CONFERENCE PROCEEDINGS

Vol.3

1st Annual International Interdisciplinary Conference, *AIIC 2013*

24-26 April 2013, Azores Islands, Portugal

(Conference place: **University of the Azores, Ponta Delgada**)

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DETERMINATION OF CLENBUTEROL IN MEAT SAMPLES WITH ELISA AND GC-MS METHOD

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Abstract:
It is essential from the public health point of view, that the Macedonian regulatory agencies implement a monitoring surveillance program that not only uses preliminary screening methods for the determination of clenbuterol, but also uses confirmatory methods such as MS techniques. Thus, the aim of this work was to monitor the presence of clenbuterol residues in bovine meat muscle collected from the veterinarian inspectors, using screening and confirmatory methods. From 55 bovine meat samples analyzed by the ELISA test, 1 (1.8%) of presented clenbuterol residues was 1.19 µg/kg, 7 (12.7%) from 0.5 to 1.0 µg/kg, 13 (23.64%) from 0.1 to 0.5 µg/kg and 34 (61.82%) were below the limit of detection (LOD) of 0.1 µg/kg. In this study, from twenty one meat samples analyzed by GC-MS only one sample was confirmed positive for clenbuterol. The obtained results indicated that clenbuterol was still illegally used as a bovine growth promoter.

Key Words: Clenbuterol, residues, EU, ELISA, GC-MS, bovine meat

Introduction

Clenbuterol is used in human and veterinary medicine for its broncholytic and tocolytic action. In 5-to 10- fold the therapeutic doses, clenbuterol acts as a repartitioning agent to improve the performance of food-producing animals. Such a growth-promoting dose of clenbuterol influences animal growth and carcass composition by increased muscle mass and decreased fat mass, presumably through a direct effect of BAA on skeletal muscle and adipose tissue, indirect effect on many other tissues, or a combination of both. The monitoring of raw meat and poultry for drugs and chemical residues is necessary to ascertain that approved compounds are not misused and are not presenting a danger to consumers. Beta-agonists are a group of veterinary drugs that have been used illegally in some countries as they have a similar action to anabolic steroids in altering body composition (1). Beta-agonists act by impeding the uptake of adrenal hormones by nerve cells and stimulation of the cardiovascular system. When the treatment is prolonged, they also induce a redistribution of fat to muscle tissues (2). The beta-agonist clenbuterol has been implicated in several food poisoning cases in European countries (3). The use of b-agonists for growth promoting purposes in farm animals is not permitted in the European Union (EU), the United States of America (USA) and most other countries. The maximum residue limits (MRL) set by the EU are 0.1 µg/kg for muscle and 0.5 µg/kg liver and the MRL recommended by the WHO and the *Codex Alimentarius* are 0.2 µg/kg for muscle and 0.6 µg/kg for liver (4). Nevertheless, several cases of food poisoning between 1989 and 1992 were reported in Spain and France when liver containing high levels of clenbuterol were consumed (3). It is essential from the public health point of view, that the Macedonian regulatory agencies implement a monitoring surveillance program that only uses preliminary methods for the determination of clenbuterol. Although immunoassay techniques are very sensitive, the potential lack of specificity is a drawback that may result in false positives since other compounds of similar chemical structures present cross-reactivities (5-6). Therefore results of screening analysis should be confirmed by gas or liquid chromatography-mass spectrometric analysis (GC-MS) (7-9). The EU suggested the use of tandem mass spectrometry, also known as MS-MS, for the acquisition of

one parent ion and two product ions for the unambiguous GC-MS identification of the forbidden b-agonists (10). It is known that MS can provide significantly more informations than standard mass spectrometry (MS) in cases where the analytes exhibit a similar primary fragmentation. This is because product ion mass spectra can be significantly different even for similar analytes, thusproviding enhanced structural information with increasing selectivity of the analytes of interest (11). Additionally, MS-MS offers a number of advantages over single MS since the former provides discrimination between analytes on the basis of chromatographic properties (retention time), parent ion (MS1) and daughter ion (s) (MS2) (12). Thus, the aim of this work was to monitor the presence of clenbuterol residues in bovine meat muscle collected from the veterinarian inspectors by using screening and confirmatory methods.

Material And Methods

Reagents

Clenbuterol HCl, potassium dihydrogen phosphate, perchloric acid, bis(trimethylsilyl)-trifluoro-acetamide (BSTFA), formic acid, ethyl acetate, methanol and n-heptane were from Sigma (St. Louis, MO, USA). Trisbuffer (hidroximetyl- aminomethane) was from Bio-Rad (Richmond, CA, USA). The immunoenzymatic test was carried out by using an ELISA kit for clenbuterol and other β2-adrenergic agonists (r-Biopharm, Darmstadt, Germany). Sodium hydroxide and hydrochloric acid were from Merck Darmstadt, Germany). All solvents were of HPLC grade.

Samples

A total of 55 bovine meat samples were screened for the presence of clenbuterol as part of national monitoring residue plan. The samples were collected within period of 6 months as they were delivered by the authorised veterinary inspectors. Samples were kept frozen at -20 °C until analysis.

Clenbuterol analysis by ELISA (screening test)

A Ridascreen clenbuterol kit for ELISA was provided by R-biopharm (Darmstadt, Germany). Each kit contained a microtiter plate with 96 wells coated with antibodies to rabbit IgG, clenbuterol standard solutions (0, 100, 300, 900, 2700 and 8100 ng/L), peroxidase-conjugated clenbuterol, anti-clenbuterol antibody, substrate/chromogen solution, stop reagent, conjugate and antibody dilution buffer, and washing buffer. The extraction and clean-up procedures were those described by the ELISA kit manufacturer (r biopharm, Darmstadt, Germany). Minced meat samples (2 g) were homogenized for 30 min with 6 mL of methanol in 50 mL centrifuge tubes by using an Ultra-Turrax homogenizer, and centrifuged for 10 min at 4000 rpm at room temperature (20-25°C). Then 4 ml of methanolic solution was transferred into a new centrifugal vial and evaporated to dryness at 50°C. The dry residues were dissolved in 2 ml *n*-hexane than was added 1 ml sample dilution buffer and mixed vigorously for 30 sec. After mixing the sample was centrifuged for 10 min at 4000 rpm at room temperature (20-25°C). The aqueous (lower) phase was transferred into a new vial.
Two 20 µL aliquots of this extract were used for the ELISA test. Data were analyzed using a special software RIDAWIN ELISA (R-Biopharm, Darmstadt, Germany). The mean absorbance values obtained for the standards and the samples divided by the absorbance value of the first standard (zero standard) and multiplied by 100 was the % absorbance. The zero standard was thus made equal to 100% and the absorbance values were quoted in percentages. The method recovery was evaluated by fortifying negative meat samples with clenbuterol standards (0.1, 0.5, 1.0 and 5.0 µg/kg).

Validation of the ELISA method

The GC-MS method for determination of clenbuterol was validated for the recovery and repeatability. Clenbuterol-free meat samples were spiked at four concentration levels in the range from 0.1 to 5.0 µg/kg.

Clenbuterol analysis by GC-MS (confirmatory method)

Briefly, minced meat samples (1 g) were homogenized with 1 mL of tris-buffer (50 mM, pH 8.5). Then, 2 mL of n-heptane were added, vortexed for 2 min and centrifuged at 10000 rpm for 15 min at 4 °C. The upper organic layer was discarded and the extraction was repeated. Concentrated perchloric acid (0.5 mL) was added to the meat extract, mixed for 20 min and centrifuged for 15 min

at 10000 rpm at 4 °C. The supernatant was collected in a tube containing 300 mL of 1M NaOH and mixed for 5 min. Then, 4 mL of 500 mM KH_2PO_4-buffer (pH 3) were added, the pH was adjusted to pH 6 and the extract was stored at 4 °C for 1 h. Finally, the entire supernatant was purified by solid-phase extraction on a C18 column. The C18 column was first rinsed with 6 mL of 100% methanol. After the methanol was eluted, the column was equilibrated with 2 mL of 1 M phosphate (KH_2PO_4) buffer (pH 3). Then, the sample was loaded, and the column was rinsed with 2 mL of 1 M phosphate (KH_2PO_4) buffer (pH 6). The sample was eluted with 1.5 mL of 100% methanol and the eluate was evaporated to dryness under continuous flow of nitrogen at 45 to 50 °C.

Derivatization

The sample residue was dried before derivatization. Then 50 µL of 1% TMCS/BSTFA was added, vortex, mixed and kept and heated at 80 °C for 60 min. Once the derivatization process was completed, the solution was evaporated to dryness under continuous flow of nitrogen and reconstituted in 50 µL of toluene. Finally, 2 µL of this solution were injected into the gas chromatograph.The series standard solutions were derivatized simultaneously.

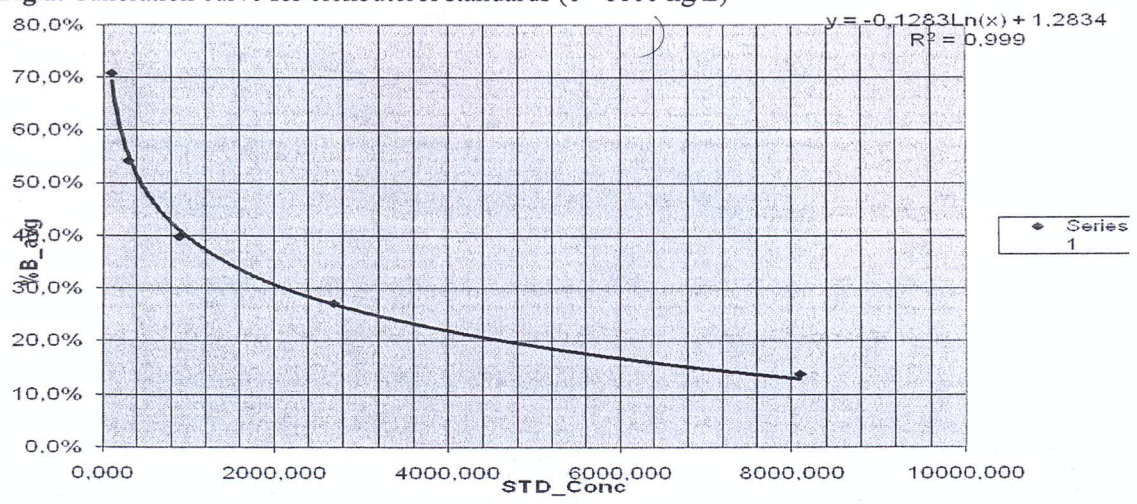
GC-MS conditions

GC-MS analysis was carried out with an Agilent 7890 A Gas chromatograph and Agilent 5975 C mass selective detector. GC was equipped with electronic pressure control unit, auto injector G 4513 A. Injection was done in splitless mode onto a 30m x 0.25 mm i.d. fused silica capillary column (HP-1 MS) with a 0.25 µm film thickness. The initial oven temperature was 70°C and was programmed to 200°C at 25°C/min, hold for 6 min and finally to 300°C at 25°C/min and hold for 5 min. Helium was used as carrier gas with a constant flow of 1.5 ml/min. Injection port and transfer zone temperatures were 250°C and 280°C, respectively. The mass spectrometer conditions were as follows: electron impact ionization voltage 70 eV for both SCAN and selected ion monitoring (SIM) mode.

Results

In this study a commercial ELISA kit was used for presumptive clenbuterol detection and quantification, however performance of the kit was not clearly described by the manufacturer and validation of the actual performance of the immunoassay with the sample matrix was carried out. The obtained calibration curves for the ELISA method in the range 100-8100 ng/L in linear and exponential form are presented on Figure 1. The curve equation y=-0.1283·Ln(x)+1.2834, where y was relative absorbance (%) and x was clenbuterol concentration in ng/L, was utilized for determining clenbuterol concentration in meat samples, obtaining high regression coefficient (R^2=0.999).

Fig 1. Calibration curve for clenbuterol standards (0 - 8100 ng/L)



The ELISA method performances were confirmed through the validation experiments for the recovery and repeatability (Table 1). Good recoveries of 72.0 to 84.2% were observed at levels of 0.1 to 5.0 µg/kg of clenbuterol fortification, followed by coefficient of variation lower than 6%.

Table 1. Validation procedure for meat samples fortified with clenbuterol standard

Validation parameter	No. of replicates	Spiked concentration (µg/kg)	Determinated concentration (µg/kg)	Mean recovery (%)	Coefficient of variation (%)
Recovery	6	0.10	0.072	72.0	0.82
	6	0.50	0.421	84.2	3.32
	6	1.00	0.799	79.9	1.50
	6	5.00	3.991	79.8	3.01
Repeatability	18	0.10	0.075	74.9	2.82
	18	0.50	0.389	77.8	1.42
	18	1.00	0.773	77.3	5.73
	18	5.00	4.015	80.3	1.60

55 meat samples were tested applying the validated screening method. Twenty one of them were considered to be potentially positive, and further were subjected to a confirmatory GC-MS method. On Figures 3 and 4 a typical selected ion monitoring (SIM) chromatogram for clenbuterol-free and positive meat sample, respectively, are presented.

Fig 3. Typical GC-MS chromatograms of TMS clenbuterol derivates – blank meat sample

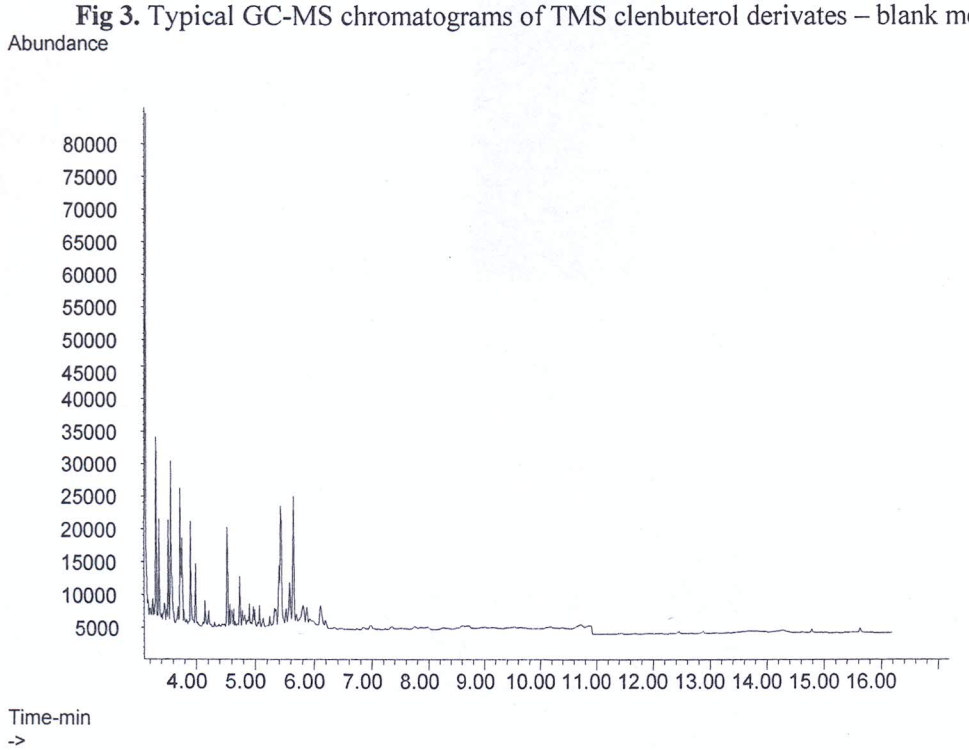
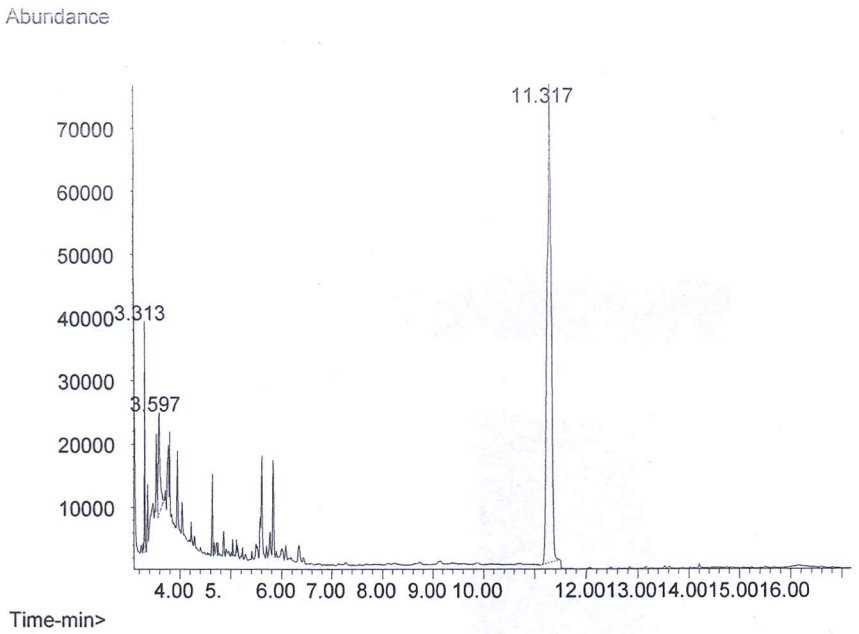
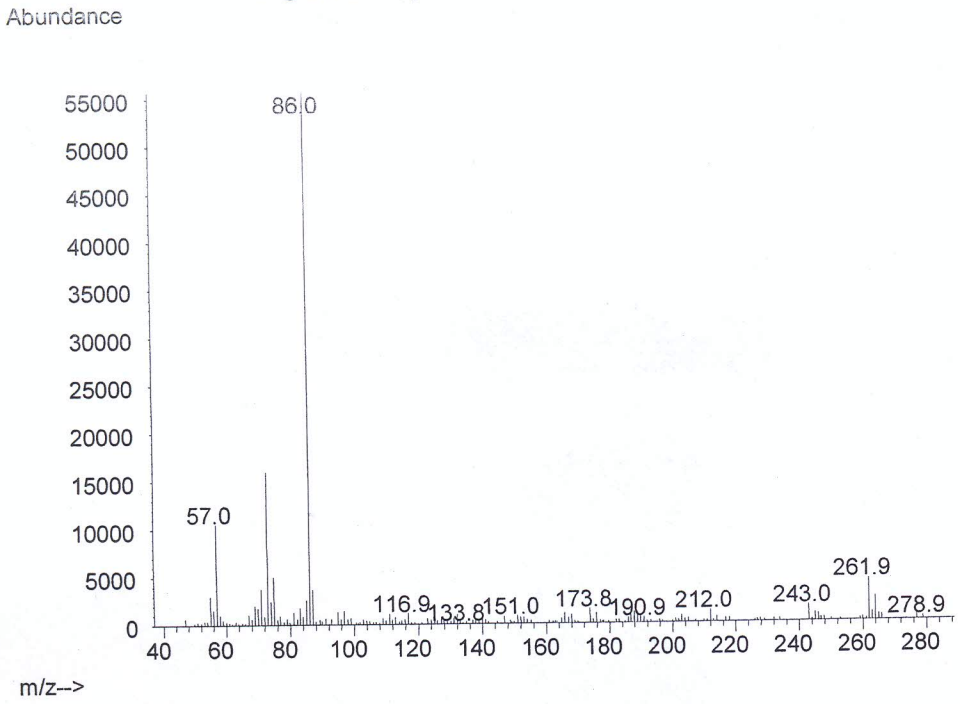


Fig 4. Typical GC-MS chromatograms of TMS clenbuterol derivates – positive meat sample



Clenbuterol was confirmed by comparison of the mass spectra of the reference standard solution of clenbuterol and analyzed samples, following the criteria laid down in Commission Decision (93/256/EEC). In electron impact mode the spectrum of the clenbuterol derivative, obtained by derivatisation with BSTFA +1 % TMCS shows the following diagnostic ions at *m/z*: 57, 86, 212, 243. (Figure 5).

Fig 5. Mass spectrum of clenbuterol mono-TMS derivate



Discussion

Determination of clenbuterol in bovine meat by ELISA (screening test)

The ELISA technique showed very high correlation between clenbuterol concentration (in µg/kg) and relative % of absorbance. The calibration curve constructed for the ELISA determinations followed a relationship with a highly significant ($P<0.01$) coefficient of multiple determination ($R^2=0.999$). The efficiency of clenbuterol extraction by the ELISA technique was evaluated by analyzing clenbuterol fortified meat samples. Good recoveries of 72.0 to 84.2% were observed at levels of 0.1 to 5.0 µg/kg of clenbuterol fortification (Table 1). The only study that reported clenbuterol recovery from meat products was much lower (63%) at a fortification level of 0.4 µg/kg and determined by LC-MS-MS (13). Similarly, clenbuterol recoveries of 44-75% from urine samples spiked with 0.2 to 1.5 µg/kg were detected by using ELISA (14). On the other hand, very good clenbuterol recoveries were (93-94%) reported previously for bovine liver samples fortified with 0.5 to 2.0 µg/kg and analyzed by GC-MS-MS (10) or those (95-113%) reported for liver samples fortified with 1.0-2.0 µg/kg and analyzed by ELISA (15). Therefore, for the quantitative determination of clenbuterol either in animal tissues or urine, it is important to perform recovery studies to ensure the accuracy of the method, since most important for the screening test is to obtain an acceptable low number of false negative results. A large number of false negative results were reported by the European residue control laboratories as a result of an interlaboratory study (16). On the other hand, other studies reported high rates of false positives in meat and urine samples, when the presumptive ELISA results were confirmed by GC-MS (17). It has been reported that commercial ELISA kits for the analysis of clenbuterol present cross reactivity with other β-agonists of similar structures (6).

Indeed, high cross reactivities were reported for the ELISA kit used in this study. Thus, confirmation of the ELISA test should always be carried out by chromatographic methods coupled to spectrometric methods (18). The limit of detection (LOD) for the ELISA test was at the lowest concentration of clenbuterol standard used in the standard curve (0.1 µg/kg), although the manufacturer reported a LOD of 0.3 µg/kg. The reproducibility of the technique was very good since coefficients of variation determined for clenbuterol standards and meat samples were lower than 0.6 and 2.5%, respectively. From 55 bovine meat samples analyzed by the ELISA test, 1 (1.8%) of presented clenbuterol residues was 1.19 µg/kg, 7 (12.7%) from 0.5 to 1.0 µg/kg, 13 (23.64%) from 0.1 to 0.5 µg/kg and 34 (61.82%) were below the limit of detection (LOD) of 0.1 µg/kg (Table 1). According to the maximum residue limited (MRL) recommended by the WHO and the *Codex Alimentarius* of 0.1 µg/kg, 21 (38.18%) samples would be considered presumptive positive samples. However, the samples analyzed in this study presented clenbuterol concentrations much lower than those reported for cases of food poisoning documented in several countries (19-22). Samples implicated in documented cases of food poisoning in different countries reported clenbuterol concentrations of 161 to 291 µg/kg (3), 375 to 500 µg/kg (22) and 19 to 5395 mg/kg (23) in veal liver or 450 µg/kg (21) and 800 to 7400 µg/kg (24) in meat samples. These documented cases presented clenbuterol concentrations much higher than the MRL set by the EC. For this reason, twenty one samples (38.18%) with the clenbuterol residues above LOD (0.1 µg/kg) found in this study were subjected to confirmatory analysis by GC-MS.

Identification of clenbuterol in bovine meat by GC-MS (confirmatory method)

Clenbuterol was derivatized with 1% TMCS/BSTFA, to form a mono-trimethyilsilyl (TMS) derivative which gave a typical most abundant precursor ion of m/z = 86. This fragment ion was selected as the primary ion, and it was utilized to confirm unequivocal presence of clenbuterol at the retention time of 11.317 min (Figure 4). The other identification product ions, when the clenbuterol-TMS was subjected to MS, were fragments with m/z = 57, 212, and 243. A clenbuterol-free meat sample analyzed by GC-MS showed the absence of the clenbuterol peak (Figure 3). On the other hand, a typical chromatogram of a clenbuterol fortified meat sample analyzed by GC-MS showed the clenbuterol peak at retention time of 11.317 ± 0.02 min (Figure 4).

In this study, the twenty one meat samples analyzed by GC-MS only one sample was confirmed positive for clenbuterol. For the confirmation of clenbuterol residues in the meat samples, the relative intensities of the product ions corresponded to those of the standard analyte (± 10%). According to the European Commission Decision 2002/657/EC (18), the relative abundances of all diagnostic ions monitored for the analyte should match those of the standard analyte or from spiked samples at comparable concentrations, preferably within a margin of ± 10%. The illegal use of clenbuterol as a growth promoting agent has been well documented to have occurred in Europe, Asia, and North and Central America, spanning a time frame of nearly a decade. Much of the detected illegal use of clenbuterol in Europe have occurred in spite of the European ban of all anabolic compounds in animal production. Extensive illegal use of clenbuterol and others b-agonists have not been halted by the ban, and the problem of unsafe residues in food remains. In fact, the most recent food poisoning outbreak caused by clenbuterol residues in meat was reported in Portugal in 2005 (20). Although samples were collected from the authorized veterinarian inspectors and mostly it comes from meat samples collected from different farms and slaughterhouses in Macedonia, but still remain questionable from meat samples who are imported from the different countries in Macedonia. Thus, this initial study should be useful as a first attempt to gain some insight in the illegal use of clenbuterol by some Macedonian meat producers.

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