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To cite this version:

Fernando Ramos, Maria Lurdes Baeta, Jorge Reis, Maria Irene Noronha da Silveira. Evaluation of the illegal use of clenbuterol in feed, drinking water, urine, and hair samples collected in bovine farms in Portugal. Food Additives and Contaminants, 2009, 26 (06), pp.814-820. 10.1080/02652030902729908 . hal-00577314

HAL Id: hal-00577314

https://hal.science/hal-00577314

Submitted on 17 Mar 2011

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Journal:	*Food Additives and Contaminants*
Manuscript ID:	TFAC-2008-320.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	05-Jan-2009
Complete List of Authors:	Ramos, Fernando; Coimbra University, Pharmacy Faculty Baeta, Maria; Faculty of Pharmacy-University of Coimbra, Laboratory of Bromatology, Hydrology and Nutrition Reis, Jorge; Portuguese Authority for Food Safety, Risk assessment Noronha da Silveira, Maria Irene; Coimbra University, Pharmacy Faculty
Methods/Techniques:	GC/MS
Additives/Contaminants:	Veterinary drug residues
Food Types:	Animal feed, Water

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Evaluation of the illegal use of clenbuterol in feed, drinking water, urine, and hair samples collected in bovine farms in Portugal

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Abstract

The recent discovery of clenbuterol contamination in Portuguese food led to a special inspection of 16 cattle farms for β-agonists, which involved the analysis of a total of 486 samples (78 feed, 106 drinking water, 168 urine and 134 hair). The samples were screened for the β-agonists bromobuterol, cimaterol, clenbuterol, clenpenterol, clenproperol, hydroxymethylclenbuterol, mapenterol, salbutamol and terbutaline. Only clenbuterol was found in all types of analyzed matrices and the most likely method of clenbuterol illegal administration to animals was through drinking water. Of the samples analysed 14.15% of drinking water were found positive in a range of 0.03 to 3.80 mg/l of clenbuterol. Inclusion of hair samples in the Portuguese plan for clenbuterol residue control in live animals is discussed.

Key words: Clenbuterol; drinking water; feeds; urine; hair

Introduction

Food with high fat content is considered one of the main causes of cardiovascular diseases. For that reason, consumers in developed countries have been avoiding the consumption of meat with higher fat content. So, animal producers have used some anabolic substances which curtail the production of this kind of meat. β-agonists were utilized because they result in animal carcasse meat with less fat tissue and, at the same time, increased muscle protein, which provides a very attractive meat to consumers' eyes (Asato et al., 1984; Mersmann, 1987; Inkster, Hovell, Kyle, Brown, & Lobley, G., 1989). The search for food sources has increased, and consequently so has competition in the

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intensive meat production sector. The demand for higher productivity, preferably at lower cost and shorter time periods, has led to several changes in cattle production, such as the improvement of animal feeding and lodging, the use of genetic breeding and the control of animal diseases. This pressure has also led to the illegal use of pharmacological agents, specifically anabolic compounds, to promote the growth of animals (Directive nº 2003/74, 2003). Clenbuterol, a β-agonist, is an authorised drug in the veterinarian therapeutics of bovines, equines and companion animals as bronchodilator or as tocolitic agent for cows. The use of β-agonists as growth promoters is a public health risk; for example, animal-derived foods containing clenbuterol have caused several outbreaks of food poisoning in Spain (Martinez-Navarro, 1990; Garay et al., 1997), France (Pulce et al. 1991), Italy (Maistro, Chiesa, Angeletti, & Brambilla, 1995; Brambilla, Loizzo, Fontana, Strozzi, Guarino, & Soprano, 1997; Brambilla et al., 2000), China (Shiu & Chong, 2001) and Portugal (Barbosa et al., 2005). In addition, the European Union (EU) has prohibited the use of growth-promoting drugs in animals destined for human consumption, and the use of such prohibited substances also constitutes an unfair practice in the market of animal production (Directive nº 96/22, 1996).

To implement the EU regulations, the EU Commission issues guidelines describing National Residue Control Plans for all Member States and these Plans involve sampling animals before and after slaughter (Directive nº 96/23, 1996). In the case of β-agonists in Portugal, the Residue Control Plan stipulates the collection of liver and muscular tissue samples from animals after slaughter, as well as samples from urine, drinking water, and feed from live animals. GC-MS was used as the analytical method for determining the presence of β-agonists, in agreement with the parameters established by Commission Decision nº 2002/657/CE (2002). The present paper is intended to contribute to a better understanding of

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the use (or misuse) of β-agonist substances in live animals in Portugal. The data obtained from samples of urine, drinking water, feed, and hair taken from cattle farms is presented and the relevance of sampling drinking water and hair samples for Portuguese plan for clenbuterol residue control is discussed

Material and Methods

Reagents and equipments

Standards of mapenterol, cimaterol, clenproperol, clenpenterol, bromobuterol, and hydroxymethylclenbuterol were kindly offered by Prof. François André (Laberca, Nantes, France); salbutamol and terbutaline were acquired from Sigma (Madrid, Spain); and clenbuterol and hexadeuterated clenbuterol (clenbuterol-d6) as an internal standard (IS) were supplied, respectively, by Interchim (Montluçon, France) and by RIVM (Bilthoven, the Netherlands). N-methyl-N-trimethylsilyl-trifluoracetamide (MSTA; Sigma, Madrid, Spain), 1,4-Dithioerythritol (DTE; Merck, Lisbon, Portugal), and trimethyliodosilane (TMIS; Sigma) in the proportion of 5 ml:10 mg:10 µl (MSTA:DTE:TMIS) were used as the derivatization reagent. Purified water was obtained from a Milli-Q Plus system (Millipore, Bedford, MA, USA) and all other reagents were from Merck, including *Helix pomatia* β-glucuronidase (30 U/ml) and arylsulfatase (60 U/ml).

For storage and sample preparation, the following materials were used: an Electrolux freezer (Coimbra, Portugal), a Mettler AG285 balance (Zurich, Switzerland), a Moulinex mixer (Lisbon, Portugal), a 40-mesh Endecotts sieve (London, UK), vortex agitators from UnimagZX (Reagente 5, Oporto, Portugal) and Edmund Bülher (KL2 model, Tübingen, Germany), and a Memmert oven (Schwabach, Germany).

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Solid-phase extraction (SPE) was performed using a vacuum manifold system involving Clean Screen DAU mixed-phase (C8+Benzyl sulfonic acid) 500 and 1000 mg columns (Interchim, Montluçon, France). Centrifugation was done on a Sigma 3-16K centrifuge (Reagente 5, Oporto, Portugal), and pH determination was done with a WPA CD7400 potentiometer (Cambridge, UK). Material for derivatization was supplied by Chromacol (Dias de Sousa, Lisbon, Portugal). β-agonist determination was achieved by GC-MS using Agilent Technologies (AT) equipment (Soquimica, Lisbon, Portugal), comprising an AT6890N gas chromatograph equipped with a HP-5MS column (30 m x 0.25 mm I.D. x 0.25 µm of film thickness), an AT7683B automatic injector, an AT5975 MSD detector, an HP Compaq computer, and an HP LaserJet 2420d printer. Nitrogen and helium gases were supplied by Sogafer (Coimbra, Portugal).

Samples

Samples of feed, drinking water, urine, and hair were collected in 16 cattle farms during the period between February 28 and August 24, 2007.

Drinking waters were collected from troughs/tanks in minimum amounts of 100 ml per sample. From farms that perform extensive growth enhancement (A, D, E, G, I and K), samples were collected from the pasture tanks. For intensive farms (B, C, F, H, J, L, M, N, O and P), samples were collected from individual bovine troughs from the cattle-shed water system.

Feed samples were collected from the bovine mangers or the feed storage silos. When sampling was made in the storage silos, appropriate equipment was used in order to collect sample aliquots at different depths. At least, 500 g of each feed were collected. Urine sampling was always taken from male animals in disposable plastic bags adequately attached

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to bovine's back, as well as by specialized technicians (Kurien, Everds & Scofield, 2004). 50 ml of urine was the minimum quantity of each sample.

Hair samples were collected using scissors and hair was cut as close as possible to the skin of the animal. A quantity of 20 g, at least, was considered appropriate. Of the 37 hair samples initially collected, only 5, one per farm B, C, D, E and F, were from animals that urine samples had also been collected. After six months, on farm B, hair and urine samples were simultaneously collected from 56 animals.

Table 1 shows the number of each sample matrices collected on each farm.

Urine and water samples were frozen at -20 ºC ± 2 ºC and hair and feed samples were stored at 4ºC ± 2ºC until further analysis.

Sample preparation

Drinking water- Samples were thawed at room temperature and, after homogenization, 15 ml were transferred to a centrifuge tube. After 10 min centrifugation at 4000*g*, an aliquot of 10 ml of clarified sample was transferred to another container and 100 µl of 1 µg/ml IS and 5 ml of 0.1 M phosphate buffer (pH 6.0 ± 0.1) were added. Finally, pH was adjusted to 6.0 ± 0.3 with 1 M potassium hydroxide (Boyd, O'Keeffe, & Smyth, 1996; Ramos, 2000).

Feed- 5.0 g of ground and sieved sample were weighed into a centrifuge tube and 25 ml of chloroform and 100 µl of 1 µg/ml IS were added. After a 30 min agitation period at 420 rpm, everything was filtered into a decantation funnel already containing 10 ml of 1 M hydrochloric acid. The decantation funnel was mixed manually for 1 min and allowed to rest until complete phase separation was achieved. The organic phase was discarded. Another 10 ml of chloroform were added to the decantation funnel and the previous step repeated. The

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aqueous phase was recovered and 5 ml of 0.1 M phosphate buffer (pH 6.0 ± 0.1) were added. After homogenization, pH was adjusted to 6.0 ± 0.3 with 2 M potassium hydroxide (Ramos, Ribeiro, Saltão, Costa, & Silveira, 1999)

Urine- Urine samples, collected each one for a different animal, were thawed at room temperature and, after homogenization, 15 ml were transferred into a centrifuge tube. After 10 min centrifugation at 4000*g*, pH was adjusted to 5.2 ± 0.3 with glacial acetic acid. Then, 10 ml of clarified sample were taken and the following were added: 100 µl of 1 µg/ml IS, 4 ml of 0.2 M acetate buffer (pH 5.2 ± 0.1) and 50 µl of *Helix pomatia*. The mixture was vortexed for 20 sec, and then incubated overnight in an oven at 48 ºC ± 2 ºC. Samples were left to cool at room temperature. Then 5 ml of 0.1 M phosphate buffer (pH 6.0 ± 0.1) were added and the pH was adjusted to 6.0 ± 0.3 with 1 M potassium hydroxide (Boyd et al., 1996).

Hair- Hair was washed with an aqueous solution of 5% Tween 80. The sample was then rinsed with purified water, as often as necessary, until the washing water was clear. Hair samples were dried overnight in an oven at 80 ºC, and subsequently cut into small pieces 1-2 mm in length. Pieces with a total mass of 1.0 g were transferred to a centrifuge tube to which 3 ml of 2 M sodium hydroxide and 100 µl of 1 µg/ml IS were added. Following homogenization, the sample was digested at 80 ºC for 1 h, with agitation every 15 min (Fente, Vázquez, Franco, Cepeda, & Gigosos, 1999; Ramos et al., 2001). After cooling to room temperature, 4 ml of 0.2 M acetate buffer (pH 5.2 ± 0.1) were added and the pH was adjusted to 5.2 ± 0.3 with 2 M hydrochloric acid. The mixture was centrifuged for 5 min and the supernatant was filtered. The sediment was resuspended in 5 ml of acetate buffer and centrifuged again for 5 min. The second supernatant was also filtered and mixed with the previous one (Cristino, Ramos, & Silveira, 2003). Then, 50 µl of *Helix pomatia* were added, the mixture was vortexed for 20 sec, and incubated overnight at 48 ºC ± 2 ºC. Samples were

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left to cool to room temperature. Then 5 ml of 0.1 M phosphate buffer (pH 6.0 ± 0.1) were added and pH was adjusted to 6.0 ± 0.3 with 1 M potassium hydroxide (Boyd et al., 1996; Ramos, 2000).

Solid-phase extraction

The sample extracts prepared as described above were purified by SPE using mixed-phase columns. A 500 mg column was used for urine and water samples, while a 1000 mg column was used for feed and hair; the volumes used for the former were one-half the volumes used for the latter. Sorbent was activated with methanol, water, and phosphate buffer (2 or 4 ml of each solvent), after which the extract was loaded. Acetic acid (1 ml or 2 ml) was added to the columns and left for 10 min before being eluted. The washing step was performed using 6 or 12 ml of methanol. After each of the previous two steps, the sorbent was dried using centrifugation at 4000*g*. Analyte elution was carried out using 5 or 10 ml of ethyl acetate: 32% ammonia (97:3, v/v) (Montrade, le Bizec, Monteau, Siliart, & André, 1993; Ramos, Bañobre, Castilho, & Silveira, 1999)

Derivatization

Eluates were evaporated to dryness under a nitrogen stream at 65 ºC. The residue was dissolved in methanol and transferred to a derivatization vial. Methanol was also evaporated to dryness under the conditions mentioned above for the eluates, and the resulting dry residue was redissolved with 50 µl of derivatization reagent. After vial encapsulation, derivatization was performed at 65 ºC for 20 min (Ramos et al., 2003).

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Chromatography

An aliquot of 2 µl was injected into the GC-MS system in splitless mode (1 min), using helium as carrier gas with a constant flow of 1.4 ml/min through the column. The column was submitted to the following temperature gradient: 100 °C (0.4 min) -------› 10 °C/min ----› 200 °C (3 min) ----› 10 °C/min ----› 300 °C (5 min). Injector and detector temperatures were 270 and 280 ºC, respectively, and detection was done in electron impact (EI) mode with selective ion monitoring (SIM).

Table 2 shows m/z ions detected for each β-agonist, as well as for the IS (clenbuterol-d6).

Figures 1 – 4 show chromatograms of a standard solution and of positives drinking water, urine and hair samples.

Results and Discussion

Method validation

Validation data was acquired for clenbuterol determination in urine, feed and hair samples. Urine data was used for drinking water samples. Specificity, linearity, decision limit (CCα), detection capability (CCβ), precision and accuracy were determined according to Commission Decision 2002/657/EC (2002). Specificity was demonstrated by analysing 20 blank of each samples before and after spiking at a concentration level of 1.0 µg/l or 5.0 µg/kg, respectively for urine and water samples and for hair and feed samples. No interfering peaks were observed at the retention time window for clenbuterol in the four analysed matrices. Linearity of the chromatographic response was tested for clenbuterol with five calibration points in the concentration range of 0.5–5.0 µg/l and of 5.0–50.0 µg/kg, respectively for urine and for hair and feed samples. The regression coefficients (r^2) for the calibration curves used in the method validation study were always above 0.993.

CCα and CCβ were calculated as stated by Commission Decision 2002/657/EC (2002) and by

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ISO 11843 (2000). For CCα, with α = 1%, the calculation was performed with the application of the following formula:-

$$CC\alpha = b + 2.33 S.D._b$$

where b is the concentration of the intercept of the regression line and $S.D._b$ is the standard deviation from regression line.

CCβ at β = 5%, was then calculated as:-

$$CC\beta = CC\alpha + 1.64 S.D._{CC\alpha}$$

wherein $S.D._{CC\alpha}$ is the standard deviation at CCα concentration.

Clenbuterol accuracy, in terms of recovery, was measured using blank samples spiked at 0.5, 1.0, and 2.0 µg/l and at 5.0, 10.0 and 20.0 µg/kg respectively for urine and for hair and feed samples. Precision, in terms of repeatability and intra-laboratorial reproducibility, was evaluated in particular the within- and between days variation, by calculating the coefficient of variation (CV) of clenbuterol mean concentrations at previous referred concentration levels and matrices. For each level, six samples of each matrix were prepared and analysed every day for 3 days. Obtained data at 1.0 µg/l and at 10.0 µg/kg levels, respectively for urine and for hair and feed samples, were shown in table 3 and lead to the conclusion that the used methodology could satisfy required reference parameters for the purpose.

Samples investigated

No β-agonists were found in any matrix from 12 of the 16 farms under inspection for using growth-enhancing drugs. No β-agonists were found in any analyzed feed samples. In the other

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matrices analyzed, only one β-agonist was found, clenbuterol, in concentrations between 0.03 – 3.80 mg/l, 0.3 – 11.6 µg/l and 32.9 – 57.0 µg/kg, respectively for drinking water, urine and hair samples. The results for the positive samples are shown in Table 1.

Comparing the incidence of positive urine samples and positive hair samples, a discrepant value could be observed. However, one of the two hair samples where clenbuterol was detected was black and was the only hair sample collected from farm C. The urine sample collected from the same animal was also declared positive with a clenbuterol concentration of 3.9 µg/l. The other positive hair sample was brown and part of a group of 13 collected at farm B where 1 hair was white, 3 were blond, and 9 were brown. The urine sample collected from the same animal was also considered positive for clenbuterol with a concentration of 4.8 µg/l. Clenbuterol was detected in the two positive samples at the levels of 57.0 µg/kg (black) and 32.9 µg/kg (brown), which is according to previously related melanin relevance in hair clenbuterol detection (Cristino et al., 2003; Gallo, Brambilla, Neri, Fiori ,Testa & Serpe, 2007, Salquèbre, Bresson, Villain, Cirimele & Kintz, 2006; Sauer & Anderson, 1994). Effectively, melanin, the main hair pigmented component was responsible for clenbuterol accumulation, due to chemical binding between their –SH radicals and the substituted aromatic ring of the β-agonist. (Howells, Godfrey & Sauer, 1994).

Of the remaining hair samples, the great majority (97) were collected in two cattle farms that had been ordered to cease clenbuterol administration six months before, when clenbuterol had been detected in 50% (Farm A) and 63% (Farm B) of the analyzed urine samples. In the 97 samples (1 white, 7 black, 40 brown, and 49 blonde), no clenbuterol was detected, which indicate, as previously related by Fente and co-workers (1999), that after six months, clenbuterol is no longer detectable in hair samples.

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A total of 168 urine samples were tested. All of the 24 samples found to be positive had been collected in 3 farms, and they constituted 58.5% of such samples.

A high number (15 of 22) of drinking water samples were positive in the farms where the β-agonist was detected. All positive samples were collected from individual troughs of intensive bovine production, where the animals were normally maintained in cattle-sheds during the greater part of the day. It was also observed that clenbuterol was only put in the watering system inside each cattle-shed. Thus, clenbuterol was pumped from a 50-liter water barrel into the pipe that supplies bovine individual troughs. Indeed, clenbuterol water solubility allows an easy and homogeneous mixture in the water that was supplied by the farms' wells and then adequately managed and stored. The pump system was automatically activated by animals when they drank water and the system could control clenbuterol concentration in drinking water, according to animal size, age and feed and forage intake that were more or less uniformly allocated in each cattle-shed. It was also confirmed that this pump system was easy to make inoperative in order to difficult authority inspections.

Our data suggests that a new methodology for illegal clenbuterol administration to the animals is possible to use and should be inspected by authorities. However, clenbuterol was also illegally used in extensive bovine growth promotion. Farm A that performs extensive bovine growth promotion presented clenbuterol-negative drinking water samples. Samples from farm A were collected from two different paddocks and the four animals that were declared positive for urine analysis were from the same paddock. Clenbuterol administration to these animals could not be found, because drinking water and feed samples collected in the same paddock were negatives.

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Our data for urine and drinking water samples reinforces the usefulness of these matrices for screening β-agonists in live animals, which is confirmed by the Canadian Food Inspection Agency (Dickson, MacNeil, Lee & Fesser, 2005) or by the National Plans for Residue Control in EU countries (Directive nº 96/23, 1996). Nevertheless, and with respect to live animals and to the Portuguese Residue Control Plan, the hypothesis of analyzing hair samples should also be considered. As it was observed in the present study, it would be relevant to include hair samples. So, the present data will be shared by all the Portuguese authorities involved in the official residue control plan in order to discuss the eventual inclusion of hair samples for controlling β-agonists in bovines suspected of illegal fattening. Finally, this study shows that clenbuterol still remains one of the most illegally used β-agonist substances on farms that practice bovine growth promotion.

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Figure 1 – TIC chromatogram of β-agonist standards

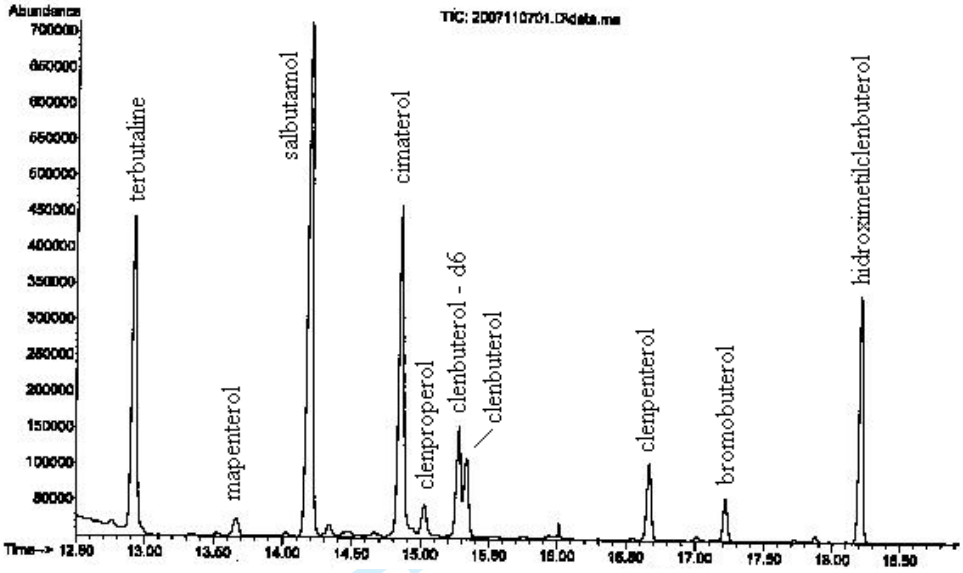

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Figure 2 – Chromatogram of a drinking water sample with 0.28 mg/l of clenbuterol

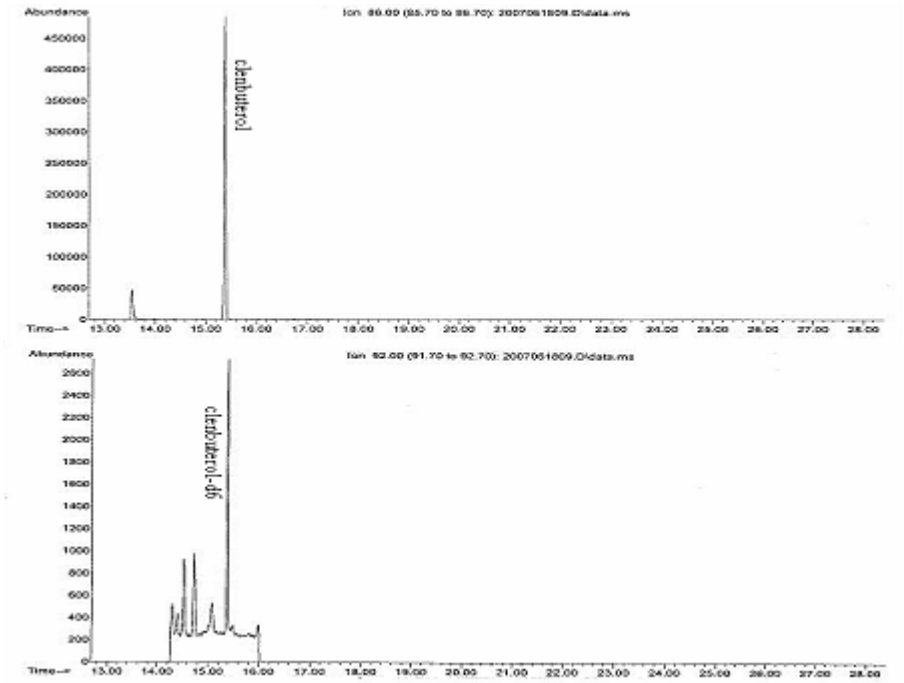

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Figure 3 – Chromatogram of a urine sample with 1.1 µg/l of clenbuterol

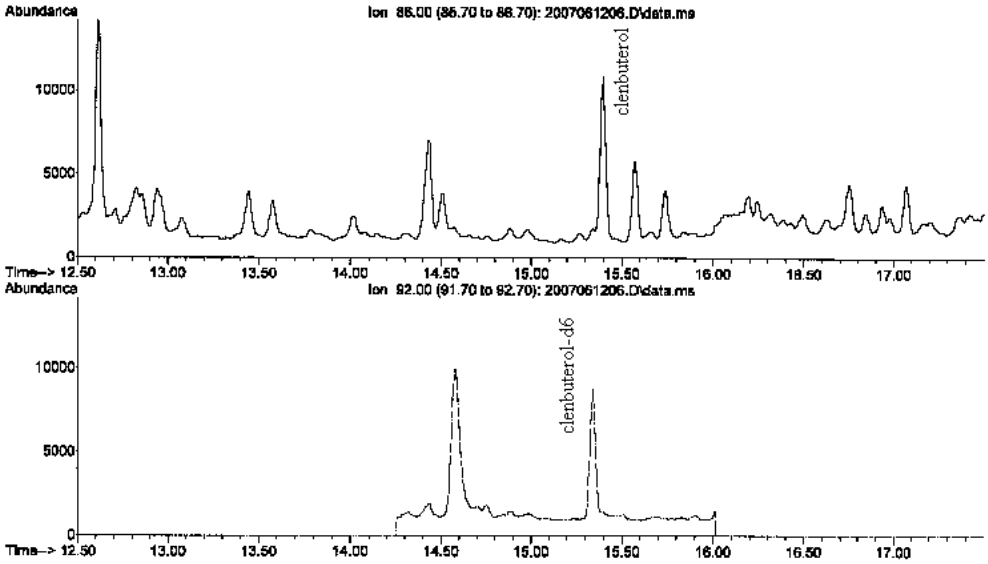

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Figure 4 – Chromatogram of a hair sample with 57.0 µg/Kg of clenbuterol

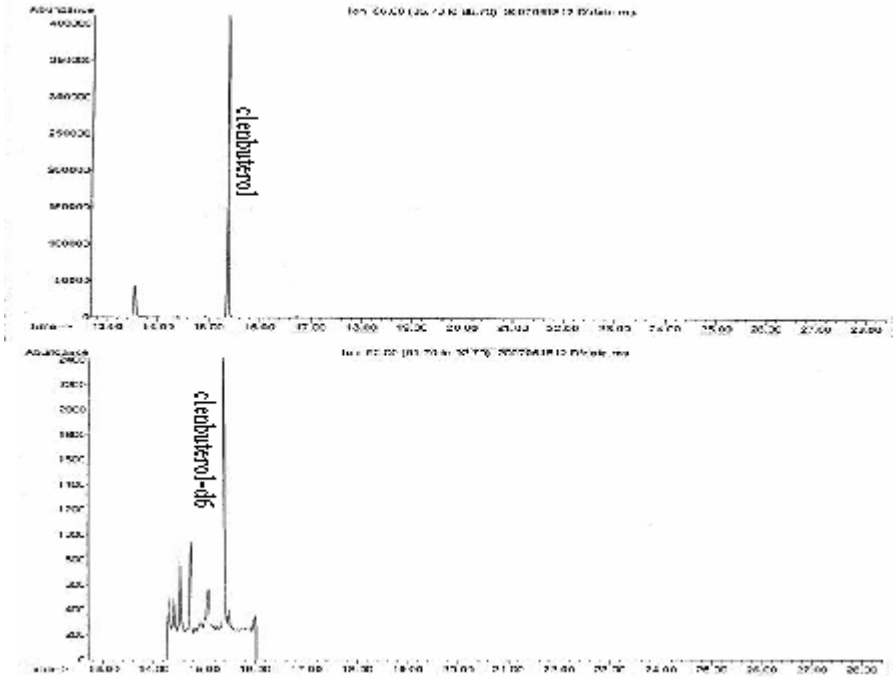

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Table 1 – Number of samples collected on each farm

Farm	Water	Feed	Urine	Hair
A	2	3	8 (4)	2 + 41
B	7 (6)	2	22 (14) + 56	13 (1) + 56
C	4 (2)	2	11 (6)	1 (1)
D	1	2	1	5
E	3	2	6	1
F	11	11	25	6
G	2	4	1	9
H	8	8	14	-
I	1	1	6	-
J	14	-	5	-
K	2	3	13	-
L	11 (7)	-	-	-
M	8	-	-	-
N	6	-	-	-
O	18	-	-	-
P	8	-	-	-
TOTAL	**106 (15)**	**78 (0)**	**168 (24)**	**134 (2)**

Note 1 – Data in brackets are the number of clenbuterol positive samples

Note 2 – Data separated by a + mark indicate that samples were collected with a interval of six months

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Table 2 – Detected m/z ions for each β-agonist

Time window	β - agonists	m/z ions
12.50 – 13.30	Terbutaline	86; 336; 356; 426
13.30 – 14.50	Mapenterol	100; 313; 369; 453
	Salbutamol	86; 350; 369; 440
14.50 – 16.00	Cimaterol	72; 219; 291; 418
	Clenproperol	72; 300, 335; 391
	Clenbuterol-d6 (IS)	92; 411
	Clenbuterol	86; 300; 335; 405
16.00 – 17.70	Clenpenterol	100; 300; 335; 421
	Bromobuterol	86; 344; 425; 495
17.70 – 19.00	Hydroximethylclenbuterol	145; 174; 315; 493

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Table 3 – Performance characteristics of the method for clenbuterol

Matrices	**CCα**	**CCβ**	**Accuracy** (n=6)	**Repeatability** (n=6)	**Reproducibility** (n=18)
	[µg/l] or [µg/kg]	[µg/l] or [µg/kg]	[%]	[% (CV)]	[% (CV)]
Urine	0.12	0.25	98.45	2.31	9.93
Feed	0.73	1.92	92.10	6.97	12.23
Hair	1.05	2.53	93.31	5.82	13.55

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