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Study of 17β-estradiol-3-benzoate, 17α-methyltestosterone and medroxyprogesterone acetate fixation in bovine hair

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Abstract

The detection of steroid residues in hair is a powerful strategy to demonstrate long-term administration of these growth promoters in meat production animals. A fast and reliable method was developed for monitoring anabolic steroids and their esters in hair. A 100 mg hair sample was converted into powder and extracted at 50 °C with methanol (sebum fraction). The remaining hair was digested with 1 M NaOH for further extraction of bound steroids. The two fractions were separately purified onto an aminopropyle solid-phase extraction column and onto a silica SPE cartridge. Steroids were detected either by gas chromatography–tandem mass spectrometry after silylation using N-methyl-N-(trimethylsilyl)-trifluoroacetamide/trimethyliodosilane/dithiothreitol or liquid chromatography–tandem mass spectrometry. This method was applied to hair samples collected over a three months period after treatment of three cows respectively with 17α-methyltestosterone, medroxyprogesterone acetate and 17β-estradiol-3-benzoate. The fixation kinetic into hair of the three steroids have been deeply examined and discussed; relation in-between concentration and distance from the injection site, influence of hair colour and sample treatment consequences have been discussed.

Keywords: Anabolic; Steroid; Esters; Hair; GC–MS/MS; LC–MS/MS

1. Introduction

The use of xenobiotic agents for improving the growth and feed conversion rates of food producing animals has been banned in the European Union since 1988 and no residues of these anabolic substances should be present in animal products. Many biological matrices such as tissue or urine samples can be used for the control of the illegal use of anabolic steroids but hair can be considered as amongst the most judicious matrix due to its easiness of collection and sometimes the long-term detectability of administered substances.

Originally used to evaluate human exposure to heavy metals [1], hair analysis is used for criminal court proceedings, clinical purposes and doping control [2]. While urine analysis provides a detectability time window of hour or day range for drugs, hair testing permits a long-term detection sometimes up to several months [3]. In 2000, Gaillard et al. [4] published a study on the compared interest between hair analysis and urine analysis in doping controls. Thirty cyclists were sampled and tested both in the two matrices. For corticosteroids, urine analysis was proved to be an efficient tool. For anabolic steroids and amphetamines, conversely, hair appears as a sensitive matrix. The interest of hair testing was also demonstrated for revealing drugs treated horses. It was proved that measurement of clenbuterol in tail [5] and diazepam in mane [6] are respectively possible for up to 13 months and at least 85 days after the last administration. Only few papers have been reported concerning bovine hair. Analyses of corticosteroids [7], clenbuterol [8-10] and steroids [11-15] have been performed. This lack of steroid studies does not permit to answer to many questions: how long are

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the steroids fixed in the bovine hair? What are the mechanisms of steroids fixation? Drug incorporation into hair is a complex phenomenon and many factors are involved. Different major pathways may be followed [16,17]: incorporation from blood via the follicle, incorporation from skin excretions, and transfer from the external environment. Many factors [18,19] can influence the drug incorporation, the mains being: melanin amount in hair, basicity, neutral or acidic character of the substance, lipophilicity . . . what is the influence of melanin? The study of the relation between steroid concentration and hair colour should provide information. How long are the steroids detectable? Medroxyprogesterone acetate, 17β-estradiol-3-benzoate and 17α-methyltestosterone were selected as model steroid for progestagens, estrogens and androgens, respectively, to answer to these questions. What are the consequences of the hair washing? To avoid external influences [20] and inter-animal contamination, we can wonder if a washing step of hair samples is efficient. Last question we planned to answer: is it possible to detect the ester form of administered steroid? If yes, it would be an unambiguous approach to prove the illegal use of natural hormone.

To study the mechanisms of steroid incorporation in hair, two sample preparation approaches were developed as well as two detection techniques [21] authorising the measurement of all steroid residues whatever their polarity and their hair incorporation mechanism.

2. Experimental

2.1. Reagents and chemicals

Most of the reagents and solvents were of analytical grade quality and provided by VWR International (Pessac, France) and Solvants Documentation Syntheses (SDS, Peypin, France). The solid-phase extraction (SPE) columns were from SDS (Peypin, France) (silica: 1 g, aminopropyle: 0.5 g). The derivatization reagents *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), dithiothreitol (DTE) and trimethyliodosilane (TMIS) were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). The reference steroids were from Interchim (Montluçon, France), Sigma–Aldrich (St. Quentin Fallavier, France) and RIVM (Bilthoven, The Netherlands).

2.2. Animal experiments

A cow (Prim'Holstein, 7 years old) was treated by one intramuscular (IM) injection in the shoulder with a solution of 400 mg of 17α-methyltestosterone (MT). A second one (crossbreed Prim'Holstein/Normande, 18 months old) was treated with 400 mg of medroxyprogesterone acetate (MPA) and a third one (Prim'Holstein, 6 years old) with 400 mg of 17β-estradiol-3-benzoate (Ebz). Hair samples were collected with scissors from day 0 (D0) to day 84 (D84). Five different locations have been studied; black and white hairs have been collected when available at same location (1: head, 2: neck, 3: abdomen, 4: rump, 5: tail; b: black hair, w: white hair) (see Fig. 1).

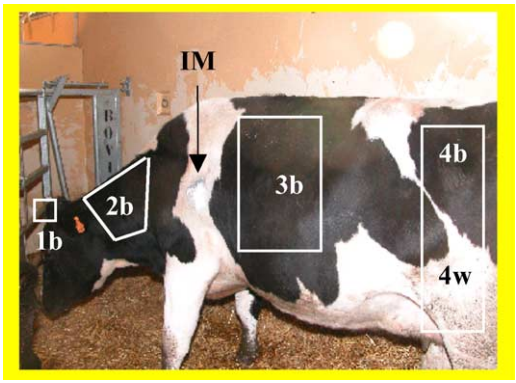

Fig. 1. Picture of the MT-treated cow: (1) head, (2) neck, (3) abdomen, (4) rump, (5) tail; b: black hair, w: white hair.

2.3. Extraction and purification procedure

2.3.1. Hair sample preparation

A 100 mg incurred hair samples from treated bovines were washed two times with 5 mL of dichloromethane. Washing solvent was removed from the tube with a Pasteur pipette and evaporated. The hair was grinded, sonicated 1 h with 5 mL of methanol and incubated one night at 50 °C for "external" steroid extraction. After centrifugation the methanolic extract was removed, evaporated and prepared for further purification (1). The remaining hair was digested by adding 4 mL of 1 M NaOH and incubating for 25 min at 85 °C before further purification (2) (Fig. 2).

2.3.1.1. Purification of non-bound residues (washing solvent and methanolic extract). After adding 4 mL of ethyl acetate, 2 mL of water and 100 μL of 0.1 M NaOH, a liquid-liquid extraction was realized. The organic phase was applied onto a NH_2 SPE column activated with 6 mL of ethyl acetate. Samples were deposited onto the cartridges and immediately collected. The elution was completed with 4 mL of ethyl acetate. After evaporation under a nitrogen stream at 50 °C, extract was reconstituted with 1 mL of chloroform. The last purification was performed onto silica cartridges. The SPE columns were conditioned with 15 mL of chloroform. The samples were applied onto the stationary phase, which was further washed with 1.5 mL of chloroform. The steroid esters and non-esters were eluted with 4.5 mL of chloroform and 8 mL of chloroform/ethyl acetate (75:25; v/v) mixture. The final residues were evaporated to dryness. For liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis, the residues were dissolved in 50 μL of water/methanol/glacial acetic acid (60:40:0.5; v/v/v). For gas chromatography–tandem mass spectrometry (GC–MS/MS) analysis, the residues were derivatized

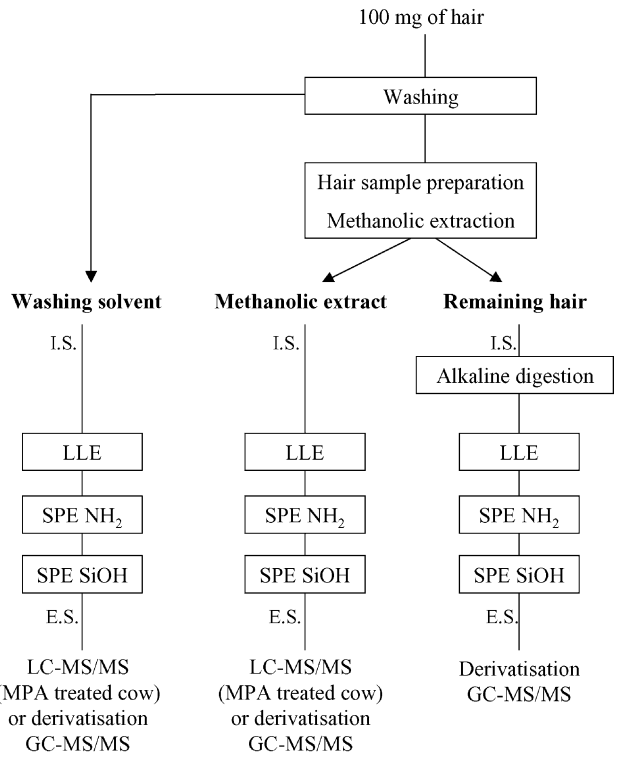

Fig. 2. General analytical procedure (SPE: solid phase extraction; LLE: liquid liquid extraction; IS: internal standard; ES: external standard).

in 20 μL of MSTFA/TMIS/DTE (1000:5:5; v/v/w) during 40 min at 60 °C.

2.3.1.2. Purification of bound residues (remaining hair). A specific liquid-liquid extraction [22,23] allowed the separation of phenolic (Ebz) and Δ4–3–one (MT and MPA). For MT and MPA containing hair samples, the extraction was realized two times with 5 mL of hexane/diethyl ether (70:30; v/v). For Ebz containing hair samples, 1 mL of 0.2 M, pH = 5.2 acetate buffer and 400 μL of glacial acetic acid were added to the hair sample; the extraction was realized twice with 5 mL of diethyl ether. The organic phases were evaporated and the samples were dissolved with 4 mL of ethyl acetate. The purification was performed by the same way as that for the methanolic extract (NH_2 and SiOH SPE cartridges). The eluted fractions were evaporated and derivatized with 20 μL of MSTFA/TMIS/DTE (1000:5:5; v/v/w) during 40 min at 60 °C.

2.4. Gas chromatography–mass spectrometry method

HP 6890 gas chromatograph was coupled to a VG Quattro II (Micromass, Manchester, UK) triple quadrupole mass spectrometer and a OV-1 (OHIO VALLEY, 30 m × 0.25 mm, 0.25 μm) was used. Helium was used as the carrier gas at a flow rate of 1 mL/min in the constant pressure mode and the interface was maintained at 320 °C. Pulsed splitless injection was done at 250 °C and 60 kPa during 1 min. The initial oven temperature was 120 °C for 2 min and increased to 250 °C at 15 °C/min then to 310 °C at 5 °C/min and hold for 10 min. The chromatographic run time was 33 min. Injection volume was 2 μL. The mass spectrometer was operated in multiple reaction monitoring (MRM) acquisition mode after electronic impact ionisation of the analytes. In the collision cell, argon was used as collision gas at 4.5×10^{-4} mbar. Table 1 summarizes the retention times (T_R values), transitions and collision energies of screened molecules and standards.

2.5. Liquid chromatography–mass spectrometry method

The LC separation was achieved on Nucleosil C18AB stationary phase (50 mm × 2 mm, 5 μm) (Nucleosil C18 AB-Interchim-N5AB#5QK) with a guard column (Nucleosil C18 AB, 10 mm × 2 mm, 5 μm), coupled to an Alliance® 2690 pump (Waters, Milford, MA, USA). Elution solvents were acetic acid in water (0.5:99.5, v/v) (A) and methanol (B). The mobile phase composition (A:B, v/v) was 60:40, 0:100 (10–15 min) and 60:40 (20–30 min). Gradient was linear and flow rate was set at 0.22 mL/min. The injected volume was 10 μL.

The detection was performed on a QuattroLC® triple quadrupole analyser (Micromass, Manchester, UK) operating in positive electrospray ionisation (ESI+) and multiple reaction monitoring acquisition mode (MRM). Nitrogen was used as nebulisation and desolvatation gas, at 90 and 600 L/h flow rates respectively. Potential applied onto the capillary was 4 kV. Cone potential and collision energy were optimised for each molecule. In the collision cell, argon was used as collision gas at 4.5×10^{-4} mbar. Transitions used for detection of analytes and standards are reported in Table 2.

2.6. Validation

Specificity has been studied on 20 "blank" hair samples: under the chromatographic conditions used, there was no interferences with the analytes by any extractable endogenous material present in hair. The detection limit (LOD) and identification limit (LOI) of analytes were determined for a signal/noise ratio of 3 (Table 3). Analytes were identified and quantified on the basis of their retention time and at least two transitions, according to the criteria of the European union decision 2002/657/EC. Medroxyprogesterone-d_3, 17α-methyltestosterone-d_3 and 17β-estradiol propionate were used as internal standards for medroxyprogesterone, 17α-methyltestosterone and 17β-estradiol-3-benzoate, respectively.

3. Results and discussion

We developed a strategy consisting in measuring:

1. the steroid profile in the superficial layer of the hair sample (the washing solvent);

Table 1
Acquisition method for analysis by GC–MS/MS

Analytes	Transition	Collision (eV)	T_R (min)
17α-Estradiol	416.3 > 285.2	45	15.98
	416.3 > 129.1	45	
5β-Androstane-17α-methyl-3α,17β-diol	143.1 > 73.0	10	16.00
	255.2 > 199.2	15	
	270.2 > 255.2	10	
	270.2 > 199.2	15	
Estrone	414.2 > 399.2	10	16.15
	414.2 > 309.2	20	
	414.2 > 231.2	25	
17β-Estradiol-d_3	419.3 > 285.2	45	16.39
17β-Estradiol	416.3 > 285.2	45	16.41
	416.3 > 129.1	45	
5α-Androstane-17α-methyl-3β,17β-diol	143.1 > 73.0	10	17.11
	435.3 > 255.2	15	
	345.3 > 255.2	10	
	255.2 > 199.2	15	
17α-Methyltestosterone-d_3	449.3 > 301.2	35	17.56
17α-Methyltestosterone	446.3 > 301.2	20	17.58
	446.3 > 169.1	35	
	446.3 > 341.2	15	
17β-Estradiol propionate	400.2 > 244.1	15	18.09
Norgestrel	456.3 > 301.3	25	18.35
Medroxyprogesterone-d_3	563.4 > 318.2	30	20.35
Medroxyprogesterone	560.4 > 315.2	30	20.37
	560.4 > 328.2	15	
17β-Estradiol-3-benzoate	358.2 > 105.0	20	25.91
	448.2 > 105.0	25	
	317.2 > 105.0	10	

Table 2
Acquisition method for analysis by LC–MS/MS

Analytes	Transition	Collision T1 (eV)	Cone (V)	T_R (min)
Norgestrel	313.2 > 109.1	30	30	6.52
Medroxyprogesterone-d_3	348.2 > 126.0	30	35	7.17
Medroxyprogesterone	345.2 > 123.0	30	35	7.20
	345.2 > 97.0	35	35	
Medroxyprogesterone acetate	387.2 > 123.0	30	25	7.42
	387.2 > 327.2	15	25	
	387.2 > 97.0	35	25	
	387.2 > 285.2	20	25	

Table 3
Evaluation of the LOD et LOI for the administered steroids

Analytes	Fraction	Transition 1	LOD ($\mu g\,kg^{-1}$)	Transition 2	LOI ($\mu g\,kg^{-1}$)
17α-Methyltestosterone	Methanolic extract	446.3 > 301.2	0.2	446.3 > 169.1	1.6
	Remaining hair	446.3 > 301.2	0.4	446.3 > 169.1	0.8
17β-Estradiol-3-benzoate	Methanolic extract	448.2 > 105.0	4.1	358.2 > 105.0	5.0
Medroxyprogesterone acetate	Methanolic extract	387.2 > 123.0	0.4	387.2 > 327.2	0.6

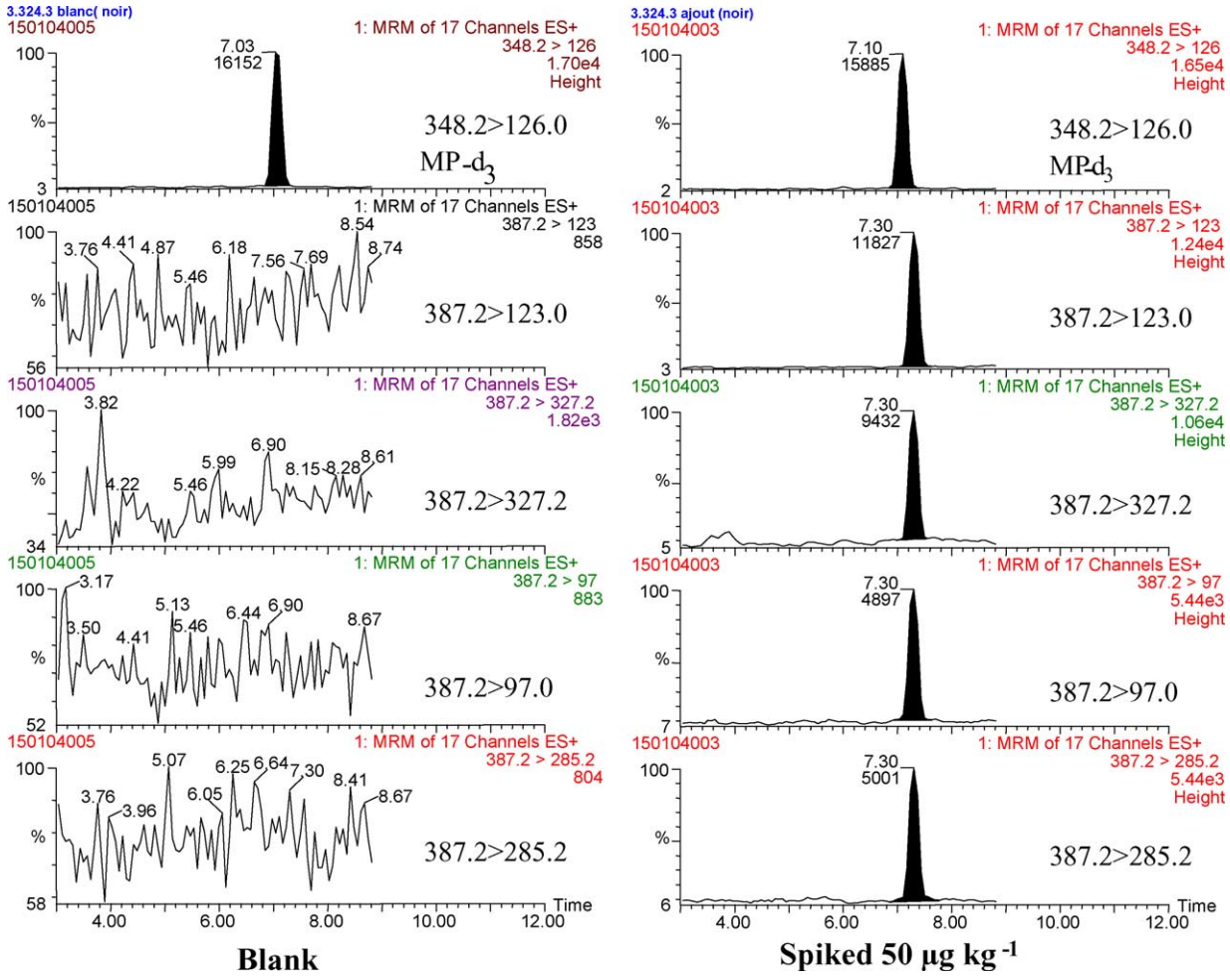

Fig. 3. Ion chromatograms of a blank hair sample (100 mg of hair, 5 ng of MP-d_3) and a spiked hair sample (100 mg of hair, 5 ng of MP-d_3, 5 ng of MPA); LC–MS/MS, MRM acquisition, ESI+ ionisation, methanolic extract fraction.

2. the steroid residues closely bound to hair and inaccessible by the simple action of solvent on non-grinded hair (the methanolic extract);
3. the steroids strongly fixed into the hair and sometimes bound to endogenous bio-molecules (the remaining hair).

This strategy (Fig. 2) permitted to study the influence of the washing and to investigate the mechanisms of drug incorporation into hair by the knowledge of which molecules are superficially fixed on the hair shaft, weakly or strongly incorporated, and when.

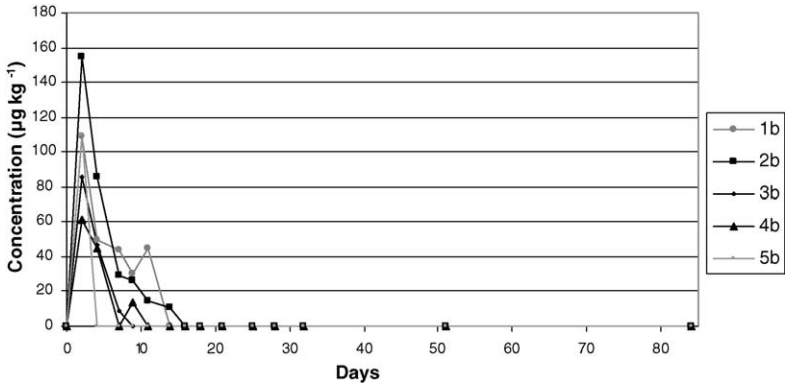

Fig. 4. Kinetic of 17β-estradiol-3-benzoate in hair after single intramuscular injection of 400 mg of 17β-estradiol-3-benzoate.

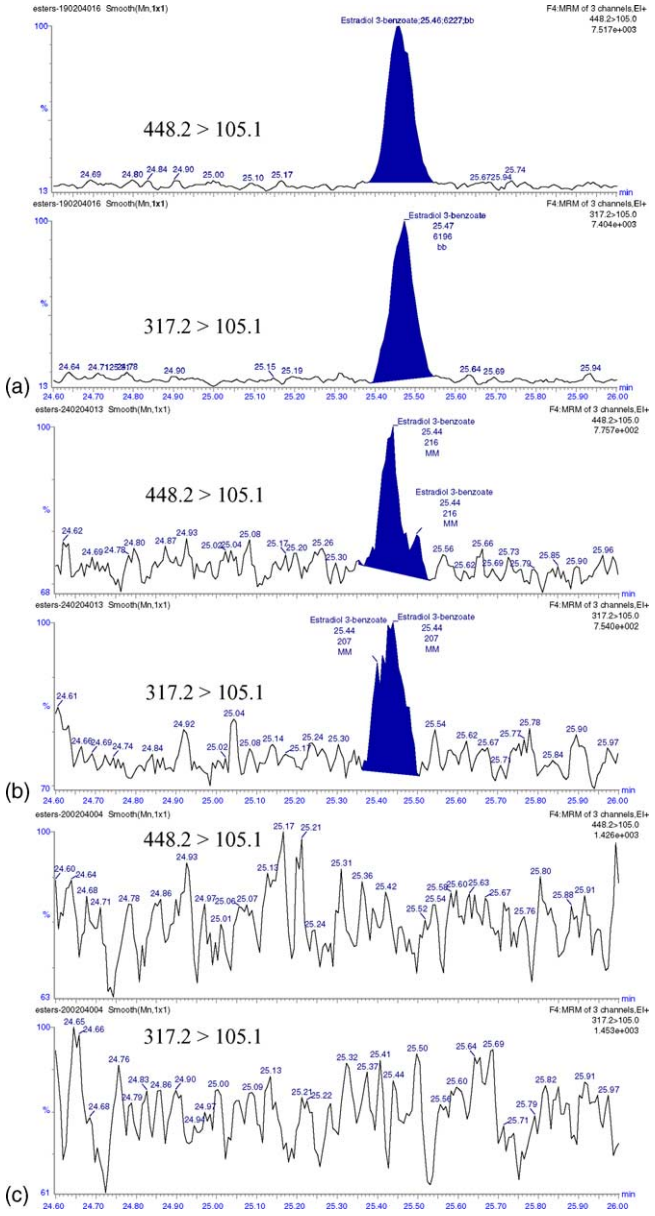

Fig. 5. GC–MS/MS, MRM acquisition, methanolic extract fraction; ion chromatograms corresponding to the 17β-estradiol-3-benzoate: (a) site 2b at D4 (Ebz: 85.6 μg kg^{-1}); (b) site 2b at D14 (Ebz: 10.2 μg kg^{-1}); (c) blank hair sample (100 mg of hair).

3.1. *Medroxyprogesterone acetate treated cow*

Hair samples obtained from the medroxyprogesterone-treated cow were analyzed without any washing steps. The methanolic extract was measured by LC–MS/MS because more adapted to MPA that GC approach; the remaining hair after alkaline hydrolysis was analysed by GC–MS/MS, the desesterified MPA, i.e. medroxyprogesterone, being separable by GC. Five different locations of hair collection have been studied as well as, when possible, black and white hair.

The LC–MS/MS ion chromatograms of medroxyprogesterone acetate (MPA) are shown on Fig. 3. Four transitions have been recorded for MPA. Examples of blank hair sample (non treated animals, 6 days before injection), and spiked are given. Medroxyprogesterone-d_3 (MP-d_3) was used as internal standard. One can see the good specificity of the signals; no interference occurs at the expected time of MPA. Sensitivity was judged acceptable; even at 50 μg kg^{-1} (5 ng in 100 mg of hair), four transitions were detected with a good signal/noise ratio. Despite this good sensitivity, no residues of MPA, nor MP, were found in hair whatever the collection location. Excretion of MPA was monitored into faeces to check if the steroid diffused from the injection site. Indeed, MPA was identified by LC–MS/MS (ESI+) very rapidly after IM administration and over several days, demonstrating that the observations done in hair can be taken into account. These data would indicate that hair sample for progestagen ester control would not be a strategic approach.

3.2. *17β-Estradiol-3-benzoate treated cow*

The kinetic of 17β-estradiol-3-benzoate in different collection sites is presented on Fig. 4; the five curves are almost perfectly matching. Hair and neck were found to be the most efficient sites because of the concentration level and the detectability delay. 17β-Estradiol-3-benzoate has been detected and identified during 2 weeks after the administration. This is probably one of the first times that an ester of natural hormones is demonstrated to be fixed in bovine hair opening the doors of a new strategy for gonadic steroid survey in cattle.

Fig. 5 illustrates the signals obtained for blank and incurred hair samples. We observed a good specificity for the recorded transitions but a limited sensitivity. The improvement of the LOD for instance by LC-atmospheric pressure photoionization–MS/MS would increase the detectability of such residue in hair.

Then, we wonder how Ebz was fixed into the hair. To answer this question we analysed the washing solvent, the methanolic extract and the remaining hair contents in term of Ebz concentration (see Fig. 2). The Ebz kinetics obtained are shown on Fig. 6.

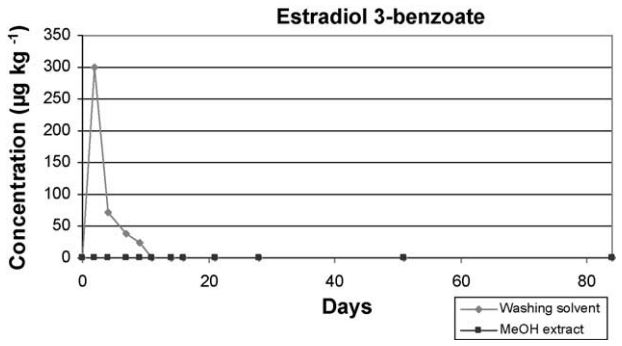

Fig. 6. Kinetic of Ebz in 2b site of incurred hair and corresponding washing solvent after single intramuscular injection of 400 mg of Ebz.

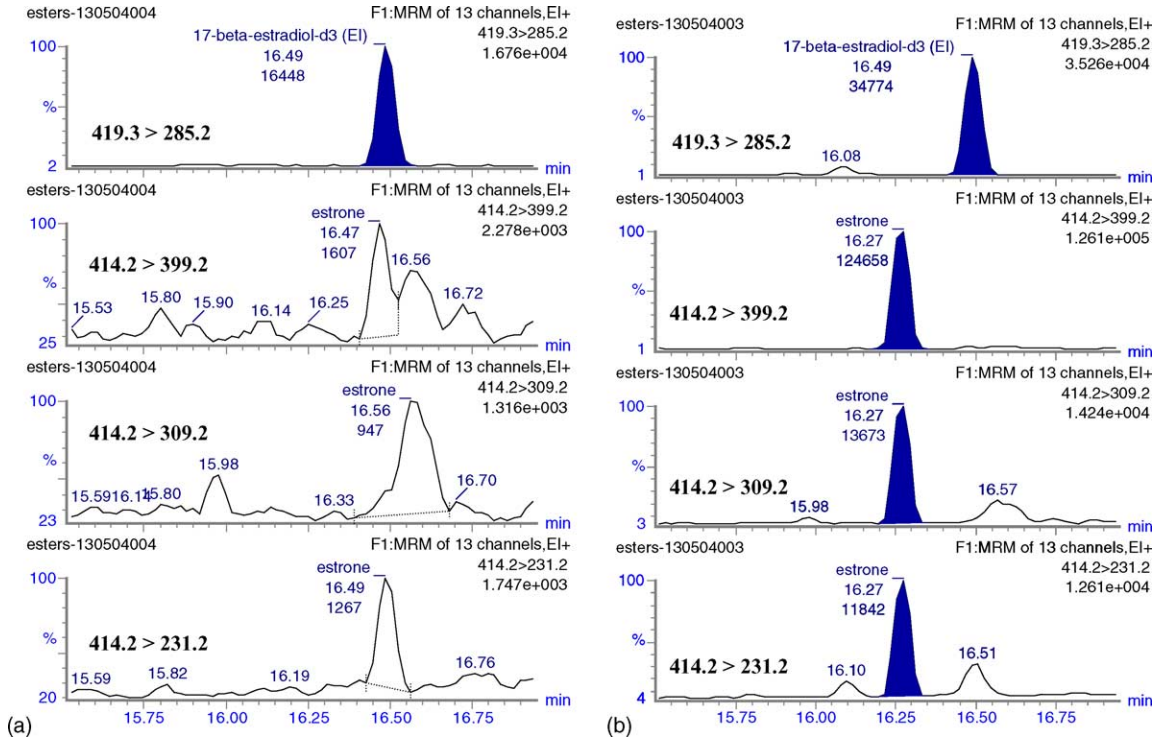

Fig. 7. GC–MS/MS, MRM acquisition, methanolic extract fraction; ion chromatograms corresponding to the 17β-estradiol-d_3 and estrone: (a) blank hair sample (100 mg of hair, 1 ng of 17β-estradiol-d_3); (b) spiked hair sample (100 mg of hair, 1 ng of 17β-estradiol-d_3, 5 ng of estrone).

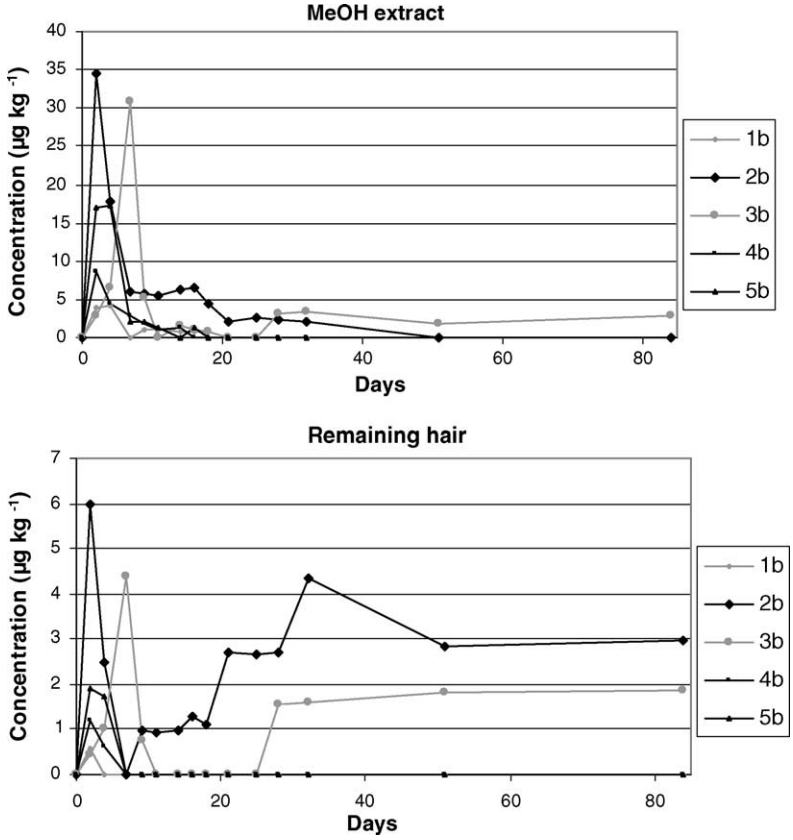

Fig. 8. Kinetic of MT in hair after single intramuscular injection of 400 mg of MT.

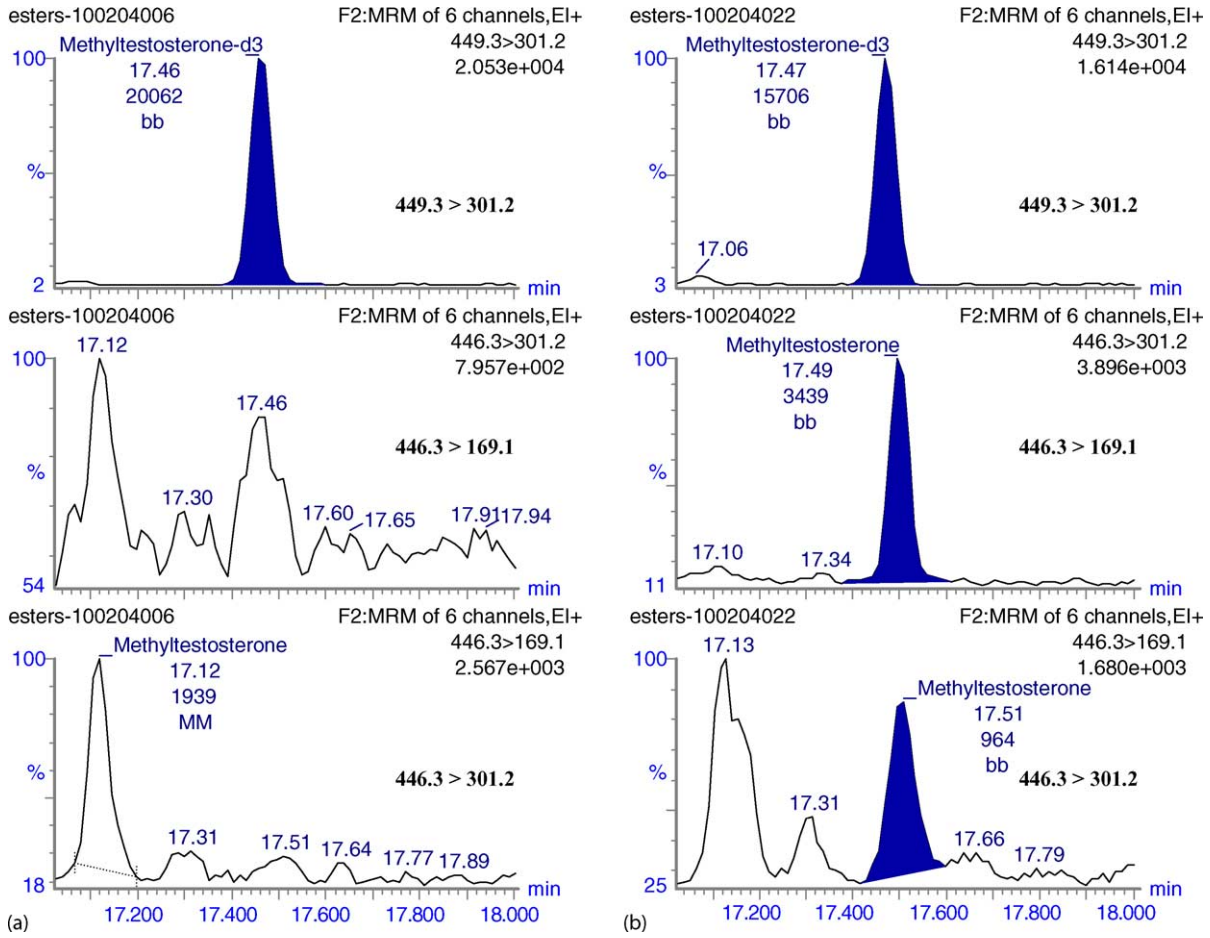

Fig. 9. GC–MS/MS, MRM acquisition, methanolic extract fraction; ion chromatograms corresponding to the MT and MT-d_3: (a) blank hair sample (100 mg of hair, 5 ng of MT-d_3); (b) site 3b at D84 (100 mg of hair, MT-d_3: 5 ng, MT: 2.7 ng).

17β-Estradiol-3-benzoate was identifiable in the washing solvent during the first ten days but was not detectable in methanolic extract fraction. It indicates a superficial fixation of Ebz in hair. Moreover, the consequence of this observation is the unability to wash the hair sample to eliminate the hypothesis of cross-contamination in-between animals.

Finally, three potential metabolites were monitored: 17α-estradiol, 17β-estradiol, and estrone. The GC–MS/MS ion chromatograms of estrone are shown on Fig. 7. Examples of blank hair sample and spiked, for the methanolic extract fraction, are given. One can see the good specificity of the signals and the correct sensitivity obtained. Despite the low $\mu g\,kg^{-1}$ range detection, no residues of estrone were found in hair and no significant variation of α-and β-estradiol concentrations were observed. These data would indicate that hair monitoring for these metabolites is useless.

3.3. 17α-Methyltestosterone treated cow

The kinetic of 17α-methyltestosterone for the different black collection sites is presented on Fig. 8. The sites, which permit the longer detection of this anabolic steroid, are the neck and the abdomen. One can observe for these locations the possibility of detection and identification of 17α-methyltestosterone 3 months after the intramuscular injection.

Fig. 9 shows the good specificity and sensitivity obtained for an incurred sample. This illustrates the possibility to identify unambiguously remaining residues with a good signal/noise ratio; two MRM signals were proved to fulfil official analytical criteria (concentration = 2.7 $\mu g\,kg^{-1}$, site 3b). 17α-Methyltestosterone-d_3 was used as internal standard for accurate quantification.

A comparative study of MT residues in black and white hair was also realized for the rump location (Fig. 10). The MT concentration decreases in the hair superficial fraction from D1 to D20 and then remains constant over a period of 84 days, whatever the colour of hair. After 40 days, some residues remained in this so-called fraction when hair colour was black and in opposition to white hair. In the "remaining hair", i.e. in the fraction corresponding to deeply incorporated steroids in hair, the phenomenon is more obvious. Indeed, the maximum concentration of MT fixed into the hair was about 10-fold

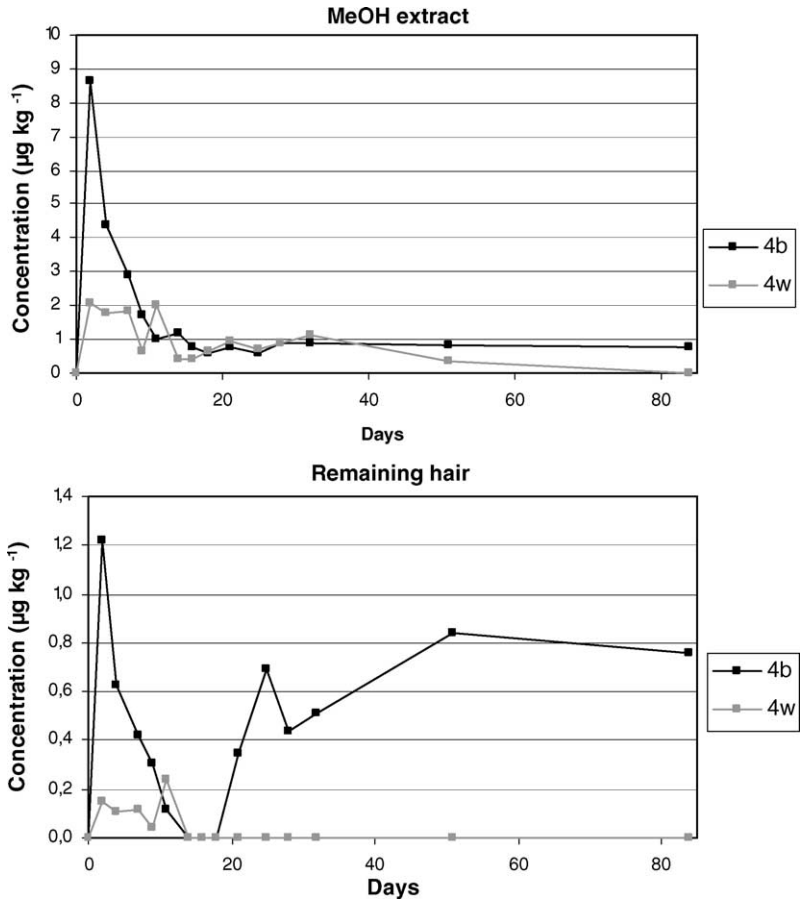

Fig. 10. Kinetic of MT in hair after single intramuscular injection of 400 mg of MT.

higher in black hair than in white hair. Residues in white hair completely disappeared after 2 weeks, whereas MT in black hair remained constant from 3 weeks to 3 months (1 $\mu g\,kg^{-1}$ level). These data would show that the MT residues are incorporated during the hair growth and preferentially in black hair. This is a supplementary proof of the importance of the melanin factor on the steroid incorporation into hair.

On a second time, we analyzed the incurred hair sample of the 3b site with washing. The location was chosen because of the long detectability of MT. The analysis of washing solvent, methanolic extract and remaining hair would permit a better knowledge of steroid fixation in hair. The MT kinetic obtained is shown on Fig. 11and the corresponding concentrations in Table 4 .

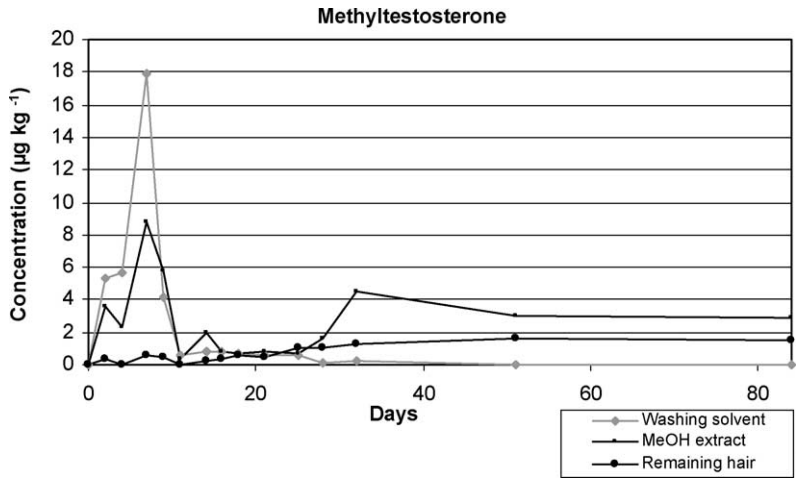

Fig. 11. Kinetic of MT in 3b site of incurred hair and corresponding washing solvent after single intramuscular injection of 400 mg of MT.

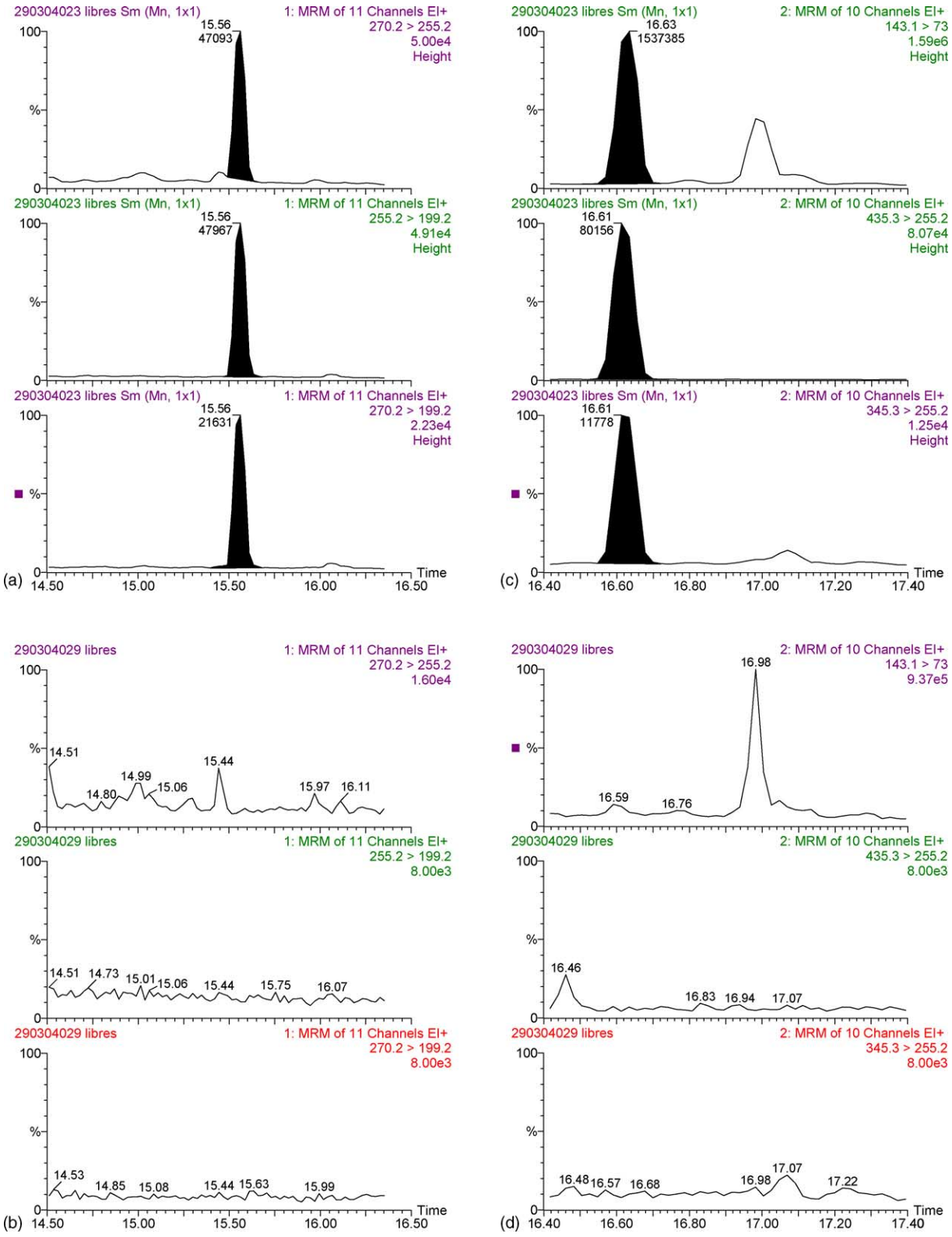

Fig. 12. GC–MS/MS, MRM acquisition, remaining hair fraction; ion chromatograms corresponding to the 5β-androstan-17α-methyl-3α,17β-diol and the 5α-androstan-17α-methyl-3β,17β-diol; (b) and (d) Site 3b at D7 (100 mg of hair); (c) spiked hair ample (100 mg of hair, 5 ng of 5α-3β); (a) spiked hair sample (100 mg of hair, 5 ng of 5β-3α).

Table 4
Concentrations ($\mu g\,kg^{-1}$) of 17α-methyltestosterone in the different analyzed fractions of 3b incurred sample of the MT-treated cow; in italic: detection; in bold: identification

	Day														
	0	2	4	7	9	11	14	16	18	21	25	28	32	51	84
Washing solvent	*0.0*	**5.4**	**5.6**	**17.9**	**4.2**	*0.6*	*0.8*	*0.8*	*0.7*	*0.6*	*0.6*	*0.1*	*0.2*	*0.0*	*0.0*
MeOH extract	*0.0*	**3.6**	**2.3**	**8.7**	**5.7**	**0.4**	*2.0*	**0.8**	**0.7**	**0.8**	**0.7**	**1.6**	**4.6**	**3.0**	**2.9**
Remaining hair	*0.0*	*0.3*	*0.0*	*0.6*	*0.4*	*0.0*	*0.3*	*0.3*	*0.6*	*0.5*	*1.0*	**1.1**	**1.3**	**1.6**	**1.5**

The washing step of the intact hair removed was between 50 and 70% of the total MT during the first ten days. After this period, less and less MT was found in this fraction compared to the two others. Despite the washing phase, 17α-methyltestosterone residues were easily detected and identified, all over the 3 months after the IM injection either in the methanolic extract or the remaining hair fractions. The apex of the excretion was observed at day 7 for the methanolic extract fraction. We observed a fast decreasing of MT until $0.7\,\mu g\,kg^{-1}$, a period during which the concentration remained constant (D11–D25) and finally increased until 3–5 $\mu g\,kg^{-1}$ (D84). The 17α-methyltestosterone levels in the remaining hair fraction were quite low (below $0.6\,\mu g\,kg^{-1}$) and stable until D21 but increased from D21 to D84 (until 1–5 $\mu g\,kg^{-1}$). These results would demonstrate that 17α-methyltestosterone is incorporated into hair according to two successively predominant phenomena:

- D0–D10: 17α-methyltestosterone would be absorbed or transferred to the keratinized hair from skin excretions (the endogenous-exogenous pathway). The washing step removed the majority of 17α-methyltestosterone residues. This stage authorises a rapid detectability of the anabolic steroid.
- From D25: 17α-methyltestosterone is strongly incorporated into hair by an endogenous pathway during the hair growth. Very small amount of 17α-methyltestosterone was then found in the washing phase. Despite two washings with dichloromethane and a methanolic extraction, 17α-methyltestosterone was still detected in the remaining hair fraction.

Finally we wonder whether or not the native steroid was the main incorporated form into hair or if its metabolites were the most predominant. Two 17α-methyltestosterone metabolites were monitored: 5α-androstane-17α-methyl-3β,17β-diol (5α-3β) and 5β-androstane-17α-methyl-3α,17β-diol (5β-3α). The GC–MS/MS ion chromatograms of these metabolites are shown on Fig. 12. Examples of blank hair sample and spiked, for the remaining hair fraction, are given. Three transitions are showed for each analyte. The specificity as well as the sensitivity of the two monitored signals was found acceptable. Despite the low $\mu g\,kg^{-1}$ range performance, no residues of 5α-3β, nor 5β-3α, were found in hair. It would mean that the fixed residues are mainly and directly coming from the injection site after the resorption, without any liver passage. It can as well explain that the more contaminated hair samples were located near the administration site.

4. Conclusion

Despite a good specificity and a decision limit guaranteed at the low ppb range, no residue of medroxyprogesterone acetate nor medroxyprogesterone has been found in hair which would indicate that hair monitoring for progestagen esters misuse is not efficient. The non-incorporation of MPA into the hair, at least through skin excretion (sebum), is still unexplained. 17β-Estradiol-3-benzoate has been detected and identified during 2 weeks after the administration in the superficial hair fraction. These data would indicate that hair samples can be considered as an efficient way to prove the illegal administration of natural steroid through the monitoring of the injected ester. Nevertheless, because Ebz is rapidly hydrolysed in the blood circulation into 17β-estradiol, the ester form is not detected in the deeply bound fraction (with melanin). The direct consequence for the control is the impossibility to wash the hair sample during confirmatory analysis to eliminate the hypothesis of inter-animal contamination. Three months after intramuscular injection of 17α-methyltestosterone, residues were still identifiable by GC–MS/MS. The analysis of different fractions allowed a better knowledge concerning the mechanisms of incorporation of 17α-methyltestosterone into the hair: after a first incorporation due to elimination via skin excretions, the residues would be incorporated during the hair growth. The presence or lack of melanin seems to be an important parameter for this second way of fixation in hair. Finally, no metabolite was detected; this observation translating that fixed residues are probably directly coming from the injection site before any hepatic passage.

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