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Confirmatory analysis of 17β-boldenone, 17α-boldenone and androsta-1,4-diene-3,17-dione in bovine urine, faeces, feed and skin swab samples by liquid chromatography–electrospray ionisation tandem mass spectrometry

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Abstract

The origin, i.e. natural occurrence or illegal treatment, of findings of 17α-boldenone (α-Bol) and 17β-boldenone (β-Bol) in urine and faeces of cattle is under debate within the European Union. A liquid chromatographic positive ion electrospray tandem mass spectrometric method is presented for the confirmatory analysis of 17β-boldenone, 17α-boldenone and an important metabolite/precursor androsta-1,4-diene-3,17-dione (ADD), using deuterium-labelled 17β-boldenone (β-Bol-d3) as internal standard. Detailed sample preparation procedures were developed for a variety of sample matrices such as bovine urine, faeces, feed and skin swab samples. The method was validated as a quantitative confirmatory method according to the latest EU guidelines and shows good precision, linearity and accuracy data, and CCα and CCβ values of 0.1–0.3 and 0.4–1.0 ng/ml, respectively. Currently, the method has been successfully applied to suspect urine samples for more than a year, and occasionally to faeces, feed and swab samples as well. Results obtained from untreated and treated animals are given and their impact on the debate about the origin of residues of 17β-boldenone is critically discussed. Finally, preliminary data about the degree of conjugation of boldenone residues are presented and a simple procedure for discrimination between residues from abuse versus natural origin is proposed.

Keywords: Boldenone; Androsta-1,4-diene-3,17-dione

1. Introduction

Boldenone (1,4-androstadiene-17β-ol-3-one, Fig. 1) is one of the anabolic steroid growth promoters banned by the EU for cattle fattening [1]. Illegal administration of boldenone and related compounds might occur by intramuscular injection, via pour-on treatment on the skin, or even orally, using structurally related precursors. Effective monitoring of the ban requires the availability of adequate analysis methods for screening and confirmation of boldenone and its metabolites in urine and other sample matrices [2]. Recently, new validation criteria concerning the performance of analytical methods and the interpretation of results were set by the European Commission [3]. As a consequence, methods for banned substances should be re-validated according to this decision by September 2004. However, enforcement of the ban on boldenone is complicated: the origin of findings of 17α-boldenone (α-Bol) and 17β-boldenone (β-Bol) residues in urine and faeces of cattle is under debate since 1996 [4]. Based on residue analysis by gas chromatography–mass spectrometry (GC–MS) the main metabolite, α-Bol was reported to be present in reference blank urines up to 2.6 ng/ml, while β-Bol itself was not observed in levels exceeding 0.1 ng/ml [4]. Since then α-Bol is considered as a naturally occurring steroid in cattle and findings of only the α-Bol metabolite do not provide sufficient evidence for illegal treatment. The excretion of boldenone metabolites has been studied in human- and equine urine, and also in bovine urine and

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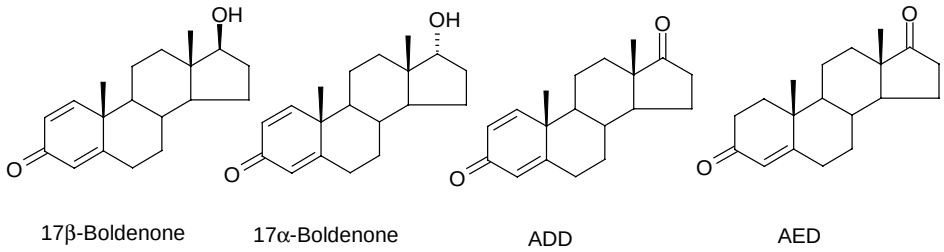

Fig. 1. 17β-boldenone (β-Bol), the major metabolite 17α-boldenone (α-Bol) and related substances 1,4-androstadiene-3,17-dione (ADD) and AED.

faeces [5–7]. In the latter study microsomes and hepatocytes from liver tissue were used to investigate metabolic pathways of boldenone in cattle in vitro and, combined with data from in-vivo findings, used in order to identify new metabolites in urine and faeces [7]. Oxidation of β-Bol to androsta-1,4-diene-3,17-dione (ADD) was found to be the most prominent in vitro metabolite in microsomal incubations, together with some minor 6-hydroxy metabolites of both β-Bol and ADD. 5β-Androst-1-ene-3,17-dione (5β-AED) was not observed in vitro. In contrast, in urine samples from animals treated with 17β-boldenone undecanoate α-Bol was detected as a major metabolite together with smaller quantities of β-Bol, ADD and 5β-AED and a prominent unidentified reduction product; in faeces on the other hand no β-Bol nor ADD was found.

Faeces samples found positive for α-Bol in routine screening also showed the presence of ADD and androst-4-ene-3,17-dione (AED), but only some of these samples also contained β-Bol [7]. AED, being both a precursor and a conversion product of the natural hormone testosterone, was also observed in some faeces samples found negative on α-Bol and ADD. Recently, preliminary data were reported that oral administration of ADD to veal calves yields ADD, α-Bol and β-Bol residues in urine, while oral administration of some phytosterols did not [8]. Different studies, aiming at finding a link between intake of specific feed components such as phytosterols and the occurrence of residues of boldenone and related substances in bovine sample matrices, are still on-going. Anyway, ADD should be considered not only as a metabolite but also as a precursor of β-Bol and analysis methods should preferably include ADD as well.

So far most studies applied derivatisation and gas chromatography–mass spectrometry with electron impact ionisation to the residue analysis of boldenone and its metabolites in urine and faeces. Very recently, it was shown that β-Bol could be detected in horse mane samples even up to 12 months following administration using a GC–MS^n approach [9]. However, in many cases liquid chromatography–tandem mass spectrometry (LC–MS/MS) can be a robust, easy and sensitive alternative as shown previously for the anabolic steroids trenbolone [10], stanozolol [11–13] and nortestosterone [14]. Two multi-analyte LC–MS/MS methods for the detection of anabolic steroids in bovine urine have been reported [15,16]. The first one includes trenbolone, 4-chloroandrost-4-ene-3,17-dione (CLAD), 16β-OH-stanozolol, α-Bol and β-Bol, and 17β-19-nortestosterone-d3 as internal standard [15], and the second one reports the screening of even 22 steroids at one time, including α-Bol and β-Bol and methylboldenone-d3 as internal standard [16]. Both methods were validated according to EU criteria [3] and show a detection capability (CCβ) at the 1 ng/ml level. Very recently Draisci et al. [17] described a confirmatory LC–MS/MS analysis method for boldenone and ADD in bovine urine using atmospheric pressure chemical ionisation (APCI) and 17β-testosterone-d2 as internal standard. The method was validated and successfully applied to the Italian Official Residue Control program.

In this work, we present an alternative method based on LC–MS/MS with positive electrospray ionisation (ESI) for the determination of β-Bol, its major metabolite α-Bol, and the precursor and metabolite ADD, using 17β-boldenone-d3 (β-Bol-d3) as internal standard. The method is designed with emphasis on a very high versatility towards different types of sample matrices. Following specific adaptations in sample preparation the method is applicable to urine, faeces, feed and skin swab samples. The method was validated as a quantitative confirmatory method following the latest EU guidelines [3] and applied to suspect urine and feed samples. Urine, faeces and skin swab samples from stated untreated veal calves were analysed in order to assess whether boldenone residues are from endogenous or exogenous origin. In addition, samples from controlled animal treatment were analysed for free boldenone and free plus conjugated boldenone in order to assess the role of conjugation in monitoring for boldenone abuse.

2. Experimental

2.1. Chemicals

Chemicals and solutions used were of analytical-reagent grade. Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). 17β-boldenone (β-Bol) and ADD were obtained from Steraloids (Newport, RI, USA). 17α-Boldenone (α-Bol) and the deuterated internal standard 17β-boldenone-d3 (β-Bol-d3) were obtained from the Community Reference Laboratory RIVM (Bilthoven, The Netherlands). AED was obtained from Sigma (St. Louis, MO, USA). β-Glucuronidase/arylsulfatase (from *suc* Helix

Pomatia) was from Merck (Darmstadt , Germany). Isolute NH_2 (100 mg) extraction columns were from IST (Hengoed, UK) and Bond Elut C18 (500 mg) solid phase extraction columns were from Varian (Harbor City, CA, USA).

2.2. Samples

In the validation study, 24 independent blank bovine urine samples from different animals were used. Incurred bovine urine and feed samples were obtained from a suspect sampling programme. Sample materials from stated untreated veal calves were obtained as follows: veal calves ($n = 46$) were under the control of an official Dutch experimental farm, fed by regular milk replacer according to current practice in veal calve industry, and not treated by intention with any steroidal veterinary drugs. Only antibiotics were applied on an individual basis and under the strict control of a veterinarian. Urine and rectal faeces samples from these veal calves were collected at the farm at the age of approximately 27 weeks. Skin swab samples were obtained by wiping the skin with cotton wool previously wetted with ethanol. Extracts of dried faeces scraped-off from the skin during slaughter (approximately 28 weeks) were kindly provided by RIVM.

2.3. Sample preparation

Urine: Two milliliters urine samples, blanks and controls were spiked with the internal standard β-Bol-d3 to a level of 2 ng/ml. The control urine samples were prepared by spiking blank urine with α-Bol and β-Bol at 1, 2 and 3 ng/ml. The samples were adjusted to pH 4.8, 20 μl Helix Pomatia was added and enzymatic deconjugation was carried out overnight in a water bath at 37 °C. Next, 2 ml of 0.25 M sodium acetate buffer pH 4.8 was added and the hydrolysed sample was subjected to solid phase extraction (SPE) on a C18 column, previously conditioned by methanol and sodium acetate buffer. The column was washed subsequently with 10% sodium carbonate solution, water, sodium acetate buffer, water and finally with 2 ml of methanol/water (50:50). The dried C18 column was eluted with 4 ml of methanol and the eluate was applied to an NH_2-column, previously conditioned with methanol. The methanol eluate thus obtained was evaporated to dryness under a stream of nitrogen gas at 40 °C.

Faeces: Two grams faeces was spiked with the internal standard β-Bol-d3 at 5 ng/g and 1 ml of 10% (w/v) sodium carbonate solution was added. Following mixing on a Vortex mixer 1 g of hydromatrix was added and mixing was repeated. Thirty-five milliliters diethylether was added and the mixture was shaken for 30 min in a head-over-head apparatus. Next, the mixture was put in a freezer and the upper layer was decanted in a centrifuge tube and evaporated to dryness in a rotavapor apparatus at 35 °C. The sample was redissolved in 2 ml of a mixture of methanol/ethanol/*i*-octane (27:8:60) and 0.4 ml of 0.2 M acetic acid was added. Following mixing on a vortex the tube was centrifuged during 15 min at 3400 rpm. The lower aqueous phase was transferred to a test tube and further subjected to SPE as described for urine samples.

Feed: One gram homogenized feed was spiked with the internal standard β-Bol-d3 at 10 ng/g and 4 ml of 0.25 M sodium acetate buffer pH 4.8 and 4 ml of methanol were added. The tube containing the entire mixture was placed for 5 min in an ultrasonic bath, followed by 5 min shaking in a head-over-head apparatus. Next, the tube was centrifuged during 10 min at 3000 rpm and finally 4 ml of the supernatant was transferred to a test tube and further subjected to SPE as described for urine samples.

Skin swabs: The swabs (cotton wool) were transferred into a 500 ml tube, spiked with 4 ng of the internal standard β-Bol-d3 and soaked in methanol overnight. The liquid layer was filtered over a plug of glass wool and the solid material was re-extracted with methanol. The combined methanol was evaporated to dryness in a rotavapor apparatus at 45 °C. The sample was redissolved in 3 ml methanol and 7 ml of 0.25 M sodium acetate buffer pH 4.8 was added. In case of turbidity, the extracts were centrifuged at this stage. Finally, the samples were subjected to SPE as described for urine samples.

2.4. LC fractionation of extracts

The extracts obtained from SPE were redissolved and fractionated by preparative reversed phase gradient LC on a 250 mm × 3.0 mm i.d. Lichrocart (Merck) column packed with 5 μm C18 RP-Select B material and equipped with a Lichrocart 4-4 guard column packed with similar material. The two mobile phases used consisted of (A) methanol/water (10:90) and (B) methanol/water (90:10) and the flow was 0.4 ml/min. Following an isocratic period of 10 min at 65% B a steep linear gradient was started towards 95% B at 12 min, followed by a final isocratic step of another 9 min. Under the conditions of the specific gradient system applied α-Bol and β-Bol and the internal standard β-Bol-d3 were collected in a window of approximately 7 min corresponding with a fraction volume of 2.8 ml. The fraction thus obtained was evaporated to dryness under a stream of nitrogen gas at 40 °C. The fraction was finally redissolved in 50 μl of methanol, 250 μl of water/formic acid (100:0.5) was added and 100 μl of the sample thus obtained injected into the LC–MS/MS system.

2.5. Liquid chromatography–mass spectrometry

The liquid chromatography–mass spectrometry (LC–MS/MS) system consisted of a Waters (Milford, MA, USA) model Alliance 2690 LC system equipped with a Micromass (Manchester, UK) model Quattro Ultima tandem mass spectrometer. The mass spectrometer was operated in the positive electrospray ionisation (ESI) mode at a capillary voltage of 2.7 kV, a desolvation temperature of 300 °C, source temperature 120 °C and a cone voltage of 25 V. Desolvation gas

Table 1
Positive ion MS/MS conditions for the MRM acquisition of boldenone and related components

Component	Precursor ion mass (*m*/*z*)	Product ion mass (*m*/*z*)	Collision energy (eV)
17β-Boldenone	287	121	20
		135	
17α-Boldenone	287	121	20
		135	
17β-Boldenone-d3	290	121	20
ADD	285	121	20
		147	

was nitrogen and the CID gas was argon. For each analyte, the two most abundant product ions were monitored using the conditions given in Table 1. The analytical column was a 150 mm × 3 mm i.d. 5 μm C18 Waters (Milford, MA, USA) Symmetry column, kept in a column oven at 40 °C. The two mobile phases used consisted of (A) water/formic acid (100:0.2) and (B) water/acetonitrile/formic acid (10:90:0.2) and the flow was 0.4 ml/min. Both mobile phases were filtered through a 0.45 μm Millipore membrane filter. Following a 5 min isocratic period at 0% B, a linear gradient was started towards 100% B at 22 min, kept at that composition until 24 min. The LC was connected with the ESI MS/MS using a 1:2 flow split.

In all cases, reagent blanks, matrix blanks and (spiked) control matrices were analysed in the same analysis series. The concentration of α-Bol and β-Bol in the samples were calculated using the isotope dilution method and a calibration graph constructed from results obtained with the blank and spiked matrix samples.

2.6. Validation study

The validation study was carried out at three concentration levels by the analysis of bovine urine samples spiked with 0.5, 1.0 and 1.5 ng/ml of the analytes β-Bol, α-Bol and ADD. To each sample 2.0 ng/ml of the internal standard β-Bol-d3 was added, and six replicates of each sample were analysed on three different days using two different LC–MS/MS systems and two different technicians. The analytes were quantitated using the isotope dilution method (relative peak areas versus internal standard) and calibration curves prepared each day in urine matrix. From the data thus obtained within-day repeatability, within-laboratory reproducibility and accuracy were calculated. By doing so robustness data are actually included in the reproducibility results. Furthermore, it should be underlined that the ruggedness is monitored in each individual sample analysis by monitoring the signal intensity of the deuterated internal standard. The recovery of the overall sample preparation procedure was determined on three different days by the analysis of three urine samples spiked at 1.0 ng/ml and addition of the internal standard just before injection into the LC–MS/MS system, i.e. internal standard correction for injection volume and ion suppression only. The linearity was determined for a limited concentration range since the analytes of interest are banned substances for which no safe maximum residue limits have been established. Spiked urine samples in the range of 0.25–5 ng/ml ($n = 6$) were analysed on three different days.

3. Results and discussion

3.1. General considerations

The molecular structures of boldenone and related compounds (Fig. 1) are very similar to endogenous steroids such as testosterone. In many cases, only one double bond or one oxidised hydroxyl functionality counts for the difference, others even have identical elemental compositions. According to ref. [3] at least two MRM transitions should be measured in LC-MS/MS in order to comply with the required minimum number of identification points. Moreover, the method should provide selectivity in order to prevent false positive results. The MRM transitions used should be preferably unique for a specific substance or, alternatively, the LC part of the system should provide sufficient resolution in order to differentiate between substances having the same MRM transitions in MS/MS. The fragmentation behaviour of testosterone and related components under ESI MS/MS conditions has been described before [18–20]. An important product ion in testosterone fragmentation is *m*/*z* 123 formed by B-ring fission of the steroid skeleton. The B-ring fission product still incorporates the intact A-ring of the steroid and is very useful to differentiate between A-rings having two double bonds (boldenone, ADD) and A-rings with a single double bond (testosterone, AED). The *m*/*z* 121 product ion chosen in this study (Table 1) thus guarantees a high selectivity towards the steroids of interest. The second product ion at *m*/*z* 135 is shifted to *m*/*z* 138 for the 16,16,17-d3 analogue of boldenone which supports an intact D-ring for the *m*/*z* 135 fragment ion of boldenone. Although not further studied in this work, it is postulated that this fragment ion is formed by a loss of methanol (involving the methyl- and hydroxyl substituents at the 18 and 17 position) and B-ring fission of the steroid. The significantly higher *m*/*z* 135/121 ion ratio as observed for β-Bol versus α-Bol and the very low intensity of *m*/*z* 135 in the product ion spectrum of ADD would strongly support such a loss of methanol.

It should be noted that $M + 2$ isotopes, although having a relative intensity of approximately 2.5% only, might contribute significantly to MRM channels of other compounds when M is coeluting and present in huge excess. AED has the same elemental composition as boldenone but fortunately its retention time and MRM transitions are different from β-Bol and α-Bol (cf. Fig. 2). The $M + 2$ isotope of ADD has the same molecular mass and MRM transitions as boldenone but ADD elutes in between β-Bol and α-Bol. Testosterone itself has MRM transitions different from

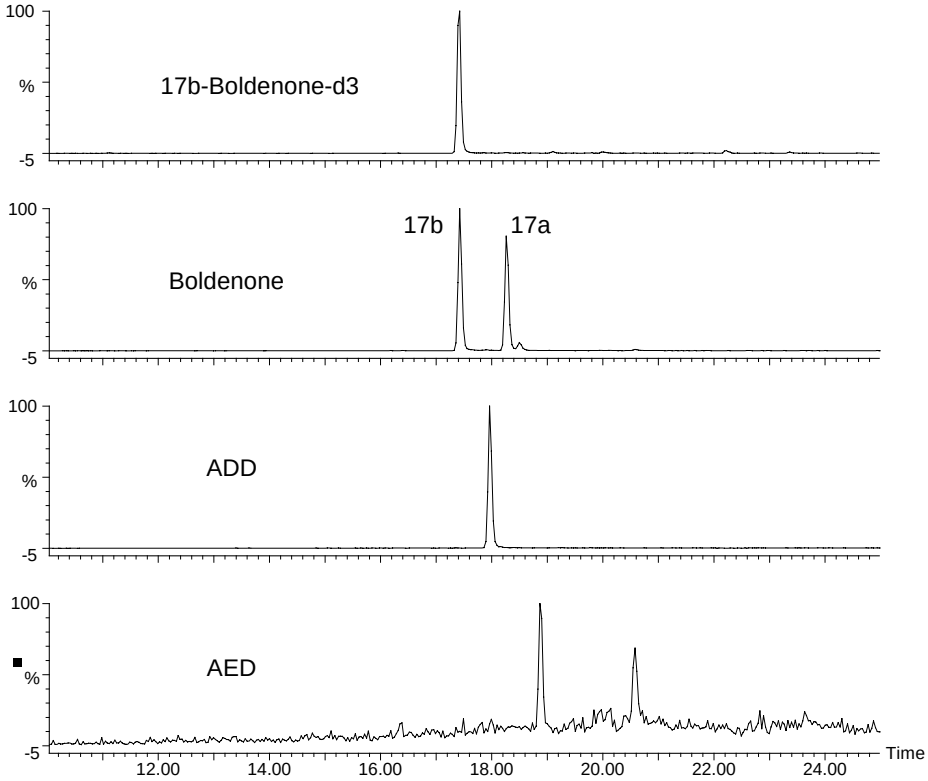

Fig. 2. Reconstructed LC–MS/MS chromatograms for the internal standard 17β-boldenone-d3 (β-Bol-d3), β-Bol and α-Bol, ADD and AED. Each chromatogram is the sum of the two MRM transitions per analyte. Note that AED was acquired at the MRM transitions of boldenone only in order to show interferences, if any, on boldenone analysis. Further explanation, see text.

boldenone as outlined above. Of course gas chromatography is an even more powerful separation method but that option would require derivatisation; the limited stability of derivatives of unsaturated steroids as boldenone and trenbolone does not favour the use of GC–MS when good alternatives without derivatisation are available.

Especially in electrospray ionisation (ESI) MS where ion suppression might occur the choice of a good internal standard is a prerequisite. In this study, we applied β-Bol-d3 which coelutes with the most relevant analyte β-Bol and will adequately compensate for both incomplete recovery and ion suppression effects. We normally add the internal standard in the very first step of the analysis procedure, i.e. for urine samples prior to enzymatic deconjugation. Occasionally, with some batches of the enzyme, we observed partial conversion of β-Bol-d3 towards the native compound β-Bol which was not observed in sample preparation procedures without the enzyme treatment. Although this unintended conversion is typically in the order of 0.1–0.2 ppb only, one should be cautious and consider to add this internal standard after the enzymatic deconjugation step.

3.2. Validation study

According to ref. [3] the following performance characteristics must be determined for a qualitative confirmatory method: detection capability CCβ, decision limit CCα, specificity and ruggedness. For banned substances such as boldenone no safe maximum residue limits have been established and a *qualitative* confirmatory method is adequate. However, one possible outcome of the on-going scientific debate on the origin of β-Bol might set a quantitative endogenous background level for bovine urine, so in this case the additional performance characteristics for a quantitative method were assessed as well. The decision limit CCα is defined as "the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant" [3]. CCα was determined by analysing 23 blank urines from 23 different young bovines and calculated using the equation $CC\alpha = 2.33\sigma_N/a$ and the procedure given in ref. [21]. The detection capability CCβ is defined as "the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β. In the case of substances for which no permitted limit has been established, the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of $1 - \beta$" [3]. CCβ was determined by the investigation of 20 blank urines from 20 different young bovines fortified with β-Bol and α-Bol at 0.4 ng/ml. For α-Bol none, and for β-Bol only one fortified sample was not confirmed and therefore false compliant. As a result, CCβ equals the fortification level of 0.4 ng/ml since one or zero of the 20 samples (=5%) investigated was false compliant. For ADD

Table 2
Validation data for the determination of 17β- and 17α-boldenone in urine by LC–MS/MS

Analyte/parameter	β-Bol			α-Bol			ADD		
Spike level (ng/ml)	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5
Repeatability (R.S.D., %)	8.0	9.0	10	10	8.0	9.3	16	10	13
Within-lab reproducibility (R.S.D., %)	10	11	12	16	9.0	9.3	20	15	17
Accuracy (%)	96	99	107	89	91	99	119	115	112
Recovery (%)		73			74			57	
Linearity (r^2, 0.25–5 ng/ml)		0.999			0.998			0.997	
CCα (ng/ml)		0.29			0.11			0.21	
CCβ (ng/ml)		0.4			0.4			<1.0	
Specificity	Passed			Passed			Passed		
Ruggedness	Passed			Passed			Passed		

β-Bol, 17β-boldenone; α-Bol, 17α-boldenone. Validation parameters according to ref. [3]; other conditions, see text.

20 blanks from six different young bovines were fortified at 1.0 ng/ml and none was false compliant, i.e. CCβ for ADD is lower than 1.0 ng/ml.

At the beginning of the validation study the specificity was checked by the analysis of 24 urine samples from different young bovines. No interference on the analysis of β-Bol, α-Bol and ADD was found due to the highly specific MRM acquisition method and the use of an appropriate deuterated internal standard. One of the urine samples was found not to be entirely blank: the presence of approximately 0.2 ng/ml of boldenone was confirmed and that particular non-compliant urine was no longer used in linearity, precision and CCα or CCβ studies. The validation results have been summarised in Table 2. From this Table it can be concluded that the method developed shows relevant CCα and CCβ values, good precision, accuracy and linearity, and fulfils the validation criteria set for quantitative confirmatory methods [3]. In comparison with the recently published LC–MS/MS work based on APCI [17] the validation data presented here are strikingly similar; only the precision data are slightly better with APCI.

The analysis procedure for the other matrices of interest, i.e. faeces, feed and skin swabs is very similar (cf. Experimental) and therefore the method was not revalidated for these matrices. Instead, quality was assured by the analysis of blank matrix and spiked matrix control samples in each analysis series and the presence of the β-Bol-d3 internal standard in each of the individual samples under investigation.

3.3. Applicability

The confirmation of identity according to ref. [3] was demonstrated by the analyses of urine, faeces and feed samples spiked with β-Bol, α-Bol and ADD, at 1.0, 1.0 and 5.0 ng/g, respectively. The precursor ion and two product ions recorded in MRM mode yielded the minimum requirement of four identification points. The results are given in Table 3 and show that all identification criteria were fulfilled, thus yielding non-compliant results [3]. The applicability is further demonstrated by the analysis of incurred urine and feed samples: the reconstructed LC–MS/MS chromatograms are shown in Figs. 3 and 4. The β-Bol and α-Bol concentrations found in the incurred urine (Fig. 3c) and feed (Fig. 4b) samples were in the order of 3 and 15, and 3 and 7 ng/g, respectively. Of course it is rather unlikely that such low levels of β-Bol and its metabolite α-Bol were added by intention to the feed in order to induce anabolic effects;

Table 3
Confirmation data of boldenone and ADD in urine spiked at 1.0 ng/ml, feed spiked at 5.0 ng/g and faeces spiked at 1.0 ng/g, and analysed by LC–MS/MS

Matrix/analyte	RRT_{exp} [a]	RRT_{req}	RRT passed	Ion $ratio_{exp}$ [b]	Ion $ratio_{req}$	Ion ratio passed
Urine						
β-Bol	1.002	0.976–1.026	Y	0.430	0.322–0.536	Y
α-Bol	1.050	1.025–1.077	Y	0.392	0.287–0.479	Y
ADD	1.035	1.008–1.060	Y	0.265	0.179–0.299	Y
Feed						
β-Bol	1.000	0.975–1.025	Y	0.466	0.347–0.578	Y
α-Bol	1.050	1.024–1.076	Y	0.390	0.283–0.472	Y
ADD	1.035	1.008–1.060	Y	0.222	0.160–0.267	Y
Faeces						
β-Bol	1.000	0.975–1.025	Y	0.495	0.371–0.619	Y
α-Bol	1.048	1.022–1.074	Y	0.409	0.321–0.535	Y

β-Bol, 17β-boldenone; α-Bol, 17α-boldenone. For conditions, see text.
[a] Relative retention time vs. internal standard.
[b] Area ratio of product ions from reconstructed MRM chromatograms.

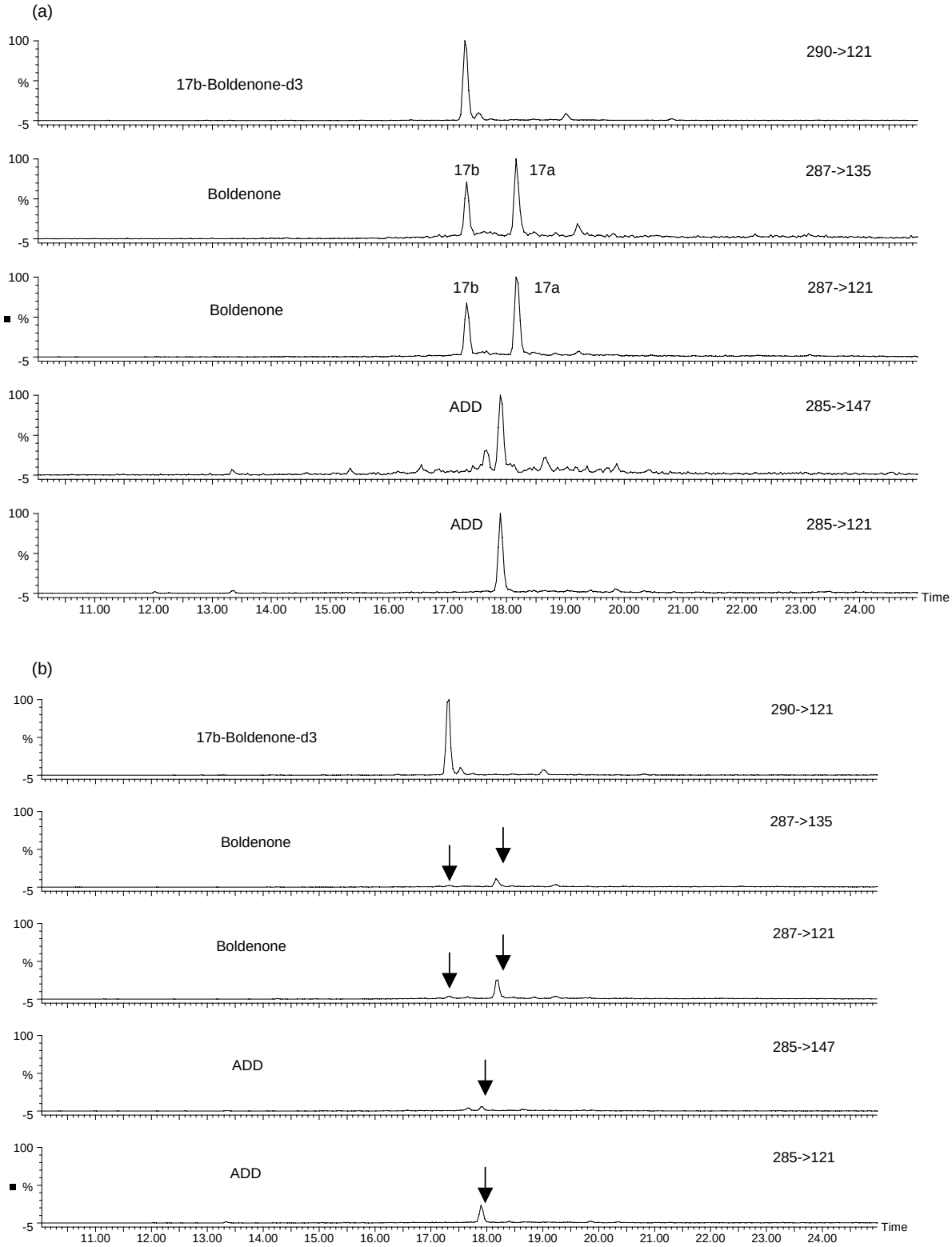

Fig. 3. Reconstructed LC–MS/MS chromatograms of the MRM transitions of the internal standard β-Bol-d3, β-Bol and α-Bol, and ADD in (a) a control urine spiked at 0.5 ng/ml, and (b) a blank urine (normalised to the same β-Bol-d3 abundance as in the spiked urine, and containing approximately 0.2 ng/ml of endogenous α-Bol and ADD, respectively). Reconstructed chromatograms of the summed MRM transitions of (c) an incurred bovine urine sample containing approximately 3 and 15 ng/ml of β-Bol and α-Bol, respectively. Further explanation, see text.

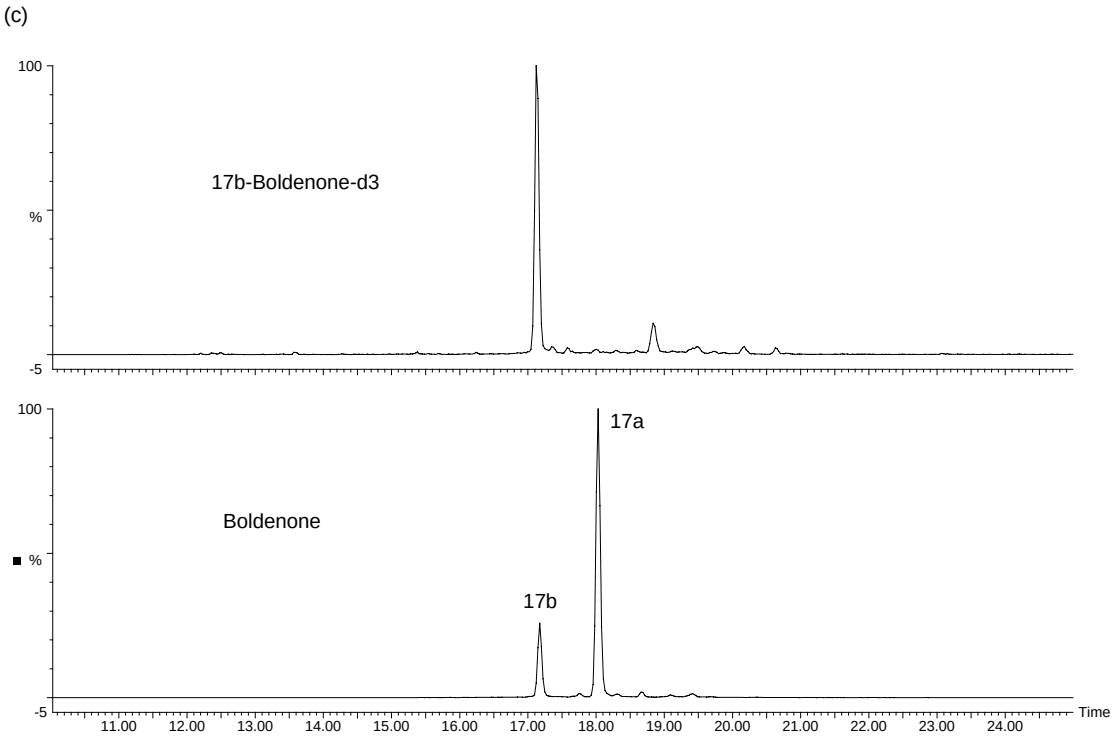

Fig. 3. (*Continued*).

most likely in this case the feed was simply contaminated, inside the stable, with bovine urine. The potential of this LC–MS/MS method for other matrices such as skin swabs is demonstrated using the incurred sample shown in Fig. 5.

3.4. Boldenone, endogenous or exogenous

The method was applied to different sample matrices from stated untreated veal calves under the control of an official Dutch experimental farm. Urine samples and rectal faeces samples yielded only compliant results for β-Bol, α-Bol and ADD (see Figs. 3b and 4a). However, skin swab samples taken from the same animals did show residues of α-Bol and ADD (Fig. 5). Also some of the dried faeces samples scraped-off from the skin were found to contain residues of α-Bol and ADD, and, in some cases, even β-Bol (in the order of 0.1–2 ng/g). In summary, stated untreated veal calves showed compliant boldenone results only for sample matrices such as urine and rectal faeces, and occasionally non-compliants when samples were collected from the outside of the animals. Several explanations might apply to this discrepancy: unintentional treatment via the skin (external contamination), or simply a concentration effect since dried faeces is a concentrate versus fresh manure thus undetectable hormone levels in the latter might yield non-compliants in dried faeces, depending on the concentration factor. An additional or other explanation might be microbiological conversion of steroidal compounds in dried faeces on the skin towards boldenone and related components. At least in rats microbiological conversion in faeces towards ADD and AED following oral administration of phytosterols has been described [22]. However these metabolites were not observed in rat liver microsomes which (provided these data would be equally applicable to veal calves) might explain the absence of β-Bol in urine of untreated calves as shown in the current work and in previous studies [4,8]. Currently, a few laboratories are investigating the relationship between the intake of specific feed components and the occurrence of boldenone-related residues in bovine urine and other sample materials. According to the present knowledge β-Bol itself has still not been detected as an endogenous substance in urine from life-time guaranteed untreated veal calves. Thus, β-Bol in urine is still applicable for tracing suspect calves which might have been treated with boldenone or related substances. Because of the apparent occasional presence of β-Bol, α-Bol and ADD in dried faeces care should be taken during sampling of urine in order to prevent contamination of the urine sample. Note that according to the levels of β-Bol in the dried faeces samples from the untreated veal calves in this study, only unrealistically high degrees of faecal contamination would theoretically yield non-compliant residue findings of β-Bol in urine up to an arbitrarily level of, let say, 0.5 ng/ml. Provided that no faecal contamination can be detected visually and the sample is properly stored at low temperature in the dark, it might be proposed that residues of β-Bol in urine at and beyond a level of 0.5 ng/ml are considered abnormal anyway and illegal treatment with boldenone or related substances will be the most likely explanation.

In literature, differentiation between endogenous and exogenous origin of nandrolone metabolites in human urine has

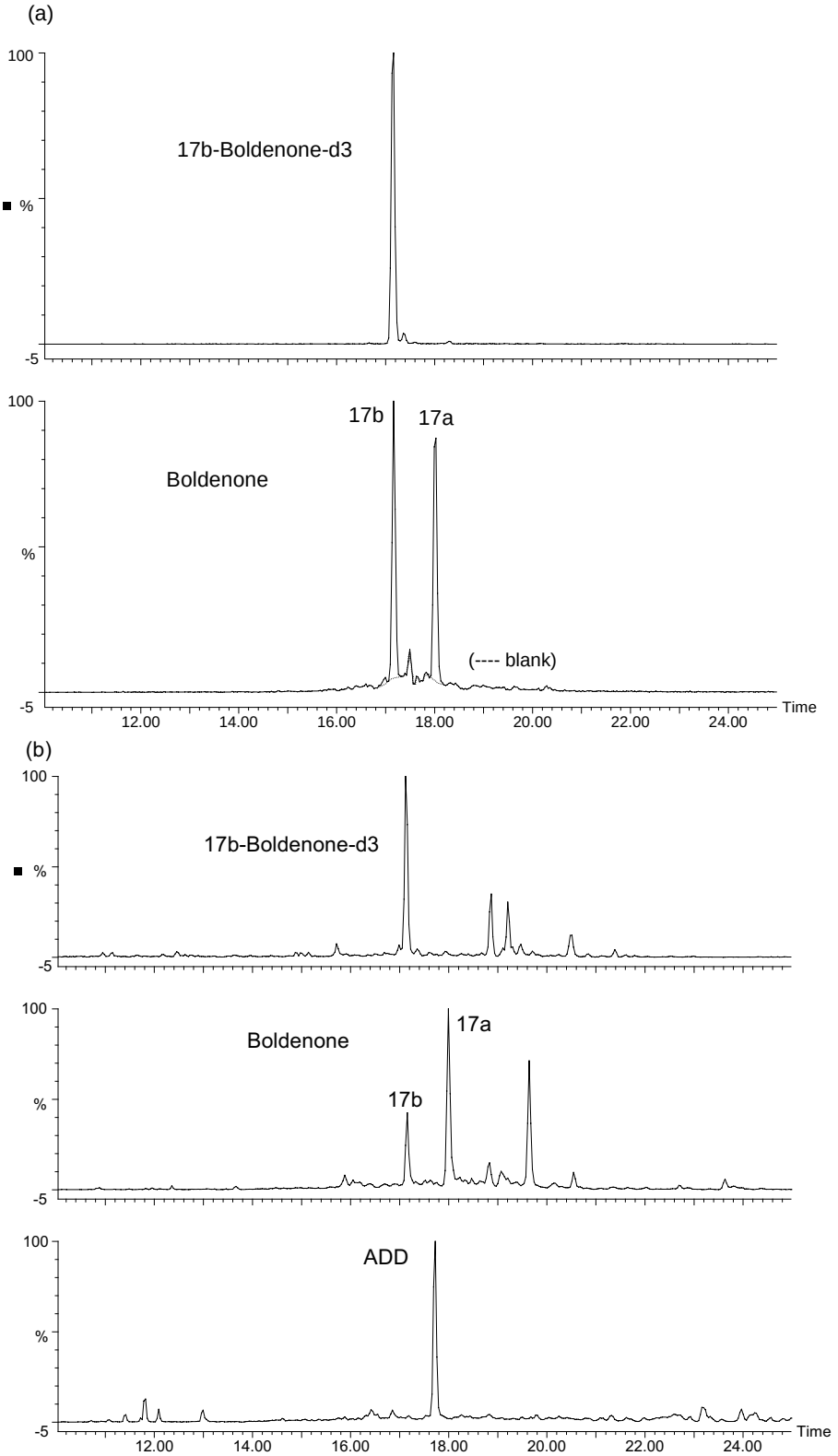

Fig. 4. Reconstructed LC–MS/MS chromatograms for the internal standard β-Bol-d3, β-Bol and α-Bol in (a) a blank and a control faeces sample spiked at 1.0 ng/g, and (b) an incurred feed sample containing approximately 3, 7 and 7 ng/g of β-Bol, α-Bol and ADD, respectively. Each chromatogram is the sum of the two MRM transitions per analyte. Further explanation, see text.

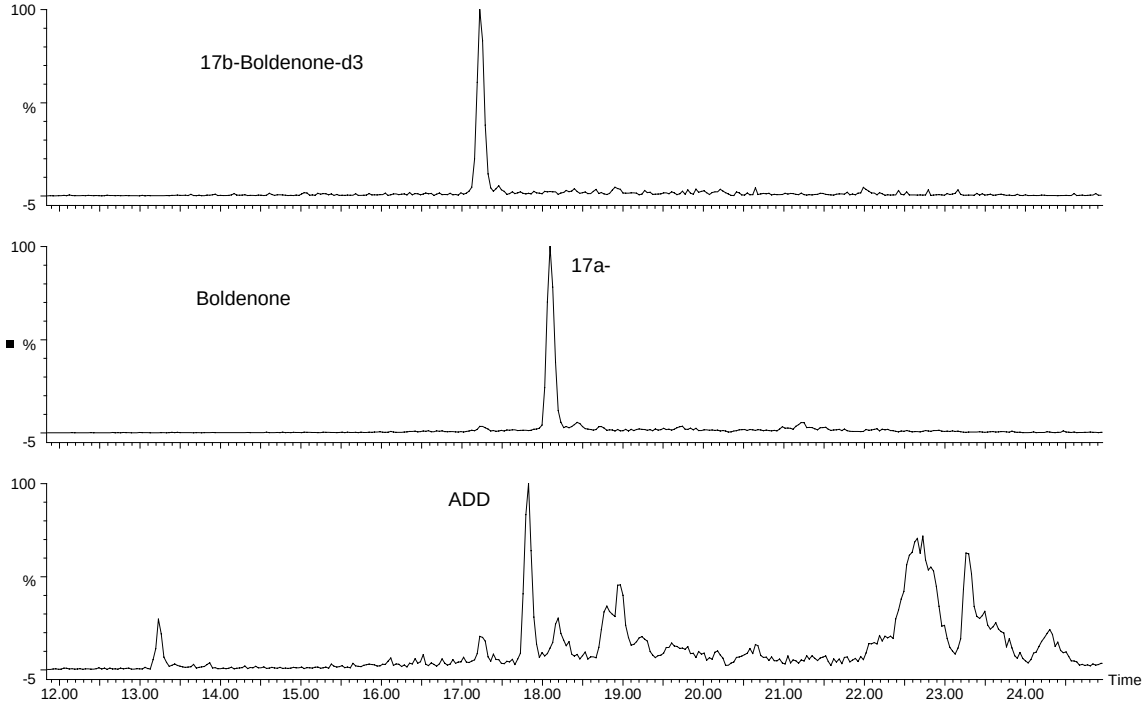

Fig. 5. Reconstructed LC–MS/MS chromatograms for the internal standard β-Bol-d3, β-Bol and α-Bol, and ADD in a skin swab sample obtained from stated untreated veal calves. Note that urine and faeces samples of the stated untreated calves were blank and given in Figs. 3b and 4a, respectively. Each chromatogram is the sum of the two MRM transitions per analyte. Further explanation, see text.

been proposed based on their different phase II metabolite compositions [23]. Similarly, differentiation between faecal contaminated and uncontaminated urine might be provided by analysis of β-Bol with and without enzymatic deconjugation of phase II metabolites. In dried faeces β-Bol occurs in the unconjugated form due to the presence of glucuronidases in the intestine. Excretion in urine following illegal treatment via intramuscular injection on the other hand will mainly occur as β-Bol conjugates. In order to support this statement incurred urine samples from veal calves in two entirely independent animal experiments with intramuscular injection of 17β-boldenone undecylenate (details will be presented elsewhere) were analysed with and without enzymatic deconjugation. Analysis without enzymatic deconjugation yields the concentration of free β-Bol, α-Bol and ADD, while analysis including the enzymatic deconjugation step shows the sum of free and conjugated analytes. Thus, the increase in concentration between these two analyses reflects the degree of conjugation of β-Bol, α-Bol and ADD in the sample matrix under investigation. The results shown in Table 4 clearly indicate that intramuscularly treatment yields almost exclusively conjugates of β-Bol and α-Bol; in contrast ADD, which is lacking similar conjugation-sites, is not much influenced by an enzymatic deconjugation step in

Table 4
Results (ng/g) of 17β-boldenone, 17α-boldenone and ADD in urine samples from two independent animal treatments (intramuscular injection of 17β-boldenone undecylenate), analysed by LC–MS/MS with and without prior enzymatic deconjugation

	Matrix/analyte					
	β-Bol		α-Bol		ADD	
Enzymatic deconjugation	No: free	Yes: free + conjugates	No: free	Yes: free + conjugates	No: free	Yes: free + conjugates
Urine 1, i.m.	0.4	7.9	0.2	23	n.c.	n.c.
Urine 2, i.m.	0.7	6.8	1.6	28	3.3	3.3
Urine 3, i.m.	0.6	9.8	0.9	32	3.4	n.c.
Urine 4, i.m.	0.0	0.5	0.2	5.3	0.2	0.5
Urine 5, i.m.	0.3	5.1	1.3	40	2.0	n.c.
Urine control untreated	Neg	Neg	0.0	0.4	Neg	Neg
Dried faeces*	21	12	520	450	790	820

For comparison, urine data from an untreated control animal and an incurred dried faeces sample are given. β-Bol, 17β-boldenone; α-Bol, 17α-boldenone; i.m., intramuscular treatment; n.c., peak present but identity not confirmed according to the criteria of ref. [3].

* Inhomogeneous sample.

the analysis procedure. Dried faeces does not show an increase in boldenone levels following enzymatic deconjugation, i.e. boldenone is present in its free form as expected, and any contamination of urine with dried faeces will only contribute to the concentration of free boldenone. Consequently, enforcement of the ban on boldenone abuse, without false non-compliants caused by faecal contamination, is still feasible and should be based on monitoring for the presence of intact 17β-boldenone *conjugates* or by differential analysis of urine samples with and without prior enzymatic deconjugation step. Very recently, this proposal has been adopted by experts from EU member states [24].

4. Conclusions

A validated method has been developed for the confirmatory analysis of residues of boldenone and metabolites in urine, faeces, feed and skin swab samples. The method complies with the latest EU requirements and is generally applicable to different sample matrices: only the primary extraction step differs for specific matrices. Thus, the method can be used in the scientific debate concerning the origin of residues of boldenone. In the present study it has been shown that urine and rectal faeces samples from animals stated to be never treated with hormonal substances indeed yield blank values for β-Bol, α-Bol, and ADD, in accordance with previous findings [4,8]. The origin of the findings of residues of α-Bol, ADD, and occasionally even β-Bol, in dried faeces on the skin of the same animals is still unknown and might be possibly explained by microbiological conversion from phytosterols or other feed components under specific conditions. Currently urine (β-Bol) and, possibly, hair [9] (intact 17β-boldenone-esters) are still the best sample matrices for detection of illegal treatment with boldenone or related substances. It has been shown that the theoretical impact of contamination of urine samples with external non-compliant faecal material can be simply excluded by differential analysis of urine samples with and without prior enzymatic deconjugation. Thus, enforcement of the ban on β-Bol is feasible and at least administration via intramuscular treatment is easily detected.

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