JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of a Rapid and Confirmatory Procedure To Detect 17β-Estradiol 3-Benzoate Treatments in Bovine Hair

PATRICIA REGAL, BEATRIZ I. VÁZQUEZ, CARLOS M. FRANCO, ALBERTO CEPEDA, AND CRISTINA A. FENTE*

Analytical Chemistry, Nutrition and Bromatology, Veterinary Faculty, University of Santiago de Compostela, 27002-Lugo, Spain

A high-performance liquid chromatography−tandem mass spectrometry method (LC-MS/MS) was developed for efficient and confirmatory surveillance of illegal use of estradiol benzoate, even when this substance is used in reproductive control. After cryogenic grinding, estradiol benzoate was extracted from hair with acetonitrile for 24 h on a rocking table. The validation of the method was based on Commission Decision 2002/657/EC using the deuterated analogue of estradiol benzoate as internal standard. Decision limit (0.81 ng/g), detection capability (1.38 ng/g), repeatability CV% (13.7), within in laboratory reproducibility CV% (15.6%), and trueness (99.3%) were calculated. Using the proposed methodology the presence of estradiol benzoate in samples obtained from animals treated to synchronize their estrous cycles can be confirmed.

KEYWORDS: 17β-Estradiol 3-benzoate; bovine hair; residues; HPLC-MS/MS

INTRODUCTION

Control of the bovine estrous cycle is used to facilitate artificial insemination and to get better birth control in cattle. For this purpose, progesterone or estradiol benzoate have frequently been used (*1, 2*). Although Directive 2003/74/EC (*3*) amending Directive 96/22/EC (*4*) permanently prohibited the use of estradiol 17β as a growth promoter, a temporary exemption was provided for the use of estradiol 17β for estrous induction in cows, horses, sheep, or goats until October 14, 2006. Because effective alternative products exist and are already used, to ensure a high level of health protection chosen in the European Community, that exemption was not renewed, and the European Parliament legislative resolution of June 5, 2008, included estradiol 17β and its ester-like derivatives as banned substances (*5*). Detection of illegally administered estradiol involves difficulties associated with differentiating between exogenous and endogenous (naturally occurring) hormones. Exogenous estradiol is usually administered as esters, and the animal's body hydrolyzes these esters to generate free steroids. It has been very difficult to detect intact steroid esters through body fluid or tissue analyses as they are rapidly metabolized to nature-identical steroids (*6*). Hair is often chosen as a matrix because the illegally used, intact steroid compound can be found (*7*). Unfortunately, drastic digestion procedures are required for liberation of the steroid from the hair matrix. These digestion procedures can completely hydrolyze anabolic steroid esters, thereby limiting the determination of esters. However, in 2005 two methods to detect intact steroid esters in bovine hair were published (*8, 9*). As steroid hormones do not occur naturally in the ester form, detection of intact steroid esters would be an unambiguous approach to prove the illegal use of a natural hormone. In 2007 (*10*), we described a method to analyze ethynylestradiol residues in hair using pulverization in a cryogenic mill to avoid digestion procedures. Using a similar methodology, in this research hair pulverization in a cryogenic mill was used to avoid digestion procedures for liberation of intact steroid esters from keratin matrix without any other step except acetonitrile incubation to remove the analyte. We propose a method that permits efficient and confirmatory surveillance of illegal use of estradiol benzoate even when this substance is used in reproductive control. The validation of the method was based on Commission Decision 2002/657/EC (*11*), which establishes criteria and procedures for the validation of analytical methods to control the presence of residues of banned substances in food-producing animals and foods.

MATERIALS AND METHODS

Chemicals and Reagents. Steroids such as 17β-estradiol 3-benzoate (EB) [1,3,5(10)-estratrien-3,17β-diol 3-benzoate] and 17β-estradiol [1,3,5(10)-estratrien-3,17β-diol] were obtained from Steraloids Ltd. (Croydon, U.K.). Deuterated 17β-estradiol (EB-d3) [17β-estradiol-16,16,17-d_3] was purchased from CDN Isotopes (Quebec, Canada). Benzoyl chloride and trisodium phosphate were obtained from VWR International (Fontenay sous Bois, France). Acetonitrile was purchased from Scharlau Chemie (Sentmenat, Barcelona, Spain). L-(+)-Ascorbic acid was obtained from Panreac Química (Castellar del Vallés, Barcelona, Spain). Water was purified using a Milli-Q water system (Millipore, Bedford, MA). Formic acid was purchased from Acros

* Author to whom correspondence should be addressed (e-mail cristina.fente@usc.es).

10.1021/jf803044b CCC: $40.75
Published on Web 12/17/2008

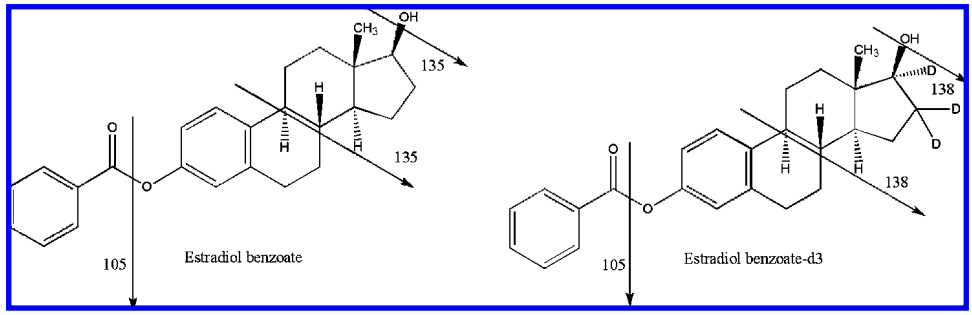

Figure 1. Structures of EB and EB-d3. Arrows indicate fragmentation points and ions produced by them.

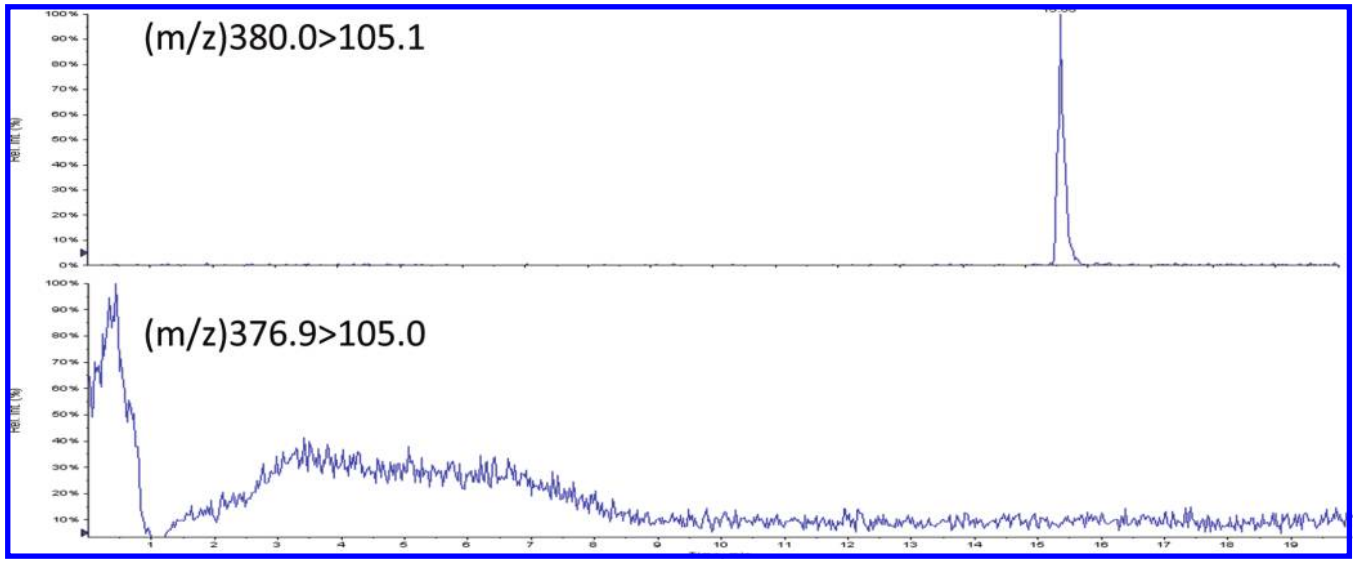

Figure 2. Chromatograms of the acquisition window for the MS/MS product ions of estradiol benzoate (*m/z* 377 > 105) and deuterated estradiol benzoate (*m/z* 380 > 105) in a blank hair sample.

Table 1. Optimized Parameters

steroid	MRM transition (Q1, Q3)	retention time (min)	dwell (ms)	DP	EP	CEP	CXP	CE
17β-estradiol 3-benzoate	376.9 > 105.0	15.63	150	66	11	12	4	25
	376.9 > 134.9	15.63	150	66	11	12	4	21
17β-estradiol 3-benzoate-d_3	380.0 > 105.1	15.55	150	66	10,5	14	4	25

Organics (Geel, Belgium). Methanol, ethyl acetate, sodium chloride, triethylamine (p.s.), hexane, and Tween 80 were supplied by Merck (Darmstadt, Germany). All chemicals and solutions were of analytical reagent grade.

Synthesis of Deuterated Estradiol Benzoate. Deuterated estradiol benzoate (EB-d3) was synthesized following the steps described by Hooijerink et al. (*9*), with very few modifications. Estradiol-16,16,17-d_3 (50.0 mg) was dissolved in 10 mL of acetonitrile. Triethylamine (60 μL) and benzoyl chloride (50 μL) were added, and the reaction progressed for 2 h. NMR analysis confirmed the formation of estradiol benzoate deuterated at positions 16, 16, and 17 of 98%. Yield of the reaction was 68.90 mg of white crystalline powder (84%).

Standards. Stock solutions of 1.0 mg/mL in methanol with 0.1% ascorbic acid were prepared for EB and EB-d3 and were stored at −20 °C in the dark. Working solutions were prepared by appropriate dilution of the stock standard solutions with methanol for EB and/or acetonitrile for internal standard and were stored at −20 °C in the dark for a maximum period of 1 month.

Instrumentation. The HPLC system consisted of a quaternary pump, degasser, and autosampler (Agilent Technologies model 1100). A hybrid triple-quadrupole linear trap (Q-Trap 2000) mass spectrometer with an Ion Source Turbo Spray (ESI) from Applied Biosystems (MSD Sciex, Toronto, Canada) was used. Nitrogen was produced by a high-purity nitrogen generator (Peak Scientific Instruments Ltd., Chicago, IL) to be used as curtain, nebulizer, and collision gas. Mass spectrometry resolution was set unit in both mass-resolving quadrupoles Q1 and Q3. Aliquots (50 μL) of standards or sample extracts were separated by HPLC on a Synergi Fusion-RP (50 × 2 mm) 2.5 μm column (Phenomenex, Torrance, CA). The mobile phase consisted of aqueous 0.1% formic acid (B) mixed in a 20 min gradient with acetonitrile 0.1% formic acid (A): 10% of A was held isocratic for 1 min and was then increased to 80% (15 min). Subsequently, the gradient was returned to the initial conditions in 1 min and held isocratic for 4 min at a flow rate of 200 μL/min. The ESI ion source was operated at 400 °C in positive ion mode. Multiple reaction monitoring (MRM) mode was used, and the transitions monitored for estradiol benzoate are shown in **Table 1** with source conditions. Data were collected using a Dell Optiplex GX400 workstation and processed by the Analyst 1.4.1 software package (MDS SCIEX).

Hair Samples. Twenty-six Holstein cows have been used for this study. Animals diagnosed as having clinical problems were excluded. The animals were fed a diet usually employed in the zootechnical practice and had ad libitum access to water. Hair samples were obtained from six cows (approximately 15 months old) treated with one intramuscular injection (5 mL) of Neonida N (Pfizer S.A., Madrid, Spain) containing 1.250 IU of chorionic gonadotropin (hCG) and 5 mg of EB. The other 20 cows were used as negative control group. Hair samples were taken of the head and shoulder of the treated cows with electric clippers in a range of 57−67 days post-treatment. Administration took place before October 2006, and the samples were

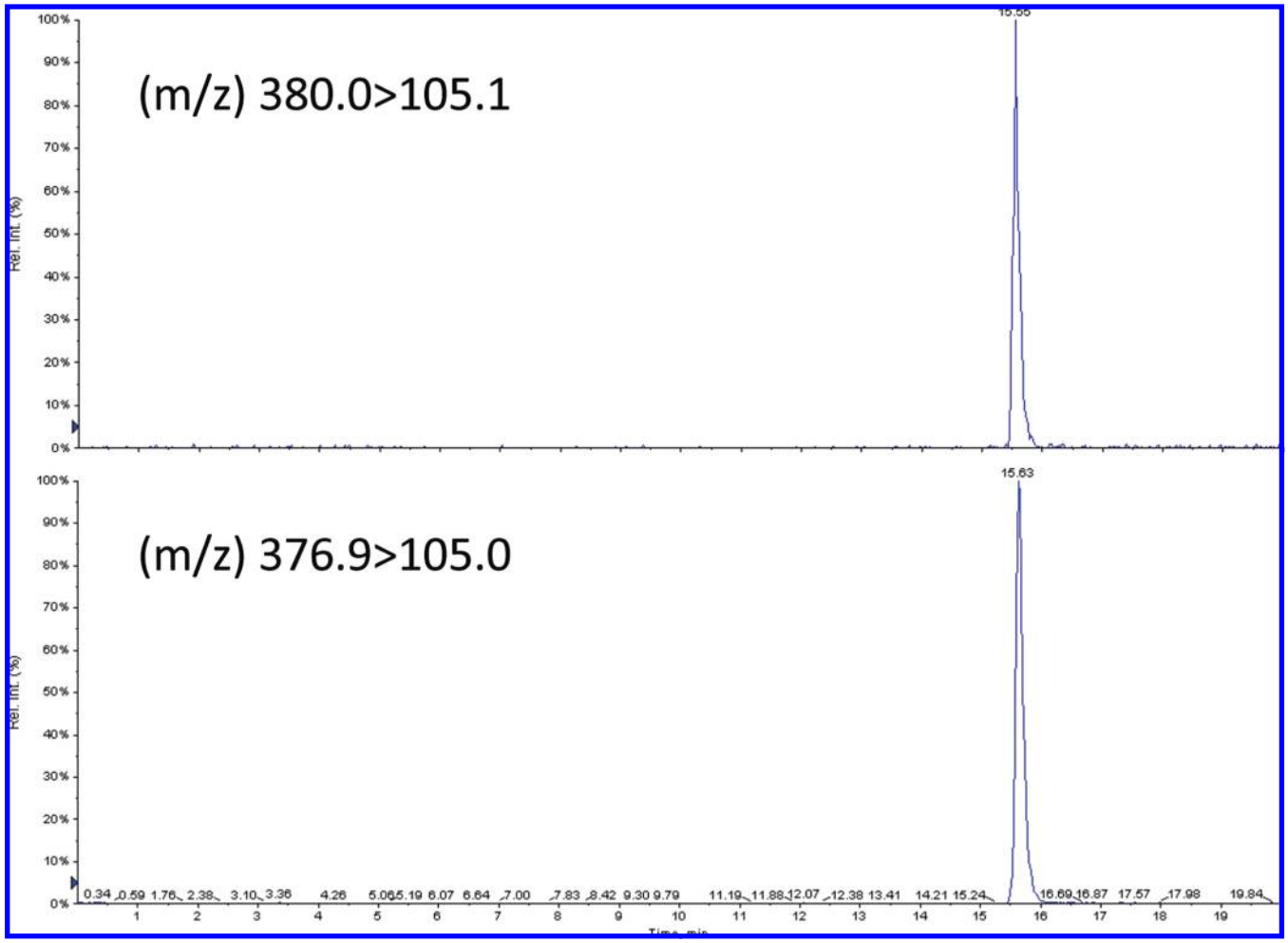

Figure 3. Chromatogram of an incurred hair sample.

frozen until analysis. To remove external contamination, hair samples were washed several times with an aqueous solution of Tween 80 (10%) in distilled water and dried in a heater at 40 °C (*12*). The absence of EB in the wash solution was verified. Hair samples were pulverized with a freezer mill (6750 Spex Certiprep Inc., Metuchen, NJ). Samples were inserted in a grinding vial (about 500 mg of hair in every grinding vial), a polycarbonate cylinder supplied with two end plugs, immersed in liquid nitrogen, and ground with a magnetically driven impactor. Fifteen minutes was required to freeze and pulverize the hair. Pulverized hair was preserved at the temperature of freezing.

Extraction of Estradiol Benzoate. Hair samples (200 mg) were placed in an Eppendorf tube with 1500 μL of acetonitrile containing 1 ng/mL of EB-d3, vortexed for 1 min, sonicated in an ultrasound bath (UCI-200) for 30 min, and incubated for 24 h on a rocker table at room temperature. Afterward, samples were centrifuged at 16100*g* for 15 min (Eppendorf centrifuge 5415D, Hamburg, Germany). The acetonitrile layer was placed into an Ultrafree-MC centrifugal filter device (Millipore, Bedford, MA), centrifuged at 16100*g* for 1 min, placed in a glass injection vial, and evaporated under a nitrogen stream at 37 °C. Samples were redissolved in 75 μL of a mixture acetonitrile/water with 0.1% formic acid (50:50) and vortex mixed for 1 min. Fifty microliters was immediately injected and assayed by HPLC-MS/MS.

Validation of Method. The method was validated on the basis of the criteria of European Commission Decision 2002/657/EC (*11*) using the program ResVal version 2.2 obtained from the Community Reference Laboratory (CRL) in hormones (RIVM, Bilthoven, The Netherlands). A homogeneous mixture of blank hair (20 g) was divided in 63 subsamples. Each day (over the course of 3 days), 21 fortified samples were analyzed: 1 unspiked sample, 6 samples spiked at 1.25 ng/g, 6 samples at 2.5 ng/g, 6 samples at 3.75 ng/g, 1 sample at 5 ng/g, and 1 sample spiked at 12.5 ng/g. Each day, we also calculated a calibration graph using standard solutions ranging from 0 to 12.5 ng/g. The concentration of the analyte in the validation and incurred samples was interpolated from calibration curves determined by calculating the area ratios of analyte peak area/IS peak area versus analyte concentration. The calibration graph was described by the equation $y = mx + b$. Additionally, 20 blank bovine hair samples were analyzed. Decision limit, detection capability, selectivity, specificity, precision, trueness (as corrected percentage recovery), and applicability/ruggedness were evaluated.

RESULTS AND DISCUSSION

Sample Preparation Procedure. Alkaline or acidic digestion of hair at high temperature can easily hydrolyze estradiol benzoate. Use of reducing agents such as dithiothreitol (*14*) can avoid ester hydrolysis. In 2005, Hooijerink et al. (*9*) used a previous pulverization followed by a reduction step with tris(2-carboxyethyl)phosphine hydrochloride to analyze intact estradiol benzoate in hair. We propose, in the present research, a methodology based on cryogenic grinding followed by incubation with acetonitrile. Therefore, we achieve the effective liberation of intact estradiol benzoate from the keratin matrix such that it can be detected in hair from cows treated during reproductive control programs. Times lower than 24 h resulted in fewer positive samples (<50%), and longer incubation does not give better results.

Liquid Chromatography−Mass Spectrometry Analysis. Deuterated estradiol benzoate synthesized in our laboratory was used as the internal standard (IS). The mass spectrometric conditions were optimized for EB and the deuterated analogue

Table 2. Method Performance Validation Data for Estradiol Benzoate in Hair

process	parameter[a]	value		
		day 1	day 2	day 3
diagnostic signal	analyte (EB) (*m*/*z*)		377 > 105	
	internal standard (EB−]d3)(*m*/*z*)		380 > 105	
blank samples	specificity (μN)		<0.001	
calibration curve	range (ng/g)	0; 1.25; 2.5; 3.75; 5; 6.25; 7.5; 10; 12.5		
	linearity (R^2)	0.9530	0.9794	0.9713
	slope	0.272	0.241	0.245
spiked samples	concentration levels (ng/g)	0; 1.25; 2.5; 3.75; 5; 12.5		
	CVr (%)	12.8	13.7	10.2
	CVR (%)		15.6	
	trueness[b] (%)		99.3	
critical limits	CCα (ng/g)		0.81	
	CCβ (ng/g)		1.38	

[a] Average of 20 blank hair noise; CVr, repeatability, greater intraday CV%; CVR, within-laboratory reproducibility, greater interday CV%. [b] Trueness (%), mean recovery of corrected concentration × 100.

in positive ion mode. The analyte was quantified in multiple reaction monitoring (MRM) mode. To obtain quantitative results, the analyte peak area was divided by the IS peak area. Two MRM transitions (one precursor and two product ions, four identification points) were monitored (150 ms dwell time/transition), according to the 2002/657/CE decision (*11*). Addition of formic acid (0.1%) was necessary to improve ionization. In Q1 we monitored $[M + H]^+$ for estradiol benzoate and the deuterated analogue. The optimized parameters to better achieve a declustering potential, entrance potential, collision cell entrance potential, collision cell exit potential, and collision energy are shown in **Table 1**. **Figure 1** shows the structure and fragmentation of EB and EB-d3. **Figure 2** shows the chromatogram of the acquisition window for the MS/MS product ions of estradiol benzoate (*m*/*z* 377 > 105) and deuterated estradiol benzoate (*m*/*z* 380 > 105) in a blank hair sample. In **Figure 3** is shown a chromatogram of an incurred hair sample.

Method Validation. A summary of method performance validation data for estradiol benzoate in hair is shown in **Table 2**. Concerning selectivity and specificity, no interference was observed in the retention time of the analyte ± 2.5% in 20 blank hair samples, which present or not endogenous β-estradiol. No interfering peaks are found in the retention time of the analyte in the chromatograms of selected ions (see **Figure 2**). **Table 2** shows the mean overview validation results. Calibration curves were calculated by linear fitting using the least-squares linear regression calculation. The calibration curves obtained each day gave a good linear correlation ($R \geq 0.95$). 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 noncompliant. The corresponding concentration at the *y*-intercept plus 2.33 times the standard deviation of the intercept equals the decision limit. CCα, the concentration level for a confirmatory method in accordance with European Decision (*11*), was 0.81 ng/g. Hooijerink et al. (*9*) reported similar values. The detection capability (CCβ) is the smallest content of the analyte that may be detected, identified, and/or quantified in a sample with an error probability of β. The corresponding concentration at the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility of the mean measured content at the decision limit equals the detection capability. CCβ was 1.38 ng/g. The α and β errors should be ≤1 or ≤5%, respectively.

Because there were not certified reference materials available in our laboratory, Commission Decision 2002/657/EC offers a means of determining the trueness as corrected recovery of spiked samples. The recovery is corrected for losses during sample preparation via the aid of a deuterated internal standard, as indicated above. Trueness, as corrected percentage recovery (99.3%), complied with the criteria of 70−110% of accuracy.

During validation, besides the pool of blank hair from different animals, 20 blank bovine hair samples, from 20 different animals, were analyzed. With the results obtained, we prove the applicability of the method in the analysis of estradiol benzoate in hair, testing relevant factors such as animal, sampling place (head or shoulder), and hair color (black, white, or mixtures).

Hair samples ($n = 6$) from animals treated with estradiol benzoate for estrus cycle synchronization were analyzed as described above. In accordance with Commission Decision 2002/657/EC, the criteria taken into account to indicate a sample as positive were as follows: the relative retention time of the analyte (RRT) should correspond to that of the estradiol benzoate, from a spiked sample, with a tolerance of ±2.5%; the relative intensities of the peak from two monitored transitions must correspond to those of the estradiol benzoate either from calibration standards or from incurred samples, at comparative concentrations, within the tolerance of ±20%. We found estradiol benzoate in amounts above the CCα in 50% of samples. Hooijerink et al. (*9*) found large amounts (8500 ng/g) of estradiol benzoate in hair from experimental animals treated by "pour-on", or 8−19 ng/g when the animals were treated twice over a period of 3 weeks with the anabolic drug. Thus, we conclude that our methodology permits the confirmatory surveillance of estradiol benzoate in animals, even when the drug is used for reproductive treatment, because the animals often require two or more applications, in a similar way to that explained under Materials and Methods.

LITERATURE CITED

(1) Cavalieri, J.; Rabiee, A. R.; Hepworth, G.; Macmillan, K. L. Effect of artificial insemination on submission rates of lactating dairy cows synchronised and resynchronised with intravaginal progesterone releasing devices and oestradiol benzoate. *Anim. Reprod. Sci.* **2005**, *90*, 39–55.

(2) Sabo, Y. G. Schemes for oestrus synchronization protocols and controlled breeding programs in cattle. *J. Appl. Sci.* **2008**, *8*, 241.

(3) Council Directive 2003/74/EC amending Council Directive 96/22/EC concerning the prohibition on the use of certain substances having a hormonal or thyrostatic action and of beta-agonists in stockfarming. *Off. J. Eur. Communities* **2003**, *L262/17*.

(4) Council Directive 96/22/EC of 29 April 1996 concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of β-agonists, and repealing Directives 81/602/EEC, 88/146/EEC and 88/299/EEC. *Off. J. Eur. Communities* **1996**, *L125/3*.

(5) European Parliament legislative resolution of 5 June 2008 on the proposal for a directive of the European Parliament and of the Council amending Council Directive 96/22/EC concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyreostatic action and of β-agonists. COM(2007)0292 - C6-0154/2007 - 2007/0102(COD).

(6) Maume, D.; Le Bizec, B.; Pouponneau, K.; Deceuninck, Y.; Solere, V.; Paris, A.; Antignac, J.; Andre, F. Modification of 17β-estradiol metabolite profile in steer edible tissues after estradiol implant administration. *Anal. Chim. Acta* **2003**, *483*, 289–297.

(7) Gratacós-Cubarsí, M.; Castellari, M.; Valero, A.; García-Regueiro, J. A. Hair analysis for veterinary drug monitoring in livestock production. *J. Chromatogr., B* **2006**, *834*, 14–25.
(8) Rambaud, L.; Bichon, E; Cesbron, N.; André, F.; Le Bizec, B. Study of 17β-estradiol-3-benzoate, 17α-methyltestosterone and medroxyprogesterone acetate fixation in bovine hair. *Anal. Chim. Acta* **2005**, *532*, 165–176.
(9) Hooijerink, H.; Lommen, A.; Mulder, P. P. J.; van Rhijn, J. A.; Nielen, M. W. F. Liquid chromatography−electrospray ionisation−mass spectrometry based method for the determination of estradiol benzoate in hair of cattle. *Anal. Chim. Acta* **2005**, *529*, 167172.5.
(10) Pedreira, S.; Lolo, M.; Vazquez, B. I.; Franco, C. M.; Cepeda, A.; Fente, C. Liquid chromatography−electrospray ionization-mass spectrometry method in multiple reaction monitoring mode to determine 17α-ethynylestradiol residues in cattle hair without previous digestion. *J. Agric. Food Chem.* **2007**, *55*, 9325–9329.
(11) 657/2002/EC Commission Decision of 12 August 2002 implementing Council Directive 96/23/ECconcerning the performance of analytical methods and the interpretationof results. *Off. J. Eur. Communities* **2002**, *L221*, 8−36.
(12) Durant, A. A.; Fente, C. A.; Franco, C. M.; Vazquez, B. I.; Cepeda, A. Gas chromatography−tandem mass spectrometry determination of 17α-ethinylestradiol residue in the hair of cattle. Application to treated animals. *J. Agric. Food Chem.* **2002**, *50*, 436–440.
(13) Blokland, M.; Herbold, H. ResVal 2.2 validation program from CRL in hormones (RIVM, Bilthoven, The Netherlands); 2003−2005.
(14) Gleixner, A.; Sauerwein, H.; Meyer, H. H. D. Accumulation of the xenobiotic anabolic steroids ethinyloestradiol and methyltestosterone in calf hair. *Food Agric. Immunol.* **1997**, *9*, 27–35.

Received for review July 29, 2008. Revised manuscript received November 13, 2008. Accepted November 13, 2008. We express our thanks for financial support received from Xunta de Galicia Programa Sectorial de Tecnoloxías da Alimentación PGIDIT07TAL025261PR.

JF803044B