multiresidue analytical method to be used in wide-range screening programs for the simultaneous detection of these two classes of drugs in bovine hair. The applicability of the whole procedure to real positive hair samples was successfully assessed for both classes of drugs.

Materials and methods

Reagent and chemicals

Clenbuterol, mapenterol hydrochloride, hydroxymethylclenbuterol, tulobuterol hydrochloride, bromobuterol hydrochloride, mabuterol hydrochloride, clenpenterol hydrochloride, cimaterol, salbutamol, ractopamine hydrochloride, isoxsuprine hydrochloride, 17α -boldenone, 17α -

nandrolone, methyltestosterone, methanol, dichloromethane, tert-butyl methyl ether were supplied by Sigma–Aldrich (St. Louis, MO, USA). 17 β -boldenone, 17 β -nandrolone, 17 β -nandrolone D3 and salbutamol D3 were supplied by Australian Government – National Measurement Institute (Sydney, Australia). Clenbuterol D6 was supplied by RIVM (Bilthoven, The Netherlands). 17 β -boldenone D3 was from CDN Isotope (Pointe-Claire, Quebec, Canada). Formic acid 85% was supplied by Riedel-de-Haën (Seelze, Germany). Ultrapure water was obtained by a Milli-Q Millipore system (Bedford, MA, USA).

Standard solutions

Stock methanol solutions were prepared at 1 mg mL⁻¹ concentration for all the analytes and internal standards (ISs), and then stored in the dark at -20°C. Working methanol solutions containing the analytes at different concentrations were prepared by mixing the stock solutions at the proper dilution. The working solutions were used to spike negative hair samples at various concentration levels. Also a proper mix solution containing all internal standards was prepared.

Negative reference hair samples

Negative reference hair samples were obtained from 20 strictly non-treated veals from three livestock located in Piedmont (Italy). Hair samples were equally distributed between males (9) and females (11). All veals were younger than 3.5 months at the moment of sample collection. Hair samples were collected from eighteen Piedmontese breed, i.e. the most popular Italian meat breed, while two samples were from "Valdostana" veals, a dual-purpose breed (milk and meat)^[19]. The aspect of different pigmentation was considered in hair selection. Out of 20 hair samples, 7 were white, 9 were black, and 4 were brown.

Sample preparation

Hair aliquots of about 200 mg were sampled, transferred into 30 mL glass tubes, and added with 3.0 mL of dichloromethane to remove the external contamination, paying attention that the whole hair lock was completely dipped into the organic solvent. To ease the complete submergence, the tube was centrifuged at 3000 rpm (model Megafuge 1.0 Heraeus from ASHI, Milan, Italy), when necessary. Otherwise, the tube was just shaken for some seconds with a vortex (model ZX3, Velp scientifica, Usmate, Italy) and then introduced into an ultrasound bath (model S 60H Elmasonic, Elma, Singen, Germany) for 10 min. After removal of the organic solvent, the decontamination step was repeated once more. The cleaned hair was dried under a nitrogen stream, and then cut into tiny fragments with clean scissors. An aliquot of 100 mg was exactly weighted, and added with the IS mixture solution. Then, an alkaline digestion was performed at 95°C for 10 min, upon addition of 2 mL NaOH 0.1 M to the hair-containing tube. Then the tube was vortexed for some seconds and centrifuged at 3000 rpm for 5 min. After pH adjustment with KH₂PO₄, 0.1 M (final pH = 8), a liquid/liquid extraction was carried out with 2.5 mL of tert-butyl-methyl-ether. The tube was shaken vigorously for 5 min by means of a vortex multimixer (Tecnovetro, Monza, Italy) and then centrifuged at 3000 rpm for 5 min. The supernatant organic phase was transferred into a 10.0 mL glass tube and evaporated to dryness under a gentle stream of nitrogen and mild heating (40°C) using a Techne Sample Concentrator (Barloworld Scientific, Stone, UK). The residue was dissolved in 50 µL of 0.1% p/v aqueous formic acid/methanol mixture 95:5 v/v and transferred into a clean vial for the final LC-MS/MS analysis.

LC-MS/MS analysis

The chromatographic separation was performed on an Agilent 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA), equipped with a vacuum degasser, a binary pump, an autosampler and a column thermostat and using an Agilent XBD C18 column (1.8 µm, 4.6 × 50 mm). The chromatographic run was carried out by a binary mobile phase of 0.1% aqueous formic

acid and methanol, using the following program: isocratic at 5% methanol for 2.5 minutes, linear gradient from 5 to 40% methanol in 2.5 minutes, isocratic at 40% methanol for 3.3 minutes, linear gradient from 40 to 70% methanol in 2 minutes, isocratic at 70% methanol for 3.7 minutes, linear gradient from 70 to 100% methanol in 1 minute, isocratic at 100% methanol for 0.5 minutes, isocratic with 5% methanol (equilibration step) for 5.5 minutes. The injection volume was 20 μL , and the flow rate was 1 mL min^{-1} . The LC was interfaced to an Applied Biosystems API 4000 triple-quadrupole mass spectrometer (Applied Biosystems Sciex, Ontario, Canada), operating in the electrospray ionization (ESI) – positive ion mode. The other MS parameters were set as follows: curtain gas: 30 psi; source gas GS1: 40 psi; source gas GS2: 30 psi; probe temperature: 400°C; gas for collisional activation: N_2 at 4 psi; ion spray voltage: 5000 V; entrance potential: 10 V. Ion acquisition was operated at unit mass resolution in the selected reaction monitoring (SRM) mode, using for each analyte three transitions from the protonated molecular ion to specific fragment ions (see Table 1).

Validation

The method was fully validated for thirteen target analytes, according to the criteria of Commission Decision 2002/657/EC for the forbidden substances confirmatory methods^[20]. The evaluated parameters included: selectivity, linearity, precision, trueness, decision limit ($\text{CC}\alpha$), detection capability ($\text{CC}\beta$), ruggedness, absolute recovery, extraction recovery, matrix effect, and evaluation of potential ion suppression or enhancement of coeluting analytes. The quantification procedure made use of four deuterated ISs: clenbuterol D6, for clenbuterol-like compounds, salbutamol D3, for salbutamol-like compounds, 17 β -boldenone D3 for 17 α - and 17 β -boldenone, and 17 β -nandrolone D3 for 17 α - and 17 β -nandrolone, and methyltestosterone. For the remaining three analytes (cimaterol, isoxsuprine and ractopamine), the recommended precision and trueness conditions could not be entirely fulfilled, and the present method was validated for screening purposes only.

Selectivity

Twenty bovine blank hair samples from as many untreated veals were analyzed as described above. The occurrence of possible interferences from endogenous substances was tested by monitoring the SRM profiles characteristic for each investigated compound, at the retention time interval expected for their elution. The S/N values were expected to be lower than 3, for all SRM transitions.

Linearity

Instrumental linearity (not prescribed by Decision 2002/657/CE) was studied in order to estimate if the method's quantification range lied within the instrumental dynamic linear range. Pure standard solutions for each target analyte (two replicates at six concentrations) were analysed by LC-MS/MS. Slope and intercept were determined by the squares regression method and their significance was evaluated with a t-test. The linear fit was verified using adjusted squared correlation coefficients (Adj R²). The Adj R² was obtained from R² by taking into account the number of observations (calibration points, n) and independent variables (replicates, k):

$$\text{Adj } R^2 = R^2 - [(1 - R^2)/(n - k)]$$

Matrix-matched calibration curves were built by analysing two replicates blank hair samples spiked with working solutions at four final concentrations. As for instrumental linearity, slope and intercept were determined by the squares regression method and their significance was evaluated with a t-test. The linear fit was verified using Adj R².

Precision–trueness

Validation levels were set at 5, 10 and 15 ng/g hair, for clenbuterol, hydroxymethylclenbuterol, tulobuterol, bromobuterol, mabuterol and clenpenterol, at 20, 40 and 60 ng/g hair for mapenterol, salbutamol and 17β-boldenone, and at 50, 100 and 150 ng/g hair for 17α-nandrolone, 17β-nandrolone and methyltestosterone. Trueness, repeatability and intra-laboratory reproducibility

were estimated by quantifying the analytes in 54 validation replicates (6 replicates for each of the three validation levels, repeated after one week and after two weeks), using the IS correction factor. ANOVA tests were set for each validation level, from which repeatability and within-laboratory reproducibility were evaluated. Precision was estimated from the percent coefficient of variation (CV%).

CC α and CC β

CC α values were calculated using the second method provided by Decision 2002/657/CE: from the twenty blank hair samples used to test selectivity, the highest average standard deviation of the background signal was considered, and CC α was calculated as the concentration yielding a signal three-times higher than this background fluctuation. The second most abundant RSM transition for each target analyte was used. S/N values were calculated by comparing the peak height at the expected retention time for each analyte (signal) and the extremes of fluctuation of the baseline in the 12 s following each peak (noise). An extrapolation from the S/N values of the samples spiked at the three lowest levels was executed to calculate CC α concentrations. These calculated CC α were rounded up to slightly higher values (i.e., 1, 2, 5 ng/g), which were then experimentally tested and confirmed.

Also CC β values were calculated using the second method provided by Decision 2002/657/CE: twenty blank materials were fortified with the analytes at the decision limit. The value of the CC α plus 1,64 times the standard deviation of the within-laboratory reproducibility of the measured content provided the CC β concentrations.

Ruggedness

A ruggedness test was conducted by introducing slight variations ($\pm 10\%$) to potentially critical analytical parameters. The resulting changes in the quantitative response were determined on blank

hair samples spiked at the lowest validation level. A Youden approach was used, in which the simultaneous change of several experimental parameters was introduced within a 8-experiments scheme. The selected parameters were: digestion NaOH volume (1.8-2.2 mL), digestion temperature (85-105 °C), digestion time (9-11 min), extraction solvent volume (2.25-2.75 mL), multimixer time (9-11 min), centrifugation time (4-6 min) and drying temperature of the extract (36–44 °C).

The significance of the test was verified by calculating the two averages of the results obtained from the high- and the low-value setting for each experimental parameter; each average difference (D_i) was tested against one another to verify their homogeneous distribution. A F-test was applied to compare the standard deviation of the differences (S_{D_i}) with the standard deviation of the method carried out under within-laboratory reproducibility condition (S_r).

Absolute recovery, extraction recovery and matrix effect

Absolute recovery (that evaluates the contribution of all sample treatments, including the alkaline digestion) was calculated by comparing the experimental results of two sets of samples. In the first set (a), five blank hair samples were spiked with all analytes at the lowest concentrations used in precision-trueness experiments (C_0 , see above) before the alkaline digestion, while in the second set (b) spiking at the same concentration was made on blank hair extracts. For extraction recovery (that evaluates only the contribution of the extraction step), the first set (c) consisted of five blank hair samples spiked with all analytes at C_0 concentration before the extraction step, but after the hydrolysis step. Matrix effect was calculated by comparing the second set (b) with a standard solution at the same concentration (d).

Ion suppression/enhancement effect of coeluting compounds

The following analytes showed partial coelution in the chromatographic run: tulobuterol, bromobuterol, mabuterol, clenpenterol, and isoxsuprine (see Figure 2). In order to consider the

possible presence of more than one analyte in real samples, evaluation of ion suppression/enhancement from these co-eluting compounds was made by comparing the experimental results from sample set (b) with two further sets of samples. In the first one (e), five blank hair extracts were spiked with tulobuterol, mabuterol and isoxxsuprine (not coeluting) at C0 concentration. In the second set (f), five blank hair extracts were spiked with bromobuterol and clenpenterol (not coeluting) at C0 concentration.

Results and discussion

Method development

MS optimization

Three SRM transitions were acquired for the identification of each analyte, so as to exceed the four identification points required by Commission Decision 2002/657/CE. The epimers 17 α -/17 β -nandrolone and 17 α -/17 β -boldenone were characterized by the same SRM transitions, so that the ability to distinguish each epimer was based on their chromatographic peaks separation, which proved sufficiently large to go beyond the retention time uncertainty.

Chromatographic optimization

An important objective of the present work was to obtain the simultaneous determination of β_2 -agonists and anabolic steroids (Figure 1) with a single analytical method, despite their considerable structural differences. The two classes of analytes are also characterized by a highly dissimilar affinity for the most common LC stationary phases. In a previous study, we achieved effective separation of all components of both classes of drugs, but the chromatographic method required a run time as long as 33 min plus 12 min for column re-equilibration^[21]. In order to reduce the time needed for the analysis, we presently selected a different column, characterized by a wide internal

diameter (4.6 mm) and small particles size (1.8 μm). This column supported an high flow, without excessive pressure rise, so that good separation could be obtained in a relatively short time: 15 min plus 6 min for the re-equilibration step (see Figure 2). Despite the high LC flow, the use of methanol and aqueous formic acid solution as mobile phases limited costs and environmental concerns.

Sample preparation

Initial treatment of hair samples generally includes three step: decontamination, digestion and extraction. The decontamination step is addressed to prevent the chance of false positive results arising from the presence of the analytes on the hair surface due to passive (exogenous) exposure not to drug intake. In fact, animals are occasionally exposed to environmental particles, urine or feces containing drug residues from authorized veterinary therapies^[22]. The adopted procedure proved not to extract the drugs present in real positive samples (see below), while it achieved effective hair surface decontamination.

Various procedures for the digestion and extraction of veterinary drugs from hair samples have been described. For both β -agonists and anabolic steroids, alkaline digestion is most frequently used^[3,13,15, 18,23]. Other procedures include methanol^[16], acidic^[24], or aqueous buffer digestion^[22]. In our optimization of the method, all these four digestion procedures were tested: both alkaline and methanol digestions yielded satisfactory results on spiked blank samples, but several authors recognized that only alkaline treatment combined with some kind of enzymatic digestion results in virtually complete dissolution of the hair structure, while the other treatments cannot assure the total drug extraction from the keratin structure^[22]. Alkaline treatment, with relatively high base concentration and temperature, proved to completely dissolve the hair matrix and give confidence that all target analytes were extracted. On the other hand, it was highlighted that such a drastic treatment may occasionally result in the production of high background noise and interferences, and risk of some analytes' degradation^[25]. The absence of the latter inconvenience was carefully tested

by trueness experiments, while the occurrence of major interferences was avoided by submitting the digested samples to extraction with tert-butylmethylether, which also allowed to lower the detection limits. Since some β -agonists, such as salbutamol (but unlike clenbuterol), cannot be satisfactorily extracted at high pH values^[3], the pH was adjusted to 8, resulting in a quite effective recovery for all the analytes considered.

Validation results

Out of the sixteen target analytes tested, the present analytical protocol was successfully validated as a confirmation method, in agreement with Commission Decision 2002/657/EC prescriptions, for thirteen substances. The specific case of 17 α -boldenone, possibly identified as an endogenous constituent of bovine hair, is discussed in the subsequent chapter.

For the remaining three target analytes (cimaterol, isoxysuprine and ractopamine), the validation protocol of the method respected Commission Decision 2002/657/EC prescriptions for screening methods, not for accurate quantitative determinations. In particular, the latter analytes presented CV% values for precision and accuracy exceeding the 20% acceptance limit. Among these, isoxsuprine and ractopamine are salbutamol-like drugs with amphoteric properties (see Figure 1), due to the presence in their structure of a basic secondary amine group, and one or two acidic phenolic hydroxyls. Therefore, the pH has an important influence on the extraction yield. In a previous study^[21], the dramatic effect of pH on the extraction yield of some β -agonists, such as salbutamol and terbutaline, has been described in detail, even if the selected experimental pH proved to maximize this yield and reduce its variability. In addition, the presence of different matrix components are likely to undermine further the extraction repeatability for these amphoteric substances. The use of an isotopically-labeled equivalent as the IS (salbutamol-D3) compensates for these fluctuations and allowed us to include salbutamol in the quantitative method.

On the other hand, salbutamol-like β -agonists are not as frequently abused as clenbuterol-like in cattle breeding, and are in fact not targeted in most screening procedures. Moreover, the high

polarity of salbutamol-like β -agonists reduces their binding to melanin^[3], which is conversely strong for clenbuterol^[23]. Similar concepts hold for anabolic steroids, whose accumulation in hair is favored by their non-polar structure, due to different incorporation pathways occurring in the follicle and hair shaft^[26].

An overview of the validation results obtained for the analytical method is given in Table 2. For cimaterol, isoxysuprine and ractopamine the validation as a screening method includes the evaluation of the following parameters: selectivity, CC β and ruggedness. These data are reported in Table 3.

Selectivity

The SRM chromatographic profiles obtained from blank hair samples collected from 20 surely untreated animals of three reliable livestock did not show the presence of any significant signal ($S/N < 3$) at the relative retention time typical of all the studied compounds and ISs, indicating that the method is selective and no interfering substances are present in the biological matrices. An interesting result is represented by 17 α -boldenone, as 14 blank hair samples out of 20 turned out positive, despite the absence of any pharmacological treatment and the young age of the animals. A striking example is reported in Figure 3 (right), where 17 α -boldenone is apparently present at a concentration as high as 16 ng/g. In the remaining 13 samples, where 17 α -boldenone has been determined, its concentration is significantly lower: although the analyte is unequivocally identified, its quantification can only be roughly estimated in the 0.5-3.5 ng/g range, below the tested CC α value.

The hypothetical occurrence of 17 α -boldenone as a natural constituent of bovine hair is in agreement with the ascertained possibility of its endogenous production, already verified by its occasional presence in the urine matrix^[27], even if no previous information about its natural occurrence in hair is reported in the scientific literature. It is also interesting to note that hair

pigmentation apparently affects 17α -boldenone incorporation into the keratin matrix, similarly to what was observed for clenbuterol,^[13] since 100% of black hair (9/9), 50% of brown hair (2/4) and only 43% of white hair (4/7) turned out positive to 17α -boldenone.

Linearity

Calibration curves from standard solutions were built by analyzing six increasing concentrations for each analyte. The quantification range proved to fall inside the instrumental dynamic linear range. The linear matrix-matched calibration model was checked by analyzing two replicate blank hair samples spiked with the working solutions at four final concentrations. More in detail, the intervals 0–15 ng/mL (0, 5, 10 and 15 ng/mL), 0–60 ng/mL (0, 20, 40 and 60) and 0–150 ng/mL (0, 50, 100 and 150 ng/mL) were investigated for the analytes listed in Table 4. Quantitative data resulting from area counts were corrected using the respective IS signal areas. The linear calibration parameters were obtained using the least squares regression method. The squared correlation coefficient, adjusted by taking into account the number of observations and independent variables ($\text{Adj } R^2$), was utilized to roughly estimate linearity. The results reported in Table 4 confirm the appropriateness of the linear model, which was assessed further by examining the residual plots.

Precision–trueness

The intra-laboratory reproducibility was expressed by the experimental coefficients of variation, that ranged between 5.0% and 17.0%. Decision 2002/657/CE and other international guidelines indicate several ways to calculate acceptable CV% limits as a function of the analyte concentration. The concentrations used to calculate reproducibility (Table 2) ranged from 5 ng/g to 150 ng/g; acceptable CV% limits are around 35% for 5 ng/g and around 21% for 150 ng/g, according to Horwitz-Thompson equations. Thus, precision values below 20% for all three concentrations tested in the present study can be considered satisfactory. Quite similarly, limited bias from true values were recorded (-7.2%–+7.2%) at all concentrations, in agreement with Decision 2002/657/CE

prescriptions. Taking into account that the IS correction factor exactly compensated for extraction yields variability only for four substances (clenbuterol, salbutamol, 17 β -boldenone, and 17 β -nandrolone), the obtainment of reasonable and repeatable concentration assessment for the rest of the target analytes represents clear indication of constant recovery yields for these substances.

CC α and CC β

Calculated CC α values ranged between 0.44 and 34.5 ng/g (Table 2). Practical CC α values were experimentally verified at 0.5 ng/g (Clenbuterol, Hydromethylclenbuterol), 1.0 ng/g (Tulobuterol, Bromobuterol, Mabuterol), 2 ng/g (Clenpenterol), 5 ng/g (Mapenterol, Salbutamol, 17 α -boldenone and 17 β -boldenone), 30 ng/g (17 α -nandrolone and 17 β -nandrolone), and 20 ng/g (Methyltestosterone).

The CC β values reported for cimaterol, isoxysuprine and ractopamine (Table 3) represent a validation requisite for screening methods: at the concentration of 20 ng/g (established and verified CC β for cimaterol, isoxysuprine, ractopamine), the respect of identification criteria was positively ascertained. The relative retention time of the analytes corresponded to those recorded from the calibration solution with a tolerance lower than $\pm 2.5\%$. The acceptable tolerance for the relative abundance of fragment ions was respected for all analytes and SRM transitions.

Ruggedness

The quantitative results arising from the experiments planned on the basis of the Youden approach, to test the method ruggedness, were interpreted on statistical ground (Table 4). Both t- and F-tests showed that no significant changes were induced when limited alteration of the experimental parameters were introduced in the sample preparation method.

For cimaterol, isoxysuprine and ractopamine, the Youden approach was applied again, in which the same eight experiments were performed at the $CC\beta$ level, but only the fulfillment of the identification criteria was used to verify the method ruggedness. Also in this case, the method proved robust against the selected modifications for screening purposes.

Absolute recovery, extraction recovery and matrix effect

Absolute recovery, extraction recovery and matrix effect were evaluated and the corresponding values are given in Table 5. Extraction recovery values were measured in the range 85%-118%, with 6%-19% variability, for all β -agonists and anabolic steroids. Absolute recoveries were on average 0%-20% lower than extraction recoveries for β -agonists, whereas 17α -, 17β -nandrolone, and methyltestosterone showed significantly lower absolute recoveries (56%-76%).

The effect of the real hair matrix appears modest (<6%) and statistically not significant for most β -agonists tested. Slightly larger negative effect (signal suppression) is evident for ractopamine (-18%). Among anabolic steroids, significant negative matrix effect was detected only for methyltestosterone (-17%), while modest positive matrix effect was observed for both boldenone e nandrolone.

Ion suppression/enhancement effect of co-eluting compounds

The possible presence of ion suppression/enhancement effects arising from coelution was investigated for five partially coeluting analytes (data reported in Table 5). Significant ion enhancement effect was observed for bromobuterol and clenpenterol (+22% and +23% respectively), while an even more significant ion suppression effect is registered for tulobuterol (-31%). These effects have to be taken into account, whenever the animals have allegedly been treated with multiple β -agonist substances.

Application to real samples

In order to verify the complete analytical procedure on authentic positive specimens, two hair samples involved in real judicial proceedings about suspected illicit treatment were analyzed. The washing solutions used in the decontamination procedure were handled and analyzed similarly to the sample extracts, and exhibited no presence of the target analytes. Figure 4 shows the SRM profiles obtained from the first hair sample, in which clenbuterol was determined at the concentration of 11.9 ng/g.

Analogous SRM profiles are reported in Figure 5, obtained from the second hair sample found positive to 17 β -boldenone at the concentration of 39.0 ng/g. Also in this case, an excellent S/N value was obtained from all SRM transitions, allowing clear evidence of the presence of the target analyte and its estimated concentration. In the central profiles of Figure 5, also the peaks relative to 17 α -boldenone are clearly evident at the expected retention time (13.12 min). Although its estimated concentration is extremely high (227 ng/g), its questionable origin makes this finding less valuable, as a judicial evidence, than the proven presence of 17 β -boldenone, even at much lower concentration.

Conclusions

The present analytical methods allows the confirmatory (quantitative) determination of thirteen β -agonists and anabolic steroids simultaneously, and also allows the qualitative identification of further three β -agonists, in bovine hair. The method has been developed and fully validated according to Decision 2002/657/CE. Its applicability to real samples from judicial proceedings has also been demonstrated.

An advantage of the present procedure is that it is practically used as a routine screening protocol targeted to the detection of the illicit administration of both β -agonists and anabolic steroids. The

single procedure applied on hair samples allows to gain relevant information concerning the retrospective use of these drugs on living animals, at reduced costs and increased efficiency.

Among anabolic steroids, 17α -boldenone represents a unique situation, since most hair samples from untreated animals that we examined turned out positive to the presence of this steroid. Even if its presence has been repeatedly ascertained in the urines of untreated calves, the present study provides the first identification of 17α -boldenone in the hair of untreated bovines, further supporting the hypothesis that this steroid can be produced by endogenous processes.

Acknowledgements

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References

- [1] C. Juan, C. Igualada, F. Moraguesa, N. Leóna, J. Mãesb. Development and validation of a liquid chromatography tandem mass spectrometry method for the analysis of β -agonists in animal feed and drinking water. *J. Chromatogr. A*. **2010**, 1217, 6061–6068.
- [2] P. Gallo, G. Brambilla, B. Neri, M. Fiori, C. Testa, L. Serpe. Purification of clenbuterol-like β -agonist drugs of new generation from bovine urine and hair by α 1-acid glycoprotein affinity chromatography and determination by gas chromatography–mass spectrometry. *Anal. Chim. Acta*. **2007**, 587, 67–74.
- [3] A. Poletini. Bioanalysis of β_2 -agonists by hyphenated chromatographic and mass spectrometric techniques. *J. Chromatogr. B*. **1996**, 687, 27-42.
- [4] D. Courtheyn, B. Le Bizec, G. Brambilla, H.F. De Brabander, E. Cobbaert, M. Van de Wiele, J. Vercammen, K. De Wasch. Recent developments in the use and abuse of growth promoters. *Anal. Chim. Acta*. **2002**, 473, 71–82.
- [5] J. Cai, J. Henion. Quantitative multi-residue determination of β -agonists in bovine urine using on-line immunoaffinity extraction-coupled column packed capillary liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B*. **1997**, 691, 357-370.
- [6] A.G. Fragkaki, C. Georgakopoulos, S. Sterk, M.W.F. Nielen. Sports doping: Emerging designer and therapeutic β_2 -agonists. *Clin. Chim. Acta*. **2013**, 425, 242-58.
- [7] K. De Wasch, H. De Brabander, D. Courtheyn. LC-MS-MS to detect and identify four beta-agonists and quantify clenbuterol in liver. *Analyst*. **1998**, 123, 2701–2705.
- [8] J.F. Martínez-Navarro. Food poisoning related to consumption of illicit β -agonist in liver. *The Lancet*. **1990**, 336(8726), 1311-1990.
- [9] C. Pulce, D. Lamaison, G. Keck, C. Bostvironnois, J. Nicolas, J. Descotes. Collective human food poisonings by clenbuterol residues in veal liver. *Vet. Hum. Toxicol*. **1991**, 33(5), 480-481.

- [10] G. Brambilla, T. Cenci, F. Franconi, R. Galarini, A. Macrì, F. Rondoni, M. Strozzi, A. Loizzo. Clinical and pharmacological profile in a clenbuterol epidemic poisoning of contaminated beef meat in Italy. *Toxicol. Lett.* **2000**, 114, 47–53.
- [11] D. Zeng, C. Lin, Z. Zeng, X. Huang, L. He. Multi-residue determination of eight anabolic steroids by GC-MS in muscle tissues from pigs. *Agric. Sci. China.* **2010**, 9(2), 306-312.
- [12] European Commission. 1996. 96/23/EC: Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decision 89/187/EEC and 91/664/EEC. *Off. J. Eur. Comm.* **1996**, L125, 10-32.
- [13] A. Cristino, F. Ramos, M.I. Noronha da Silveira. Control of the illegal use of clenbuterol in bovine production. *J. Pharmaceut. Biomed.* **2003**, 32, 311-316.
- [14] M.W.F. Nielen, H. Hooijerink, M.L. Essers, J.J.P. Lasaroms, E.O. van Bennekom, L. Brouwer. Value of alternative sample matrices in residue analysis for stanozolol. *Anal. Chim. Acta.* **2003**, 483, 11–17.
- [15] C.A. Fente, B.I. Vázquez, C. Franco, A. Cepeda, P.G. Gigoso. Determination of clenbuterol residues in bovine hair by using diphasic dialysis and gas chromatography–mass spectrometry. *J. Chromatogr. B.* **1999**, 726, 133–139.
- [16] V. Marcos, E. Perogordo, P. Espinosa, M. Martin de Pozuelo, H. Hooghuis. Multiresidue analysis of anabolic compounds in bovine hair by gas chromatography–tandem mass spectrometry. *Anal. Chim. Acta.* **2004**, 507, 219–227.
- [17] M. Regiart, M.A. Fernández-Baldo, V.G. Spotorno, F.B. Bertolino, J. Raba. Ultra sensitive microfluidic immunosensor for determination of clenbuterol in bovine hair samples using electrodeposited gold nanoparticles and magnetic micro particles as bio-affinity platform. *Biosens. Bioelectron.* **2013**, 41, 211–217.
- [18] M.W.F. Nielen, J.J.P. Lasaroms, M.L. Essers, J.E. Oosterink, T. Mejer, M.B. Sanders, T. Zuidema, A.A.M. Stolker. Multiresidue analysis of beta-agonists in bovine and porcine

urine, feed and hair using liquid chromatography electrospray ionization tandem mass spectrometry. *Anal. Bioanal. Chem.* **2008**, 391, 199-210.

- [19] <http://www.agraria.org/zootecnia.htm> (Accessed 26 Sep 2013)
- [20] European Commission. 2002. 2002/657/EC: Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Comm.* **2002**, L221, 8-36.
- [21] M. Leporati, P. Capra, P. Brizio, V. Ciccotelli, M.C. Abete, M. Vincenti. Fit-for-purpose in veterinary drug residue analysis: development and validation of an LC-MS/MS method for the screening of thirty illicit drugs in bovine urine. *J. Sep. Sci.* **2012**, 35, 400–409.
- [22] M. Gratacós-Cubarsí, M. Castellari, A. Valero, J.A. García-Regueiro. Hair analysis for veterinary drug monitoring in livestock production. *J. Chromatogr. B.* **2006**, 834, 14–25.
- [23] F.J. dos Ramos. β -Agonist extraction procedures for chromatographic analysis. *J. Chromatogr. A.* **2000**, 880, 69–83.
- [24] M. Hernández-Carrasquilla. External contamination of bovine hair with β_2 -agonist compounds: evaluation of decontamination strategies. *J. Chromatogr. B.* 2002, 767, 235–243.
- [25] M. Hernández-Carrasquilla. Gas chromatography–mass spectrometry analysis of anabolic compounds in bovine hair: evaluation of hair extraction procedures. *Anal. Chim. Acta.* **2001**, 434, 59–66.
- [26] D. Thieme, P. Anielski, J. Grosse, H. Sachs, R.K. Mueller. Identification of anabolic steroids in serum, urine, sweat and hair Comparison of metabolic patterns. *Anal. Chim. Acta.* **2003**, 483, 299-306.
- [27] H.F.De Brabander, S. Poelmans, R. Schilt, R.W. Stephany, B. Le Bizec, R. Draisci, S.S. Sterk, L.A. van Ginkel, D. Courtheyn, N. Van Hoof, A. Macrík, K. De Wasch. Presence and metabolism of the anabolic steroid boldenone in various animal species: a review. *Food Addit. Contam.* **2004**, 21(6), 515-25.

Table 1. Target analytes are reported with their retention time, precursor and product ions involved in the SRM transition and MS experimental conditions. The underlined qualifier product ions are used for CC α calculation.

Class	Analyte	Retention time (min)	Parent ion	Quantifier product ion	Qualifier product ions (qualifier/quantifier %)	Declustering Potential (V)	Collision Energy (CE)
Clenbuterol-like β_2 -agonists	Cimaterol	4.21	219.9	202.1	<u>160.4</u> (36%); 143.3 (22%)	33	14; 23; 32
	Hydroxymethylclenbuterol	6.10	293.0	203.2	<u>132.0</u> (32%); 167.1 (12%)	41	26; 41; 37
	Clenbuterol	6.61	277.0	203.1	<u>132.1</u> (25%); 168.0 (27%)	60	27; 42; 41
	Tulobuterol	6.99	228.1	154.0	<u>172.0</u> (15%); 117.9 (6%)	93	26; 18; 33
	Bromobuterol	7.06	367.0	293.1	<u>349.2</u> (38%); 212.2 (24%)	30	25; 16; 42
	Mabuterol	7.12	311.1	236.9	<u>293.4</u> (34%); 217.1 (31%)	35	23; 18; 36
	Clenpenterol	7.28	291.0	203.0	<u>273.4</u> (20%); 132.1 (17%)	43	22; 16; 38
Mapenterol	8.19	325.0	237.2	<u>307.2</u> (68%); 217.2 (66%)	38	22; 17; 36	
Salbutamol-like β_2 -agonists	Salbutamol	4.89	240.0	148.0	<u>222.2</u> (67%); 166.2 (39%)	77	28; 22; 18
	Ractopamine	6.42	302.2	164.1	<u>284.2</u> (45%); 121.1 (23%)	92	24; 20; 35
	Isoxysuprine	7.34	302.1	284.4	<u>150.3</u> (61%); 107.4 (51%)	52	20; 31; 41
Anabolic steroids	17 α -/17 β -boldenone	13.12; 12.42	287.4	121.0	<u>135.2</u> 54%-87%); 173.1 (18%-30%)	72	33; 21; 25
	17 α -/17 β -nandrolone	13.36; 12.65	275.2	109.1	<u>257.3</u> (112%-72%); 239.5 (32%-22%)	79	39; 25; 31
	17 α -methyltestosterone	13.80	303.2	97.2	<u>285.1</u> (88%); 109.2 (25%)	76	37; 38; 23
Internal standards	Salbutamol D3	4.86	243.2	151.0	-	38	26
	Clenbuterol D6	6.59	283.0	203.0	-	45	18
	17 β -boldenone D3	12.42	290.2	121.2	-	51	34
	17 β -nandrolone D3	12.65	278.5	109.1	-	65	20

Table 2. Intralaboratory reproducibility (CV%), trueness (%), calculated and experimentally verified CC α ,(ng/g), reported for thirteen analytes at three concentration values.

Analyte concentration	Intralaboratory reproducibility (CV%) (n=18)			Trueness (%) Mean \pm Std Dev (n=18)			CC α from 20 blanks	Verified CC α	CC β
	C0 = 5 ng/g	10 ng/g	15 ng/g	5 ng/g	10 ng/g	15 ng/g	ng/g	ng/g	ng/g
Clenbuterol	8.67	6.88	5.31	105 \pm 8	95 \pm 6	102 \pm 5	0.44	0.5	0.61
Hydroxymethylclenbuterol	17.0	17.7	7.78	100 \pm 9	100 \pm 9	100 \pm 6	0.52	0.5	0.57
Tulobuterol	13.3	13.3	6.21	103 \pm 8	97 \pm 8	101 \pm 5	0.78	1	1.26
Bromobuterol	13.9	14.4	6.69	103 \pm 7	97 \pm 8	101 \pm 5	0.82	1	1.41
Mabuterol	9.77	10.2	7.56	104 \pm 6	96 \pm 7	101 \pm 7	0.48	1	1.27
Clenpenterol	8.17	10.3	6.02	103 \pm 4	97 \pm 7	101 \pm 5	1.71	2	2.76
Analyte concentration	C0 = 20 ng/g	40 ng/g	60 ng/g	20 ng/g	40 ng/g	60 ng/g	ng/g	ng/g	ng/g
Mapenterol	10.7	9.98	7.28	100 \pm 7	100 \pm 5	100 \pm 6	3.47	5	5.99
Salbutamol	13.1	14.1	6.47	101 \pm 8	99 \pm 9	100 \pm 5	4.00	5	6.11
17 α -boldenone	15.9			95 \pm 14			7.62	5	5.69
17 β -boldenone	7.38	6.68	5.34	102 \pm 5	98 \pm 5	101 \pm 5	3.38	5	5.79
Analyte concentration	C0 = 50 ng/g	100 ng/g	150 ng/g	50 ng/g	100 ng/g	150 ng/g	ng/g	ng/g	ng/g
17 α -nandrolone	9.08	8.46	5.21	101 \pm 5	99 \pm 4	100 \pm 4	33.1	30	34.6
17 β -nandrolone	9.69	9.90	5.00	101 \pm 6	99 \pm 6	100 \pm 4	34.5	30	34.1

Table 3. Verification of identification criteria from 20 hair samples spiked at 20 ng/g concentration: experimental CC β (ng/g), relative retention time, and relative abundance of SRM transitions, for cimaterol, isoxxsuprine and ractopamine.

Analyte	CC β (ng/g) verified		Relative retention time	Internal standard for relative retention time calculation	Relative abundances		
					Quantifier product ion	Qualifier product ions	
						219.9/202.1	219.9/160.4
Cimaterol	20	Reference	0.658	Clenbuterol D6	100%	38%	23%
		Mean\pmstandard deviation (n=20)	0.655 \pm 0.007			36 \pm 5%	22 \pm 2%
					302.1/284.4	302.1/150.3	302.1/107.4*
Isoxxsuprine	20	Reference	1.514	Salbutamol D3	100%	60%	47%
		Mean\pmstandard deviation (n=20)	1.510 \pm 0.013			61 \pm 7%	51 \pm 5%
					302.2/164.1	302.2/284.2	302.2/121.1*
Ractopamine	20	Reference	0.977	Salbutamol D3	100%	48%	20%
		Mean\pmstandard deviation (n=20)	0.975 \pm 0.003			45 \pm 6%	23 \pm 2%

* product ions excluded for CC β calculation

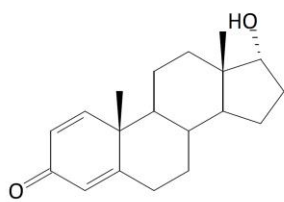
Table 4. Calibration curves obtained from pure standard solutions (instrumental response linearity) and spiked hair samples (quantitative determinations) with corresponding Adjusted R^2 values and standard deviation of the differences (S_{Di}), within-laboratory reproducibility standard deviation (S_r), calculated and critical F values ($p = 0.05$) for ruggedness evaluation.

Analyte	Standard solutions			Spiked matrix			Ruggedness (minor changes)			
	Linearity range (ng/mL)	Calibration curve	Linearity (Adj R^2)	Linearity range (ng/mL)	Calibration curve	Linearity (Adj R^2)	Standard deviation of the differences (S_{Di})	Within-laboratory reproducibility standard deviation (S_r)	Calculated F value (F_{oss})	Critical F value (F_{tab})
Clenbuterol	0 – 30	$y = 0.4260 x + 0.0649$	0.9945	0.5 – 15	$y = 0.5542 x - 0.1303$	0.9921	0.5649	0.4335	1.3033	2.9277
Hydroxymethylclenbuterol	0 – 40	$y = 0.1038 x + 0.0929$	0.9968	0.5 – 15	$y = 0.2895 x - 0.0447$	0.9907	0.4085	0.8491	0.4810	
Tulobuterol	0 – 40	$y = 0.3459 x - 0.3917$	0.9936	1.0 – 15	$y = 0.8301 x - 0.4849$	0.9853	0.6654	0.4463	1.4909	
Bromobuterol	0 – 40	$y = 0.1314 x - 0.0733$	0.9823	1.0 – 15	$y = 0.2946 x + 0.1256$	0.9927	0.4537	0.6930	0.6547	
Mabuterol	0 – 40	$y = 0.3251 x - 0.0955$	0.9952	1.0 – 15	$y = 0.7499 x - 0.2543$	0.9919	0.7067	0.4882	1.4475	
Clenpenterol	0 – 30	$y = 0.2851 x - 0.1329$	0.9932	1.0 – 15	$y = 0.5986 x - 0.2217$	0.9837	0.5772	0.4084	1.4134	
Mapenterol	0 – 120	$y = 0.2114 x - 0.8990$	0.9883	5.0 – 60	$y = 0.4661 x + 0.7085$	0.9879	2.2583	2.1462	1.0522	
Salbutamol	0 – 120	$y = 0.0054 x - 0.0317$	0.9833	5.0 – 60	$y = 0.0123 x - 0.0261$	0.9873	2.7708	2.6219	1.0568	
17 α -boldenone	0 – 120	$Y = 0.0178 x + 0.0002$	0.9921	5.0 – 60	$Y = 0.0520 x + 0.0010$	0.9874	2.6478	2.5461	1.0399	
17 β -boldenone	0 – 120	$y = 0.0179 x - 0.0280$	0.9921	5.0 – 60	$y = 0.0266 x + 0.0493$	0.9874	1.3413	1.4070	0.9533	
17 α -nandrolone	0 – 300	$y = 0.0188 x - 0.0093$	0.9915	30.0 – 150	$y = 0.0096 x + 0.0043$	0.9997	3.1549	4.5378	0.6953	
17 β -nandrolone	0 – 300	$y = 0.0154 x - 0.0005$	0.9968	30.0 – 150	$y = 0.0091 x + 0.0183$	0.9922	4.7473	4.8445	0.9799	
Methyltestosterone	0 – 300	$y = 0.0192 x - 0.1400$	0.9828	20.0 – 150	$y = 0.0099 x + 0.0130$	0.9991	3.7549	4.3138	0.8704	

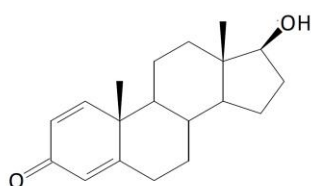
Table 5. Absolute recovery, extraction recovery, matrix effect and ion suppression/enhancement effect of co-eluting compounds evaluated on five replicates at C0 concentration.

Analyte concentration C₀ = 5 ng/g	Absolute recovery (n=5)	Extraction recovery (n=5)	Matrix effect (n=5)	Matrix coelution ion enhancement/suppression (%) (n=5)
Clenbuterol	94% ± 11%	102% ± 11%	97% ± 4%	
Hydroxymethylclenbuterol	97% ± 6%	97% ± 12%	105% ± 2%	
Tulobuterol	98% ± 7%	98% ± 10%	99% ± 4%	69% ± 5%
Bromobuterol	75% ± 12%	93% ± 8%	95% ± 4%	122% ± 6%
Mabuterol	101% ± 12%	118% ± 19%	94% ± 3%	90% ± 7%
Clenpenterol	94% ± 11%	102% ± 11%	97% ± 4%	123% ± 8%
Analyte concentration C₀ = 20 ng/g				
Mapenterol	80% ± 18%	102% ± 19%	106% ± 5%	
Salbutamol	114% ± 8%	94% ± 16%	98% ± 3%	
17 α -boldenone	104% ± 5%	93% ± 19%	112% ± 5%	
17 β -boldenone	83% ± 18%	102% ± 6%	102% ± 8%	
Analyte concentration C₀ = 50 ng/g				
17 α -nandrolone	56% ± 9%	108% ± 7%	112% ± 8%	
17 β -nandrolone	76% ± 13%	114% ± 9%	101% ± 8%	
Methyltestosterone	56% ± 10%	85% ± 15%	83% ± 9%	
Analyte concentration CCβ = 20 ng/g				
Cimaterol			94% ± 11%	
Isoxsuprine			95% ± 8%	107% ± 9%
Ractopamine			82% ± 4%	

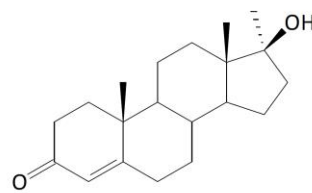
Anabolic steroids



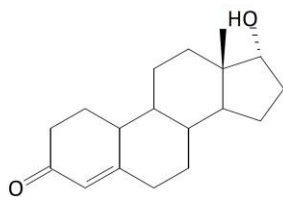
17 α -boldenone



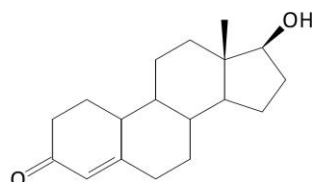
17 β -boldenone



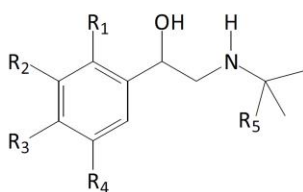
Methyltestosterone



17 α -nandrolone



17 β -nandrolone

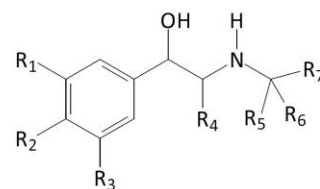


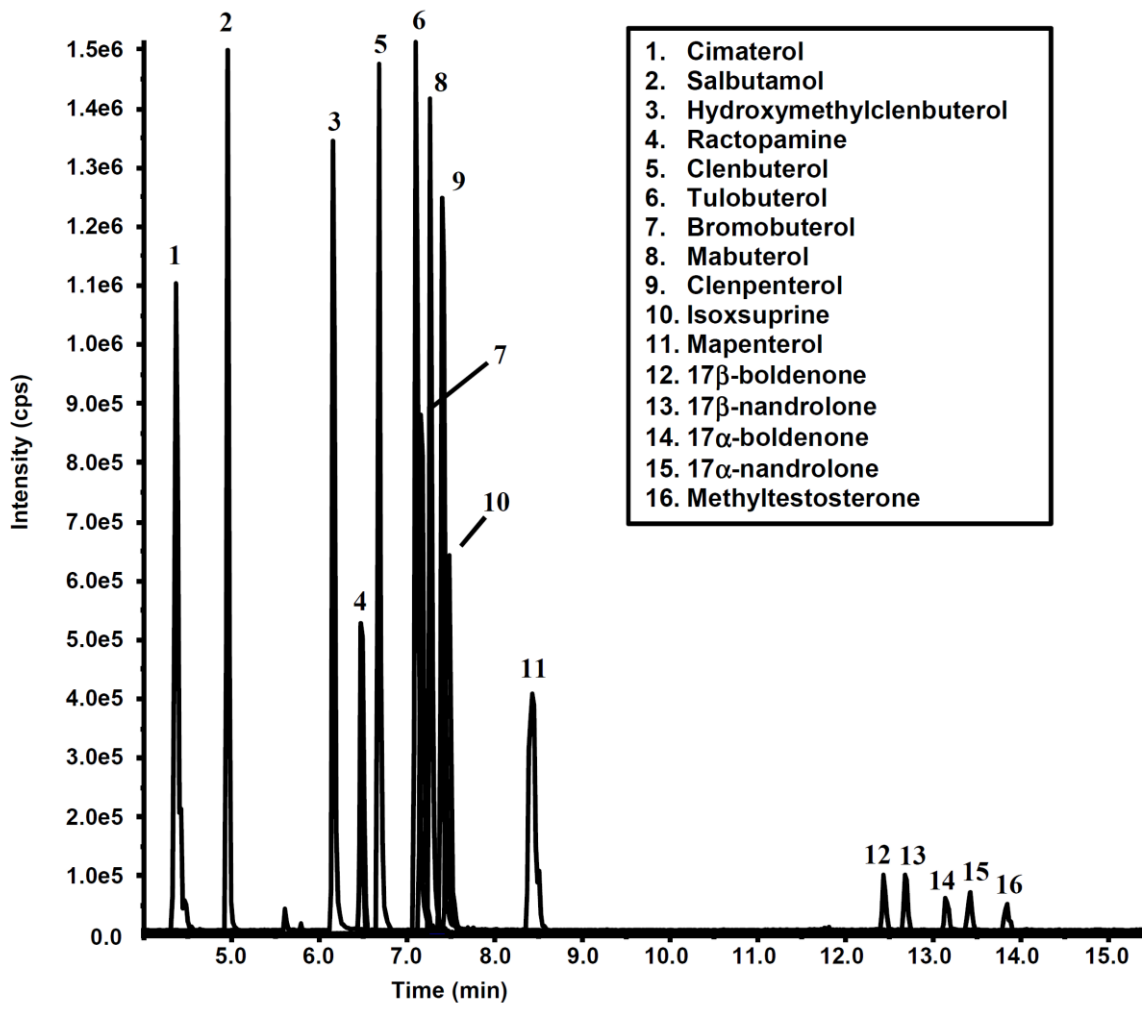
Clenbuterol-like compounds

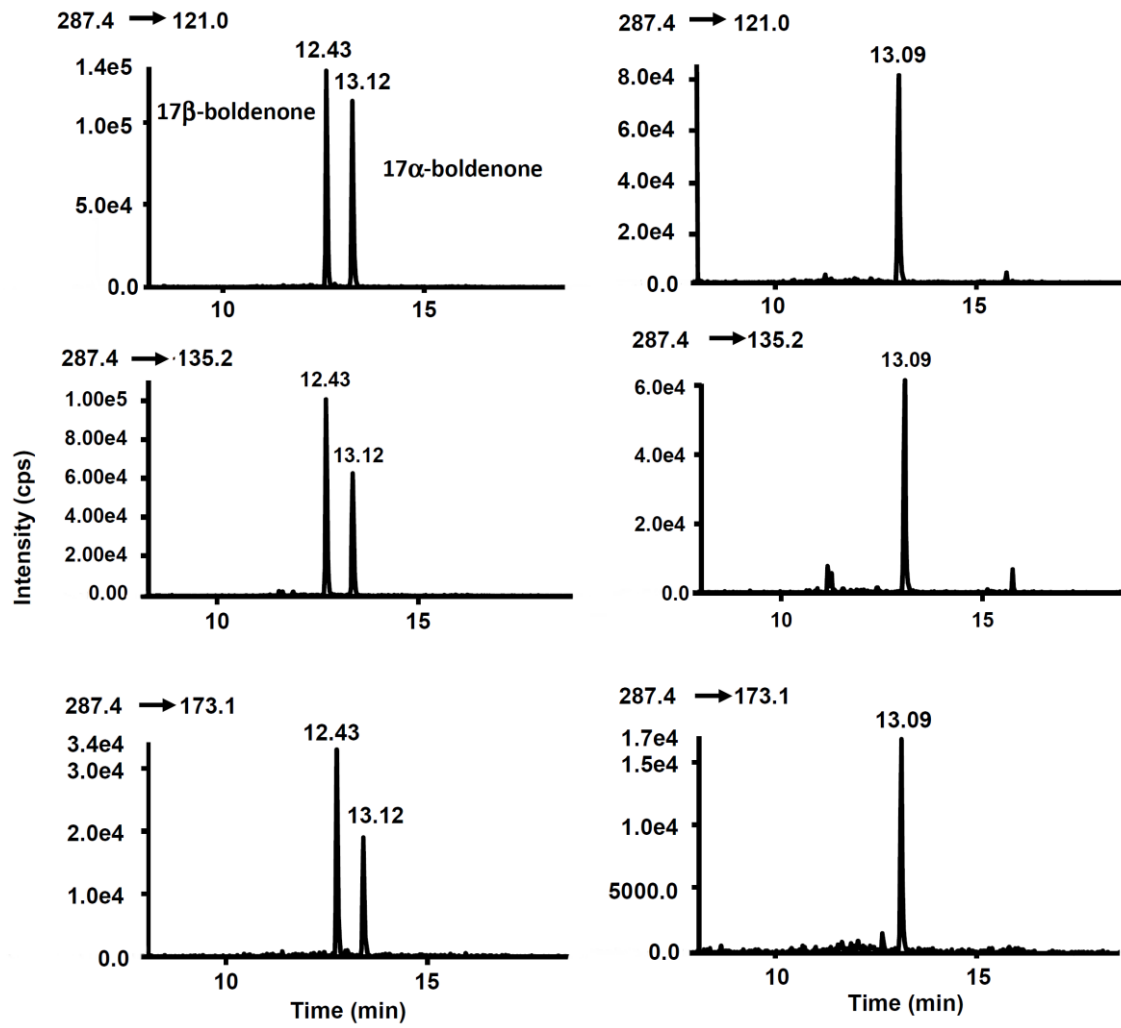
	R ₁	R ₂	R ₃	R ₄	R ₅
Clenbuterol	H	Cl	NH ₂	Cl	CH ₃
Hydroxymethylclenbuterol	H	Cl	NH ₂	Cl	CH ₂ OH
Tulobuterol	Cl	H	H	H	CH ₃
Bromobuterol	H	Br	NH ₂	Br	CH ₃
Mabuterol	H	CF ₃	NH ₂	Cl	CH ₃
Clenpenterol	H	Cl	NH ₂	Cl	C ₂ H ₅
Mapenterol	H	CF ₃	NH ₂	Cl	C ₂ H ₅
Cimaterol	H	C≡N	NH ₂	H	H

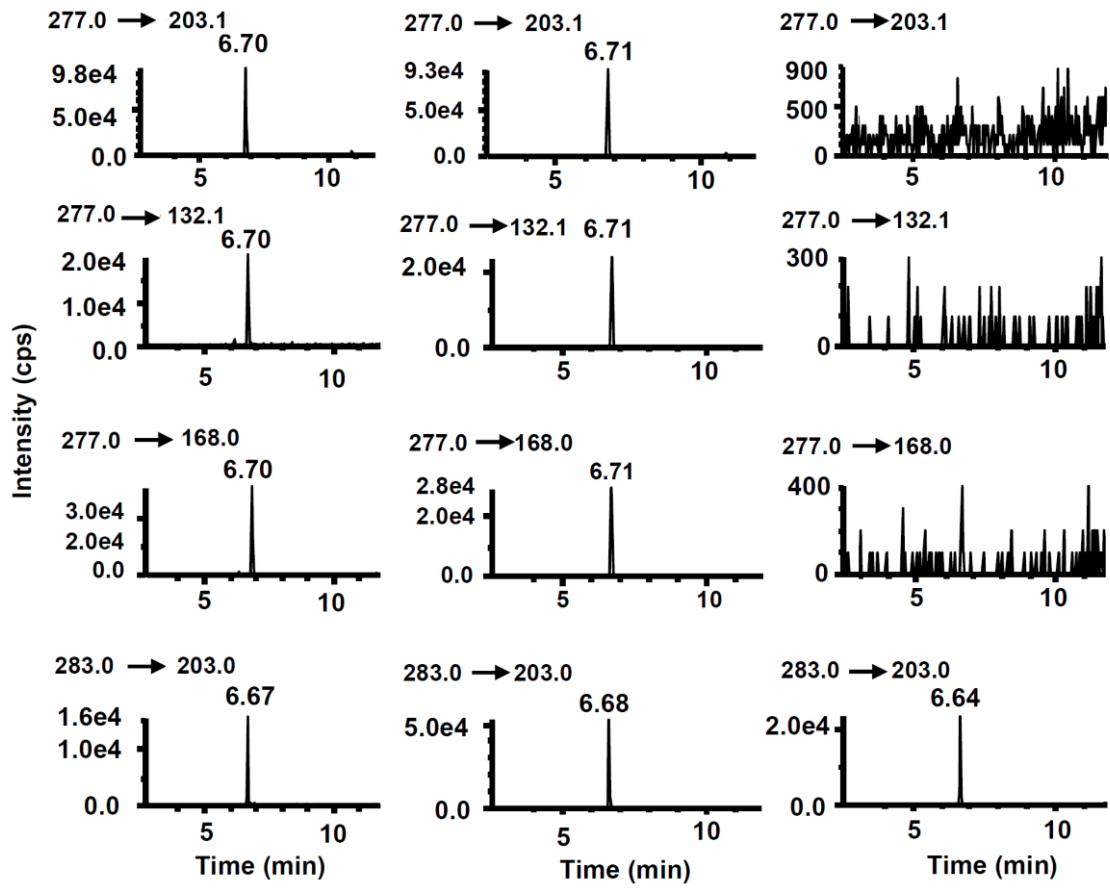
Salbutamol-like compounds

	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇
Salbutamol	CH ₂ OH	OH	H	H	CH ₃	CH ₃	CH ₃
Ractopamine	H	OH	H	H	H	CH ₃	CH ₂ -CH ₂ -Ph-OH
Isoxsuprine	H	OH	H	CH ₃	H	CH ₃	CH ₂ -O-Ph









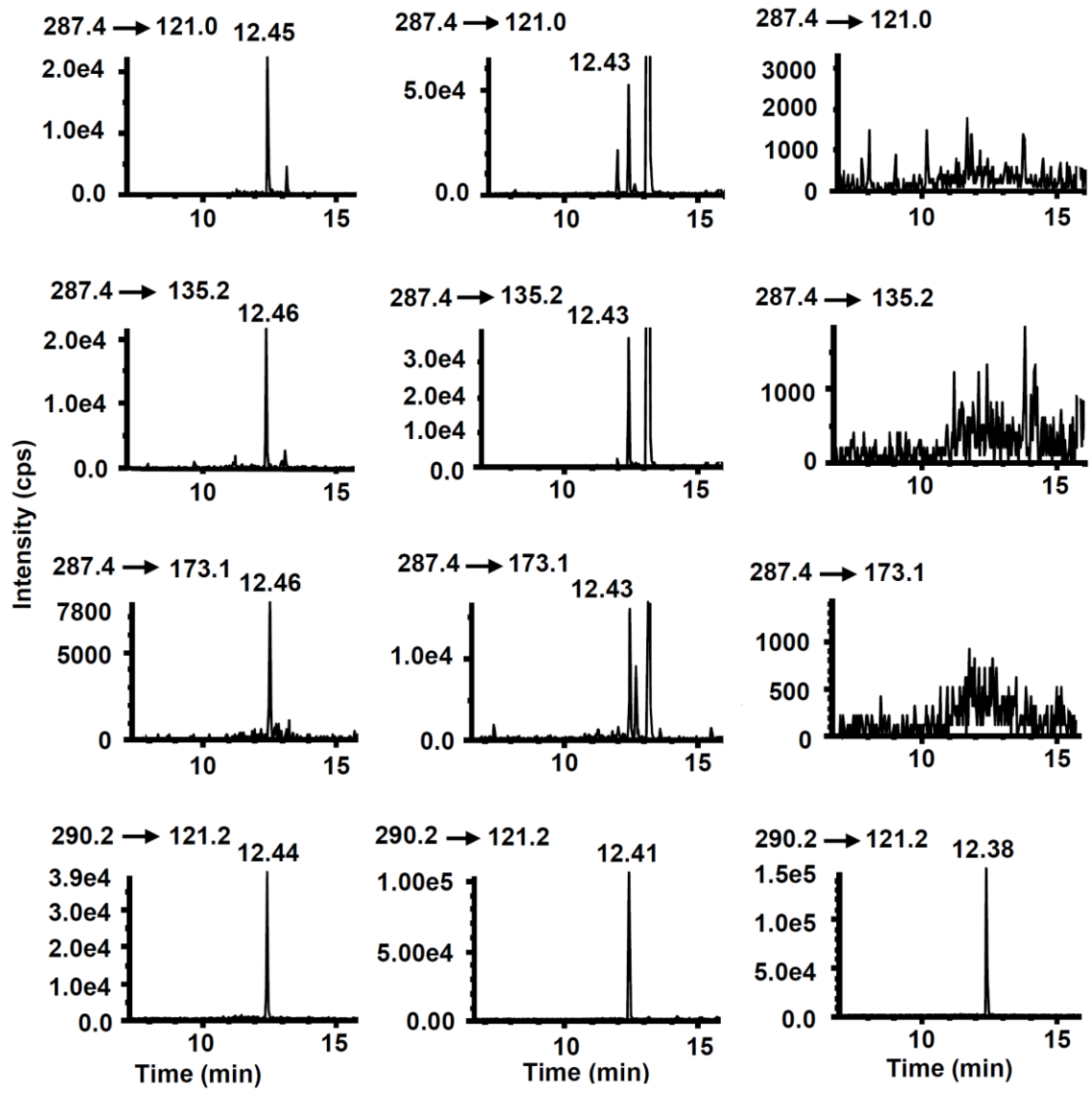


Figure Captions

Figure 1. Structure of anabolic steroids included in the method and general structure of β -agonist.

Figure 2. SRM profiles obtained from a standard solution with all the analytes at concentration of 100 ng/mL.

Figure 3. Chromatographic profiles of the three SRM transitions distinctive for 17α - and 17β -boldenone. (Left) Blank hair spiked with 17α - and 17β -boldenone at the final concentration of 20 ng/g. (Right) Unspiked hair sample from an untreated animal: the only peak in the chromatographic profile is 17α -boldenone. Estimated concentration is of 16 ng/g.

Figure 4. Chromatographic profiles of the four SRM transitions distinctive for clenbuterol and the single transition distinctive for ISTD clenbuterol D6. (Left) Blank hair spiked with clenbuterol at the final concentration of 10 ng/g. (Center) Real hair sample involved in a judicial proceeding about suspected illicit treatment (measured clenbuterol concentration: 11.9 ng/g). (Right) Blank hair.

Figure 5. Chromatographic profiles of the three SRM transitions distinctive for 17β -boldenone and that the single transition distinctive for ISTD 17β -boldenone D3. (Left) Blank hair spiked with 17β -boldenone at the final concentration of 40 ng/g. (Center) Real sample involved in a judicial proceeding about suspected illicit treatment (measured 17β -boldenone concentration: 39.0 ng/g). (Right) Blank hair.