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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  $\beta$ -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  $\beta$ -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.  $\beta$ -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, & Loble, 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 n° 2003/74, 2003). Clenbuterol, a  $\beta$ -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  $\beta$ -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 n° 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 n° 96/23, 1996). In the case of  $\beta$ -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  $\beta$ -agonists, in agreement with the parameters established by Commission Decision n° 2002/657/CE (2002). The present paper is intended to contribute to a better understanding of

the use (or misuse) of  $\beta$ -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

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## 75 **Material and Methods**

### 76 *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-trifluoroacetamide (MSTA; Sigma, Madrid, Spain), 1,4-Dithioerythritol (DTE; Merck, Lisbon, Portugal), and trimethyliodosilane (TMIS; Sigma) in the proportion of 5 ml:10 mg:10  $\mu$ l (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*  $\beta$ -glucuronidase (30 U/ml) and arylsulfatase (60 U/ml).

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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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3 95 Solid-phase extraction (SPE) was performed using a vacuum manifold system involving  
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5 96 Clean Screen DAU mixed-phase (C8+Benzyl sulfonic acid) 500 and 1000 mg columns  
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8 97 (Interchim, Montluçon, France). Centrifugation was done on a Sigma 3-16K centrifuge  
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10 98 (Reagente 5, Oporto, Portugal), and pH determination was done with a WPA CD7400  
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12 99 potentiometer (Cambridge, UK). Material for derivatization was supplied by Chromacol (Dias  
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14 100 de Sousa, Lisbon, Portugal).  $\beta$ -agonist determination was achieved by GC-MS using Agilent  
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16 101 Technologies (AT) equipment (Soquimica, Lisbon, Portugal), comprising an AT6890N gas  
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18 102 chromatograph equipped with a HP-5MS column (30 m x 0.25 mm I.D. x 0.25  $\mu$ m of film  
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20 103 thickness), an AT7683B automatic injector, an AT5975 MSD detector, an HP Compaq  
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22 104 computer, and an HP LaserJet 2420d printer. Nitrogen and helium gases were supplied by  
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24 105 Sogafer (Coimbra, Portugal).  
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34 107 *Samples*  
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36 108 Samples of feed, drinking water, urine, and hair were collected in 16 cattle farms during the  
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38 109 period between February 28 and August 24, 2007.  
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40 110 Drinking waters were collected from troughs/tanks in minimum amounts of 100 ml per  
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42 111 sample. From farms that perform extensive growth enhancement (A, D, E, G, I and K),  
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44 112 samples were collected from the pasture tanks. For intensive farms (B, C, F, H, J, L, M, N, O  
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46 113 and P), samples were collected from individual bovine troughs from the cattle-shed water  
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48 114 system.  
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53 116 Feed samples were collected from the bovine mangers or the feed storage silos. When  
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55 117 sampling was made in the storage silos, appropriate equipment was used in order to collect  
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57 118 sample aliquots at different depths. At least, 500 g of each feed were collected. Urine  
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59 119 sampling was always taken from male animals in disposable plastic bags adequately attached  
60

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

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

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

129 Urine and water samples were frozen at  $-20^{\circ}\text{C} \pm 2^{\circ}\text{C}$  and hair and feed samples were stored  
130 at  $4^{\circ}\text{C} \pm 2^{\circ}\text{C}$  until further analysis.

### 132 *Sample preparation*

133 Drinking water- Samples were thawed at room temperature and, after homogenization, 15 ml  
134 were transferred to a centrifuge tube. After 10 min centrifugation at 4000g, an aliquot of 10  
135 ml of clarified sample was transferred to another container and 100  $\mu\text{l}$  of 1  $\mu\text{g/ml}$  IS and 5 ml  
136 of 0.1 M phosphate buffer ( $\text{pH } 6.0 \pm 0.1$ ) were added. Finally, pH was adjusted to  $6.0 \pm 0.3$   
137 with 1 M potassium hydroxide (Boyd, O'Keeffe, & Smyth, 1996; Ramos, 2000).

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



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

148 Urine- Urine samples, collected each one for a different animal, were thawed at room  
149 temperature and, after homogenization, 15 ml were transferred into a centrifuge tube. After 10  
150 min centrifugation at 4000g, pH was adjusted to  $5.2 \pm 0.3$  with glacial acetic acid. Then, 10  
151 ml of clarified sample were taken and the following were added: 100  $\mu$ l of 1  $\mu$ g/ml IS, 4 ml of  
152 0.2 M acetate buffer (pH  $5.2 \pm 0.1$ ) and 50  $\mu$ l of *Helix pomatia*. The mixture was vortexed for  
153 20 sec, and then incubated overnight in an oven at  $48^\circ\text{C} \pm 2^\circ\text{C}$ . Samples were left to cool at  
154 room temperature. Then 5 ml of 0.1 M phosphate buffer (pH  $6.0 \pm 0.1$ ) were added and the  
155 pH was adjusted to  $6.0 \pm 0.3$  with 1 M potassium hydroxide (Boyd et al., 1996).

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157 Hair- Hair was washed with an aqueous solution of 5% Tween 80. The sample was then  
158 rinsed with purified water, as often as necessary, until the washing water was clear. Hair  
159 samples were dried overnight in an oven at  $80^\circ\text{C}$ , and subsequently cut into small pieces 1-2  
160 mm in length. Pieces with a total mass of 1.0 g were transferred to a centrifuge tube to which  
161 3 ml of 2 M sodium hydroxide and 100  $\mu$ l of 1  $\mu$ g/ml IS were added. Following  
162 homogenization, the sample was digested at  $80^\circ\text{C}$  for 1 h, with agitation every 15 min (Fente,  
163 Vázquez, Franco, Cepeda, & Gigosos, 1999; Ramos et al., 2001). After cooling to room  
164 temperature, 4 ml of 0.2 M acetate buffer (pH  $5.2 \pm 0.1$ ) were added and the pH was adjusted  
165 to  $5.2 \pm 0.3$  with 2 M hydrochloric acid. The mixture was centrifuged for 5 min and the  
166 supernatant was filtered. The sediment was resuspended in 5 ml of acetate buffer and  
167 centrifuged again for 5 min. The second supernatant was also filtered and mixed with the  
168 previous one (Cristino, Ramos, & Silveira, 2003). Then, 50  $\mu$ l of *Helix pomatia* were added,  
169 the mixture was vortexed for 20 sec, and incubated overnight at  $48^\circ\text{C} \pm 2^\circ\text{C}$ . Samples were

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

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#### 174 *Solid-phase extraction*

175 The sample extracts prepared as described above were purified by SPE using mixed-phase  
176 columns. A 500 mg column was used for urine and water samples, while a 1000 mg column  
177 was used for feed and hair; the volumes used for the former were one-half the volumes used  
178 for the latter. Sorbent was activated with methanol, water, and phosphate buffer (2 or 4 ml of  
179 each solvent), after which the extract was loaded. Acetic acid (1 ml or 2 ml) was added to the  
180 columns and left for 10 min before being eluted. The washing step was performed using 6 or  
181 12 ml of methanol. After each of the previous two steps, the sorbent was dried using  
182 centrifugation at 4000g. Analyte elution was carried out using 5 or 10 ml of ethyl acetate:  
183 32% ammonia (97:3, v/v) (Montrade, le Bizec, Monteau, Siliart, & André, 1993; Ramos,  
184 Bañobre, Castilho, & Silveira, 1999)

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#### 186 *Derivatization*

187 Eluates were evaporated to dryness under a nitrogen stream at 65 °C. The residue was  
188 dissolved in methanol and transferred to a derivatization vial. Methanol was also evaporated  
189 to dryness under the conditions mentioned above for the eluates, and the resulting dry residue  
190 was redissolved with 50 µl of derivatization reagent. After vial encapsulation, derivatization  
191 was performed at 65 °C 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 °C, 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<sup>2</sup>) 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

ISO 11843 (2000). For  $CC\alpha$ , with  $\alpha = 1\%$ , the calculation was performed with the application of the following formula:-

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

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

$CC\beta$  at  $\beta = 5\%$ , was then calculated as:-

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

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

Clenbuterol accuracy, in terms of recovery, was measured using blank samples spiked at 0.5, 1.0, and 2.0  $\mu\text{g/l}$  and at 5.0, 10.0 and 20.0  $\mu\text{g/kg}$  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  $\mu\text{g/l}$  and at 10.0  $\mu\text{g/kg}$  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  $\beta$ -agonists were found in any matrix from 12 of the 16 farms under inspection for using growth-enhancing drugs. No  $\beta$ -agonists were found in any analyzed feed samples. In the other

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3 247 matrices analyzed, only one  $\beta$ -agonist was found, clenbuterol, in concentrations between 0.03  
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5 248 – 3.80 mg/l, 0.3 – 11.6  $\mu$ g/l and 32.9 – 57.0  $\mu$ g/kg, respectively for drinking water, urine and  
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8 249 hair samples. The results for the positive samples are shown in Table 1.  
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10 250 Comparing the incidence of positive urine samples and positive hair samples, a discrepant  
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12 251 value could be observed. However, one of the two hair samples where clenbuterol was  
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15 252 detected was black and was the only hair sample collected from farm C. The urine sample  
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17 253 collected from the same animal was also declared positive with a clenbuterol concentration of  
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19 254 3.9  $\mu$ g/l. The other positive hair sample was brown and part of a group of 13 collected at farm  
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22 255 B where 1 hair was white, 3 were blond, and 9 were brown. The urine sample collected from  
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24 256 the same animal was also considered positive for clenbuterol with a concentration of 4.8  $\mu$ g/l.  
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27 257 Clenbuterol was detected in the two positive samples at the levels of 57.0  $\mu$ g/kg (black) and  
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29 258 32.9  $\mu$ g/kg (brown), which is according to previously related melanin relevance in hair  
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31 259 clenbuterol detection (Cristino et al., 2003; Gallo, Brambilla, Neri, Fiori ,Testa & Serpe,  
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33 260 2007, Salqu bre, Bresson, Villain, Cirimele & Kintz, 2006; Sauer & Anderson, 1994).  
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36 261 Effectively, melanin, the main hair pigmented component was responsible for clenbuterol  
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38 262 accumulation, due to chemical binding between their –SH radicals and the substituted  
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40 263 aromatic ring of the  $\beta$ -agonist. (Howells, Godfrey & Sauer, 1994).  
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46 265 Of the remaining hair samples, the great majority (97) were collected in two cattle farms that  
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48 266 had been ordered to cease clenbuterol administration six months before, when clenbuterol had  
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50 267 been detected in 50% (Farm A) and 63% (Farm B) of the analyzed urine samples. In the 97  
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52 268 samples (1 white, 7 black, 40 brown, and 49 blonde), no clenbuterol was detected, which  
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55 269 indicate, as previously related by Fente and co-workers (1999), that after six months,  
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57 270 clenbuterol is no longer detectable in hair samples.  
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272 A total of 168 urine samples were tested. All of the 24 samples found to be positive had been  
273 collected in 3 farms, and they constituted 58.5% of such samples.

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

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

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Our data for urine and drinking water samples reinforces the usefulness of these matrices for screening  $\beta$ -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 n° 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  $\beta$ -agonists in bovines suspected of illegal fattening. Finally, this study shows that clenbuterol still remains one of the most illegally used  $\beta$ -agonist substances on farms that practice bovine growth promotion.

References

Asato, G., Baker, P., Bass, R., Bentley, T., Chari, S., Dalrymple, R., France, D., Gingher, P., Lences, B., Pascavage, J., Pensack, J. & Ricks, C. (1984). Repartitioning agents: 5-[1-Hydroxy-2-(isopropylamino) ethyl] - anthranoline and related phenethanolamines; Agents for promoting growth, increasing muscle acreation and reducing fat deposition in meat-producing animals. *Agricultural and Biological Chemistry* , 48, 2883 - 2888.

Barbosa, J., Cruz, C., Martins, J., Silva, J.M., Neves, C., Alves, C., Ramos, F. & Silveira, M. I. N. (2005). Food poisoning by clenbuterol in Portugal. *Food Additives and Contaminants*, 22, 563-566.

Boyd, D., O’Keeffe, M. & Smyth, M. (1996). Methods for the determination of  $\beta$ -agonists in biological matrices. *Analyst*, 121, 1R - 10R.



- 321
- 322 Brambilla, G., Cenci, T., Franconi, F., Galarini, R., Macri, A., Rondoni, F., Strozzi, M. &
- 323 Loizzo, A. (2000). Clinical and pharmacological profile in a clenbuterol epidemic poisoning
- 324 of contaminated beef meat in Italy. *Toxicology Letters*, 114, 47-53.
- 325
- 326 Brambilla, G., Loizzo, A., Fontana, L., Strozzi, M., Guarino, A. & Soprano, V. (1997). Food
- 327 poisoning following consumption of clenbuterol-treated veal in Italy. *Journal of the American*
- 328 *Medical Association*, 278, 635.
- 329
- 330 Cristino, A., Ramos, F. & Silveira, M.I.N. (2003). Control of the illegal use of clenbuterol in
- 331 bovine production. *Journal of Pharmaceutical and Biomedical Analysis*, 32, 311–316.
- 332
- 333 Decision n° 2002/657/CE, August, 12th (2002). *Official Journal of European Community*,
- 334 L221, 8-36
- 335
- 336 Dickson, L.C., MacNeil, J., Lee, S. & Fesser, C.E. (2005). Determination of  $\beta$ -agonist
- 337 residues in bovine urine using liquid chromatography-tandem mass spectrometry. *Journal of*
- 338 *AOAC International*, 88 (1) 46 – 56.
- 339
- 340 Directive n° 2003/74/CE, September, 22th (2003). *Official Journal of European Community*,
- 341 L262, 17-21
- 342
- 343 Directive n° 96/22/CE, April, 29<sup>th</sup> (1996). *Official Journal of European Community*, L125, 3-
- 344 9
- 345



- 346 Directive nº 96/23/CE, April, 29<sup>th</sup> (1996). *Official Journal of European Community*, L125,  
347 10-32.
- 348
- 349 Fente, C.A., Vázquez, B.I., Franco, C., Cepeda, A. & Gigoso, P.G. (1999). Determination of  
350 clenbuterol residues in bovine hair using diphasic dialysis and gas chromatography-mass  
351 spectrometry. *Journal of Chromatography B*, 726, 133-139.
- 352
- 353 Gallo, P., Brambilla, G., Neri, B., Fiori, M., Testa, C. & Serpe, L. (2007). Purification of  
354 clenbuterol-like  $\beta$ 2-agonist drugs of new generation from bovine urine and hair by  $\alpha$ 1-acid  
355 glycoprotein affinity chromatography and determination by gas chromatography-mass  
356 spectrometry. *Analytica Chimica Acta*, 587, 67 – 74.
- 357
- 358 Garay, J.B., Jiménez, J.F.H., Jiménez, M.L., Sebastian, M.V., Matesanz, J.P., Moreno, P.M.  
359 & Galiana, J.R. (1997). Intoxicación por clenbuterol: datos clínicos y analíticos de un brote  
360 epidémico en Móstoles, Madrid. *Revista Clinica Española*, 197, 92 - 95.
- 361
- 362 Howells, L., Godfrey, M. & Sauer, M.J. (1994). Melanin as an absorbent for drug residues.  
363 *Analyst*, 119, 2691 - 2693.
- 364
- 365 Inkster, J., Hovell, F., Kyle, D., Brown, D. & Loble, G. (1989). The effect of clenbuterol on  
366 basal protein turnover and endogenous nitrogen loss of sheep. *British Journal of Nutrition*, 62,  
367 285-296.
- 368
- 369 ISO 11843-2, May, 1st (2000), Capability of detection-part 2: methodology in the linear  
370 calibration case. ISO, Geneva, Switzerland.

- 371
- 372 Kurien, B.T., Everds, N.E., & Scofield, R.H. (2004). Experimental animal urine collection: a  
373 review. *Laboratory Animals*, 38, 333 – 361.
- 374
- 375 Maistro, S., Chiesa, E., Angeletti, R. & Brambilla, G. (1995). - Beta blockers to prevent  
376 clenbuterol poisoning. *Lancet*. 346, 180.
- 377
- 378 Martinez-Navarro, J.F. (1990). Food poisoning related to consumption of illicit  $\beta$ -agonist in  
379 liver. *Lancet*. 336, 1311.
- 380
- 381 Mersmann, H.J. (1987). Acute metabolic effects of adrenergic agents in swine. *American*  
382 *Journal of Physiology*, 252, E85-E95.
- 383
- 384 Montrade, M-P., le Bizec, B., Monteau, F., Siliart, B. & André, F. (1993). Multi-residue  
385 analysis for  $\beta$ -agonistic drugs in urine of meatproducing animals by gas chromatography-  
386 mass spectrometry. *Analytica Chimica Acta*, 275, 253–268.
- 387
- 388 Pulce, C., Lamaison, D., Keck, G., Bostvironnois, C., Nicolas, J., Mora, M. & Colmant, A.  
389 (1991). Intoxication alimentaire collective due à la présence de résidus de clenbutérol dans du  
390 foie de veau. *Bulletin Epidemiologique Hebdomadaire*, 5, 17 - 18.
- 391
- 392 Ramos, F. (2000).  $\beta_2$  -Agonist extraction procedures for chromatographic analysis, *Journal of*  
393 *Chromatography A*, 880, 69-83
- 394

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57  
58  
59  
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395 Ramos, F., Bañobre, M.C., Castilho, M.C. & Silveira, M.I.N. (1999). Solid phase extraction  
396 (SPE) for multi-residue analysis of  $\beta_2$ -agonists in bovine urine. *Journal of Liquid*  
397 *Chromatography & Related Technologies*, 22, 2307–2320.

398

399 Ramos, F., Cristino, A., Carrola, P., Eloy, T., Silva, J.M., Castilho, M.C. & Silveira, M.I.N.  
400 (2003). Clenbuterol food poisoning diagnosis by gas chromatography–mass spectrometric  
401 serum analysis. *Analytica Chimica Acta*, 483, 207–213.

402

403 Ramos, F., González, P., Oliveira, A., Almeida, A., Fente, C., Franco, C., Cepeda, A. &  
404 Silveira, M.I.N. (2001). Optimization of diphasic dialysis procedure for clenbuterol residues  
405 extraction in bovine retina and hair. *Journal of Liquid Chromatography & Related*  
406 *Technologies*, 24, 251-263.

407

408 Ramos, F., Ribeiro, L., Saltão, R., Costa, J.M. & Silveira, M.I.N. (1999). Clembuterol em  
409 alimentos compostos para animais por cromatografia gasosa-espectrometria de massa. *Revista*  
410 *Portuguesa de Farmácia*, XLIX, 37 - 40.

411

412 Salquèbre, G., Bresson, M., Villain, M., Cirimele, V. & Kintz, P. (2006). Clenbuterol  
413 determination in calves hair by UPLC-MS–MS: Case report of a fraudulent use for cattle  
414 growth. *Journal of Analytical Toxicology*, 30, 1 – 5.

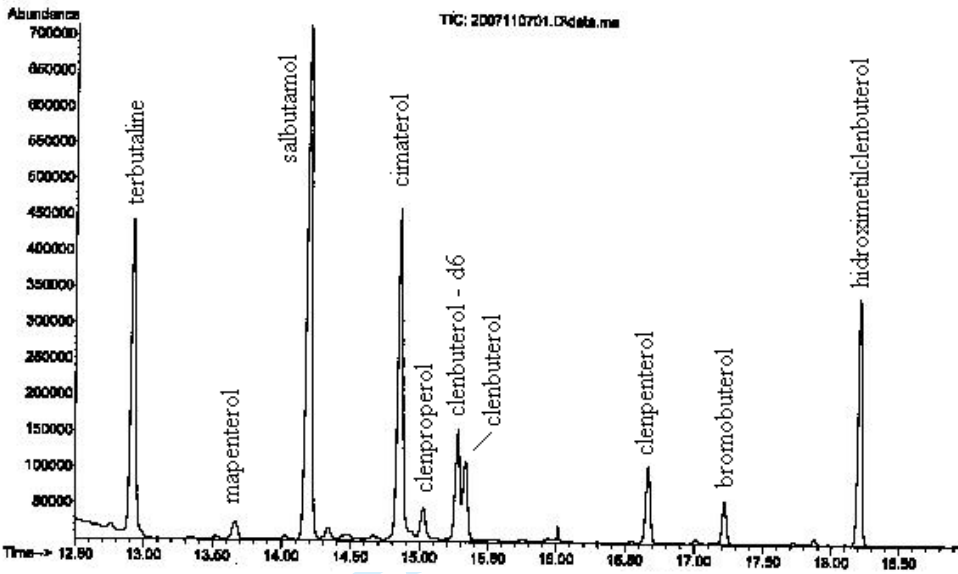
415

416 Sauer, M.J. & Anderson, S.P.L. (1994). *In vitro* and *in vivo* studies of drug residues  
417 accumulation in pigmented tissues. *Analyst*, 119, 2553 - 2556.

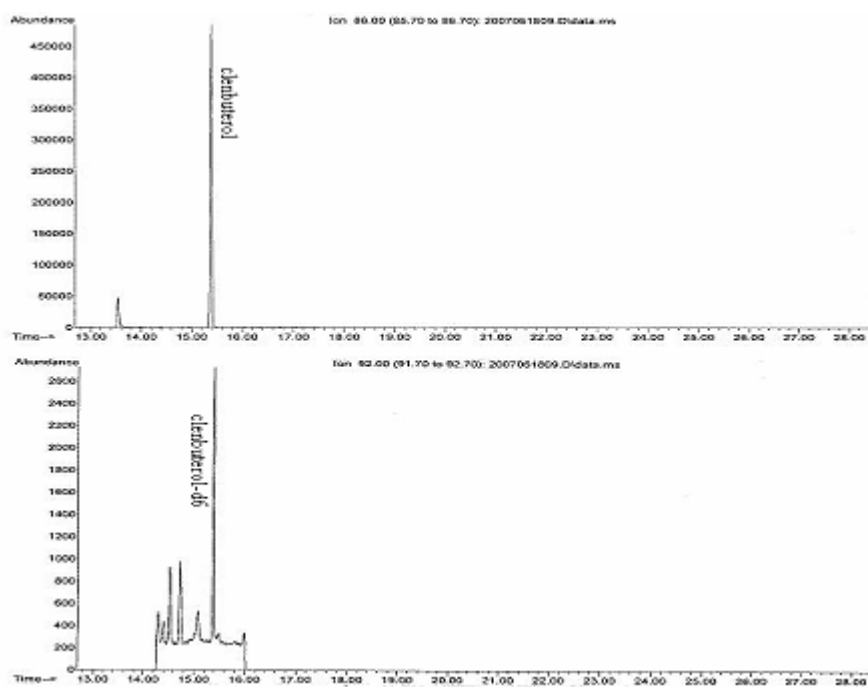
- 1  
2  
3 419 Shiu, T.C. & Chong, W.H. (2001). A cluster of clenbuterol poisoning associated with pork  
4  
5 420 and pig offal in Hong Kong. *Public Health Epidemiology Bulletin*, 10, 14–17.  
6  
7  
8  
9  
10  
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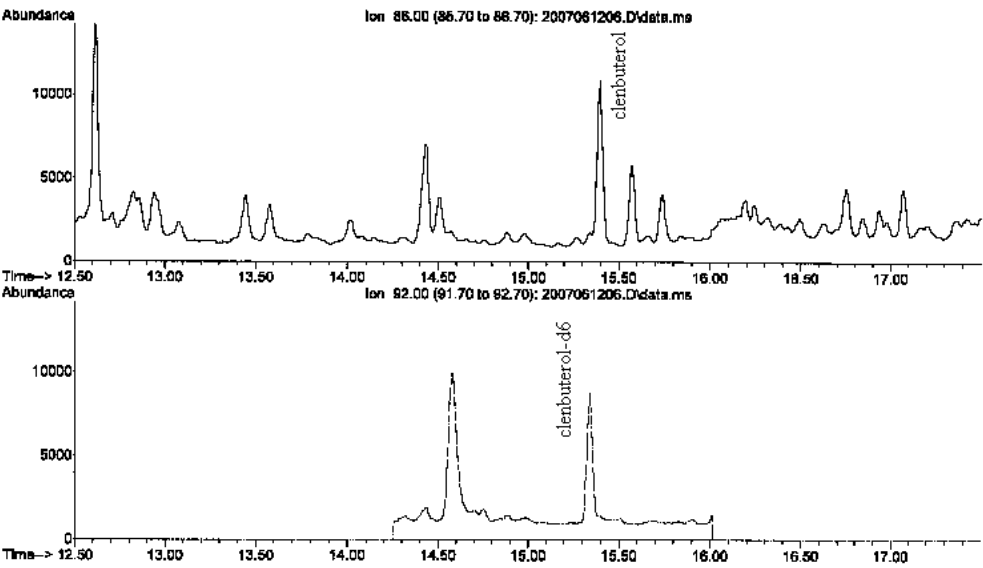
Figure 1 – TIC chromatogram of  $\beta$ -agonist standards



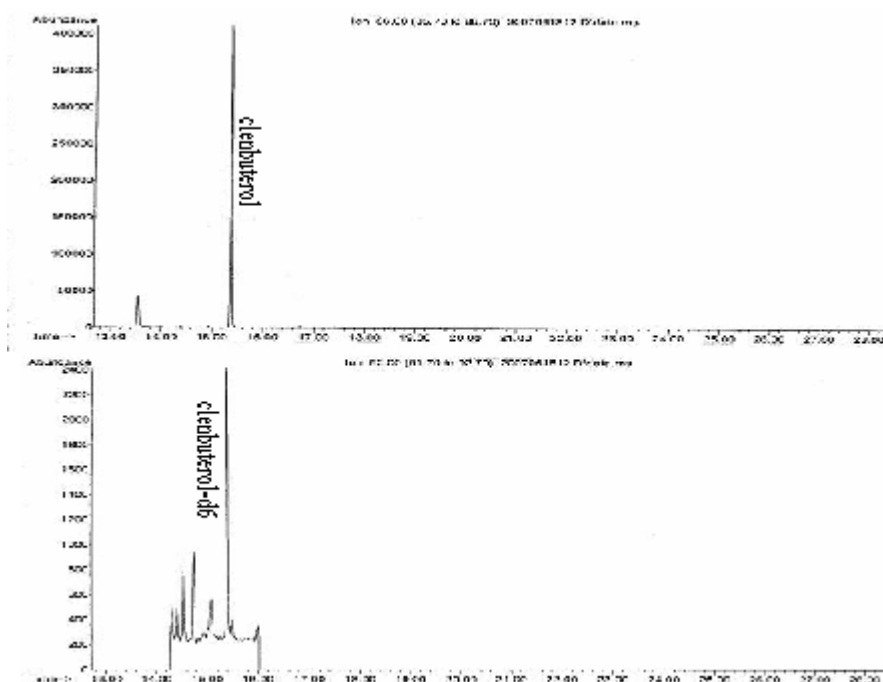
**Figure 2** – Chromatogram of a drinking water sample with 0.28 mg/l of clenbuterol



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



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





**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	-	-	-
<b>TOTAL</b>	<b>106 (15)</b>	<b>78 (0)</b>	<b>168 (24)</b>	<b>134 (2)</b>

**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

**Table 2** – Detected m/z ions for each  $\beta$ -agonist

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

**Table 3** – Performance characteristics of the method for clenbuterol

Matrices	CC $\alpha$	CC $\beta$	Accuracy (n=6)	Repeatability (n=6)	Reproducibility (n=18)
	[ $\mu\text{g/l}$ ] or [ $\mu\text{g/kg}$ ]	[ $\mu\text{g/l}$ ] or [ $\mu\text{g/kg}$ ]	[%]	[% (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