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Liquid Chromatography–Electrospray Ionization–Mass Spectrometry Method in Multiple Reaction Monitoring Mode To Determine 17α-Ethynylestradiol Residues in Cattle Hair without Previous Digestion

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A liquid chromatography–mass spectrometric (LC–MS/MS) method has been developed for determination of ethynylestradiol residues in cattle hair. Hair samples were pulverized with a cryogenic mill followed by a simple extraction with acetonitrile. A dansyl derivatization procedure to improve ethynylestradiol detection was used before the LC–MS/MS analysis in multiple reaction monitoring (MRM) mode using α-estradiol as an internal standard. The method was validated following the latest EU guidelines using blank hair samples spiked at 2 ng g^{-1}. The detection capability (CCβ) was less than 2 ng g^{-1}, and the decision limit (CCα) was 1 ng g^{-1}. Incurred samples obtained 56 days after cow treatment with ethynylestradiol were analyzed, and the presence of ethynylestradiol in the hair was confirmed in all cases.

KEYWORDS: Cryogenic grinding; hair; residues; HPLC–MS/MS

INTRODUCTION

According to European Community (EC) Directive 96/22/EC (*1*), it is forbidden to use steroid hormones as growth promoting agents. Hair has advantages over other matrixes for residue control because the drugs incorporated into the capilar fiber stays trapped for months, and this fact permits long-term detection (*2, 3*). Several authors described the analysis of steroid compounds in hair using gas chromatography or LC techniques coupled with mass spectrometry detection (*4–8*). Prior to the extraction, the liberation of the steroid from the hair matrix is necessary in all cases. Digestion methods based on alkaline (*5–7*), reductive (*8, 9*), or acidic conditions (*10*) were used for this purpose. Hair is often chosen as a matrix because the intact steroid compound used for illegal growth can be found, but these drastic procedures can completely hydrolyze anabolic steroid esters during the digestion and limits the determination of esters.

In this work, we avoid hair digestion by employing a potent cryogenic grinding that pulverizes the hair fiber, frozen with liquid nitrogen, so that complete rupture of the keratine matrix is obtained and the analyte (steroids) can contact the extraction–solvent. This method has been used in our laboratory for improved quinolone extraction from incurred chicken tissue (*11*).

Since 2000 (*12*), atmospheric pressure photoionization (APPI) ion sources can be used to ionize apolar molecules like noncombinated steroids. The signal obtained by photoionization of some of these types of compounds can be increased 3–10 times when compared with an APCI source (*13*). However, this type of source is not very common in the laboratories, and some recent papers (*14–18*) report a dansyl derivatization procedure to improve the determination of most apolar aromatic steroids (estrogens) using electrospray ionization. In this way, in this paper we describe a method to analyze ethynylestradiol residues in hair using pulverization in cryogenic mill, dansyl derivatization and multiple reaction monitoring in liquid chromatography–electrospray ionization–mass spectrometry. Also, other endogenous steroids in hair can be determined with the procedure described in this paper.

EXPERIMENTAL SECTION

Chemicals. Experimental materials: Steroids like α-estradiol [1,3,5(10)-estratrien-3,17α-diol], β-estradiol [1,3,5(10)-estratrien-3,17β-diol], ethynylestradiol [1,3,5(10)-estratrien-17α-ethynyl-3,17β-diol], estriol [1,3,5(10)-estratrien-3,16α,17β-triol], estrone [1,3,5(10)-estratrien-3-ol-17-one], and testosterone [4-androsten-17β-ol-3-one] were obtained from Steraloids Ltd. (Croydon, UK). Dansyl chloride and sodium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO). Formic acid, sodium hydroxide, acetone, HPLC-grade methanol, and acetonitrile were supplied by Merck (Darmstadt, Germany). Milli-Q organic free water from Millipore (Bedford, MA) was used. All reagents were analytical grade.

Liquid Chromatography Tandem MS Analysis. The HPLC system consisted of a quaternary pump, degasser, and autosampler Agilent Technologies Model 1100 (Minnesota). A Q-Trap

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Table 1. Mass Spectrometer Parameters and Monitorized Transitions (RT, Retention Time; DP, Declustering Potential; EP, Entrance Potential; CEP, Collision Cell Entrance Potential; CE, Collision Energy)

compd	RT	Q1 Mass (amu)	Q3 Mass (amu)	dwell (ms)	DP	EP	CEP	CE
β-estradiol	18.4	506.217	171.2	30	81	11	40	51
α-estradiol	19.0	506.217	156.2	30	81	11	40	75
ethynylestradiol	18.9	530.215	171.2	30	76	12	20	47
		530.215	156.3	30	76	12	20	75
estrone	14.0	504.122	171.2	30	101	11	20	47
		504.122	156.2	30	101	11	20	73
estriol	13.9	522.155	171.2	30	96	10.5	18	51
		522.155	156.3	30	96	10.5	18	79
testosterone	11.1	289.114	97.1	30	71	10.5	14	29
		289.114	109.2	30	71	10.5	14	35

2000 mass spectrometer with Ion Source Turbo Spray from Applied Biosystems MSD Sciex (Toronto, Canada) was used. Nitrogen produced by a high-purity nitrogen generator (PEAK Scientific Instruments Ltd., Chicago, IL) was used as curtain, nebulizer, collision, and lamp gases. Unit mass resolution was set in both mass-resolving quadrupole Q1 and Q3.

Aliquots (20 μL) of standards or sample extracts were separated by HPLC on a column Synergi Fusion-RP (150 × 2 mm) 4 μm, Phenomenex (Torrance, CA). The mobile phase was aqueous formic acid (0.1%) mixed in a gradient (10–90% in 20 min) with acetonitrile at a flow rate of 300 μL min^{-1}. The ion source was operated at 400 °C in the positive ion mode. Multiple reaction monitoring (MRM) mode was used. In **Table 1**, we show the transitions monitored for the steroids assayed. Data were collected using a Dell Optiplex GX400 workstation and processed by Analyst 1.4.1 software package (MDS SCIEX).

Standards. Stock solutions of 1.0 mg/mL in methanol were prepared for each steroid of interest and stored at −20 °C. Working standard solutions at various concentrations were prepared by dilution with methanol to obtain solutions with varying concentrations.

Samples. Fifty cows of the Rubia Gallega and Holstein breeds have been using for this study. Animals diagnosed as having clinical problems were excluded from the study. The animals were fed with a diet usually employed in the zootechnical practice and ad libitum access to water and feeds was allowed to the animals.

Five calves from these (approximately 3 months old) weighing 99 ± 2 kg were put in individual cages (see ref 5). The animals were treated with anabolic doses of ethynylestradiol (2 mg kg^{-1} of body weight) via intramuscular injection. Hair samples were taken 56 days after the treatment. Hair (old and new growth) was cut from the flanks of the animals using electric clippers.

To remove external contamination, hair samples were washed three times with an aqueous solution of Tween 80 (10%)

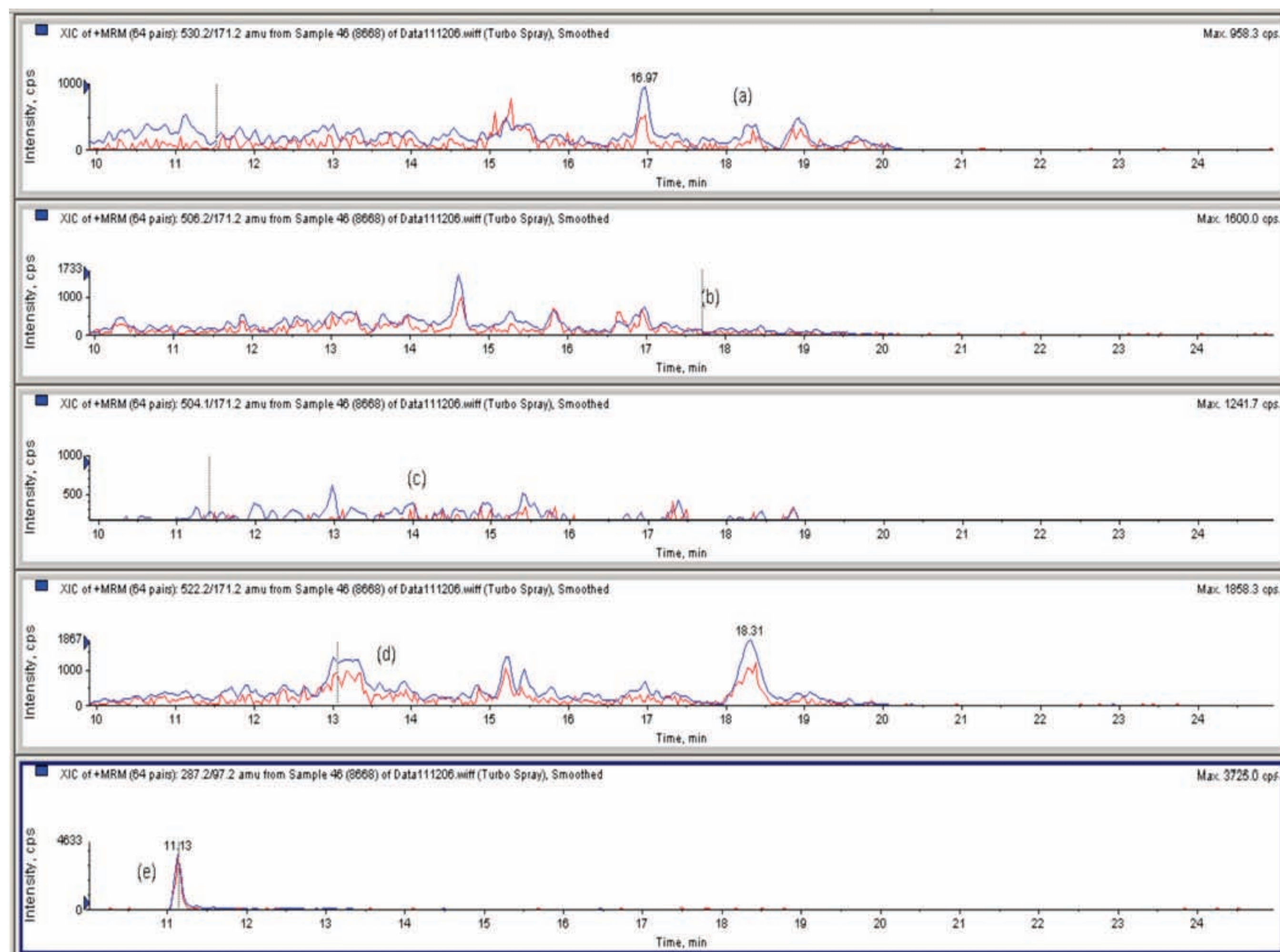

Figure 1. Chromatogram of the acquisition window for the two MRM transitions for each steroid analyzed, (a) 530.2/171.2 and 530/156.3 (ethynylestradiol, TR 18.9), (b) 506.2/171.2 and 506.2/156.2 (α and β estradiol, TR 19.0 and 18.4, respectively), (c) 504.1/171.2 and 504.1/156.2 (estrone, TR 14.0), (d) 522.1/171.2 and 522.1/156.3 (estriol, TR 13.9), (e) 289.1/97.1 and 289.1/109.2 (testosterone, TR 11.1), corresponding to a blank hair sample.

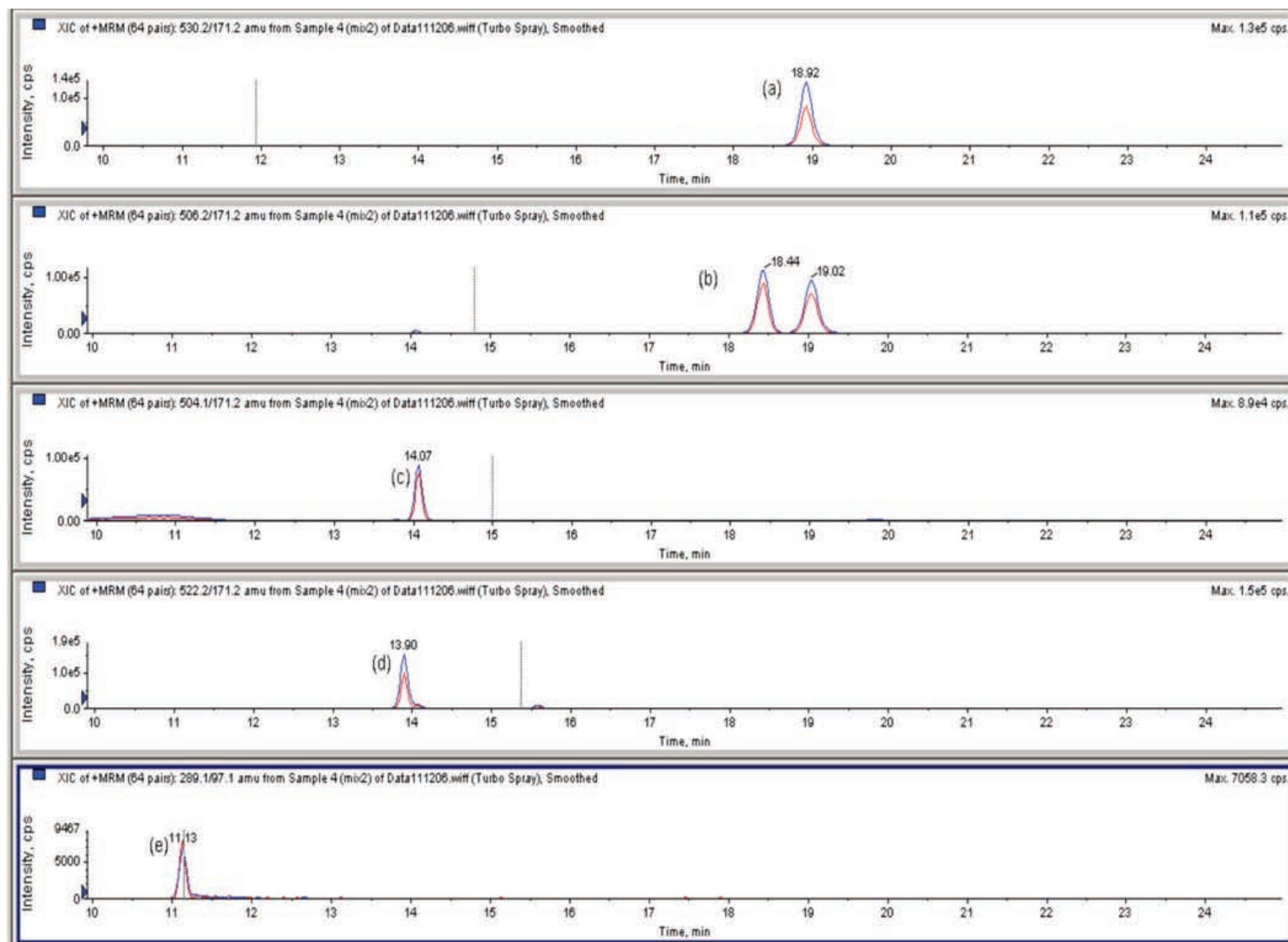

Figure 2. Chromatogram of the acquisition window for the two MRM transitions for each steroid analyzed, (a) 530.2/171.2 and 530/156.3 (ethynylestradiol, TR 18.9), (b) 506.2/171.2 and 506.2/156.2 (α and β estradiol, TR 19.0 and 18.4, respectively), (c) 504.1/171.2 and 504.1/156.2 (estrone, TR 14.0), (d) 522.1/171.2 and 522.1/156.3 (estriol, TR 13.9), (e) 289.1/97.1 and 289.1/109.2 (testosterone, TR 11.1), corresponding to 80 pg injected on the column of each steroid, corresponding to 2 ng g^{-1}, in hair processed as indicated in the Experimental Section.

(Merck, Darmstadt, Germany) and dried at 40 °C (*19*). The absence of ethynylestradiol in the final wash solutions was verified.

Freezer mill 6750 Spex Certiprep INC (Metuchen, NJ) was used for hair pulverization. A hair sample (300–400 mg) was inserted in a grinding vial, a polycarbonate cylinder supplied with two end plugs, immersed in liquid nitrogen and ground with a magnetically driven impactor. A time period of 15 min was required to freeze and pulverize the hair. An accurately weighted aliquot (100 mg) was placed in an Eppendorf tube and 25 μL of internal standard (α-estradiol, 10 ng mL^{-1}) was added. To prepare validation standards, we spiked 25 μL of appropriate working solutions into 100 mg of blank pulverizated hair.

Hair samples were vortex mixed for 1 min with 900 μL of acetonitrile, sonicated in an ultrasound bath UCI-2 for 30 min, and centrifuged at 13000 rpm for 20 min. After centrifugation, the acetonitrile layer was placed into Ultrafree-MC centrifugal filter device (Millipore, Bedford, MA) and centrifuged at 13000 rpm for 1 min. After filtration, the remainder was placed in an injection vial and evaporated under a nitrogen stream at 35 °C.

For derivatization of estrogens, we used the procedure described previously by other authors (*14–18*). The residue was redissolved in 25 μL of sodium bicarbonate buffer (100 mmol/L, pH 10.5), and an equal volume of 1g/L of dansyl chloride in acetone was also added to derivatize the analytes. Afterward, the samples were vortex-mixed for 1 min and incubated in a heating block at 60 °C for 3 min. The samples were immediately assayed with the HPLC–MS/MS system.

RESULTS AND DISCUSSION

The mass spectrometric conditions in positive mode were optimized for all steroids named before, previously treated with dansyl chloride. In order to do this, 100 μL of standard solutions containing 1 μg mL^{-1} of each steroid in methanol were evaporated to dryness under a nitrogen stream, and the residue was redissolved in 100 μL of sodium bicarbonate buffer (100 mmol/L, pH 10.5) and an equal volume of 1 g/L dansyl chloride in acetone, as decribed above. It is known that dansyl chloride can react with phenolic hydroxyls but not with alkyl hydroxyl groups. Due to this fact, only steroids with phenolic hydroxyl groups can form dansylated derivatives in our case, α- and β-estradiol, ethynylestradiol, estrone, and estriol. The derivatives obtained were easily ionizable and gave a strong protonated molecule using ESI/MS under positive mode. Analytes were quantified in multiple reaction monitoring MRM mode. Two MRM transitions for each analyte were monitored (200 ms dwell time/transition) according to the 2002/657/CE decision (*20*). The precursor product ion MRM was optimized for intensity using direct infusion of the derivatization reaction mixture. The electrospray source parameter setting was also optimized for intensity under LC conditions and also using infusion of the

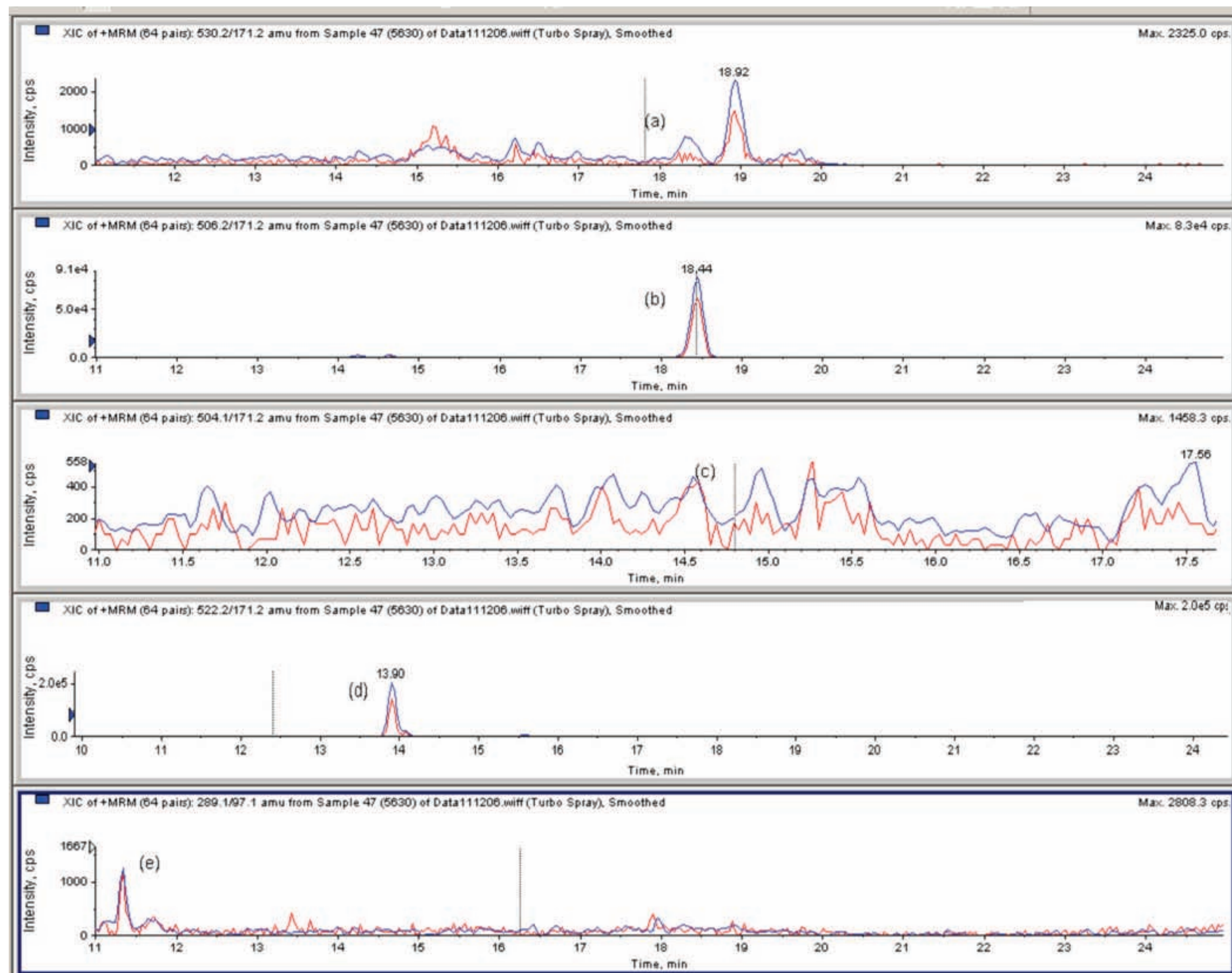

Figure 3. Chromatogram of the acquisition window for the two MRM transitions for each steroid analyzed, (a) 530.2/171.2 and 530/156.3 (ethynylestradiol, TR 18.9), (b) 506.2/171.2 and 506.2/156.2 (α and β estradiol, TR 19.0 and 18.4, respectively), (c) 504.1/171.2 and 504.1/156.2 (estrone, TR 14.0), (d) 522.1/171.2 and 522.1/156.3 (estriol, TR 13.9), (e) 289.1/97.1 and 289.1/109.2 (testosterone, TR 11.1), corresponding to an incurred hair sample taken 56 days after an intramuscular treatment with ethynylestradiol.

reaction mixture. For quantification of estrogens, the most intense product ion at *m/z* 171, which corresponds to the neutral loss of estrogen sulfate from the protonated derivative, was monitored. Another steroid compound (testosterone) that did not form dansyl derivatives was also optimized in the solution with dansyl chloride. The product ion efficiency of the precursor product ion was optimized by varying the spray, orifice, and voltages at the quadrupole using a N_2 gas atmosphere. The optimized parameters to achieve better ability as a declustering potential, entrance potential, collision cell entrance potential, collision cell exit potential, and collision energy are shown in **Table 1**.

The chromatographic separation is achieved in a column Synergi Fusion-RP filled with a hybrid polymer. Good efficiency and good peak shape were obtained in 20 min analysis time. **Figure 1** shows the chromatogram of the acquisition window for the two MRM transitions for each steroid analyzed, 530.2/171.2 and 530/156.3 (ethynylestradiol), 506.2/171.2 and 506.2/156.2 (α and β estradiol), 504.1/171.2 and 504.1/156.2 (estrone), 522.1/171.2 and 522.1/156.3 (estriol), 289.1/97.1 and 289.1/109.2 (testosterone), corresponding to a blank hair sample. In similar way, **Figure 2** shows the chromatogram of the acquisition window for the two MRM transitions obtained for 80 pg injected on the column of each steroid, corresponding to spiking with 2 ng g^{-1} of blank hair processed as indicated in the Experimental Section. **Figure 3** shows the same MRM transitions in an incurred hair sample from a cow treated with ethynylestradiol. Sample was taken 56 days after treatment. In this sample, we can detect ethynylestradiol, β-estradiol, and testosterone using α-estradiol as the internal standard. The chromatographic method can separate the five dansyl derivatives of estrogen and other one nonderivatized steroid and allows the detection of exogen ethynylestradiol and endogenous β-estradiol, estrone, estriol, and testosterone in hair of cattle. We did not detect endogenous α-estradiol in any analyzed samples from animals treated or not with ethynylestradiol.

Validation of the Method for Ethynylestradiol. Specificity, repeatability, detection capability (CCβ), decision limit (CCα), and applicability–stability were determined in order to achieve the method validation according to the 2002/657/CE decision as a confirmatory qualitative method for the forbidden compound, ethynylestradiol. We use α-estradiol as internal standard.

Concerning specificity, no interference was observed in the retention time of the analyte in 20 blank hair samples which present or not endogenous β-estradiol. For decision limits, we measure the blank signal of 20 above mentioned samples three

times, at the expected retention times for ethynylestradiol. CCα was 1 ng g^{-1}. For the CCβ determination, 20 other blank hair samples were spiked with 2 ng g^{-1}. In all samples, ethynylestradiol was detected with a signal/noise response above 3, also using the MRM transition having less response (530.2/156.2). Stability and applicability of the method described in this paper, for ethynylestradiol residues in cattle hair, can be assured with the fact that the animal treatment and collection of the samples took place 6 years before the present experiment (*5*), and the samples remained stored at −20 °C. The amount of ethynylestradiol in the samples of hair of the five calves, 56 days after treatment, had been the following six years ago: 3.64, 13.83, 3.86, 7.43, and 4.84 ng g^{-1}. In this work, we have analyzed these samples of hair with the new methodology. After 6 years at −20 °C, we are able to detect ethynylestradiol in all cases with a concentration greater than 2 ng g^{-1}. This means that for the sample with the smaller amount of ethynylestradiol, the losses possible were always under 45%.

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