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RESEARCH ARTICLE

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Detection of clenbuterol residues in beef sausages and its enantiomeric analysis using UHPLC–MS/MS: A risk of unintentional doping in sport field

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Abstract

Clenbuterol (Clb) can be present in Mexico often but not all over the world in animal tissues and organs, therefore, potentially is derived from animal sources as well. The aims of this study were to develop and validate a method for detecting traces of clenbuterol in beef sausages. A calibration curve showed linearity in the range of 20–500 pg ml^{-1}. The limit of detection (LOD) and lower limit of quantification (LLOQ) were 5 and 10 pg g^{-1}, respectively. The analyte recovery was from 95.70% to 100.40% with an intraday relative standard deviation (RSD%) of 0.99%–2.10% and an interday RSD% of 0.54%–2.34%, $R^2 = 0.9998$. The methodology developed was applied successfully in 15 samples of beef sausage, and 73.3% of the samples tested contained racemic clenbuterol in concentrations between 30 and 471 pg g^{-1}. The UHPLC–MS/MS method developed combines high sensitivity with good selectivity and short chromatographic run time. Additionally, the enantiomeric analysis of clenbuterol performed in beef sausages showed a 59% for *R*-(−)-Clb and 41% for *S*-(+)-Clb. The presence of clenbuterol in beef sausages could represent a risk of unintentional doping in sport field, because the clenbuterol is a banned substance included in the World Anti-Doping Agency's (WADA) list of prohibited substances.

KEYWORDS
clenbuterol, meat products, UHPLC–MS/MS, unintentional doping, veterinary residues

1 | INTRODUCTION

Meat products are an excellent source of protein, minerals, and vitamins.[1] Pork, chicken, turkey, and beef have also been widely used to elaborate gelatin products,[2] but the high fat content of meat products has been associated with increased risks of health problems such as obesity, hypertension, and cardiovascular disease.[3–6] Meat products are the most popular foods worldwide and, in 2019, sausages were the world's 479th most commercialized product with total trade amounting to $5.23B, though between 2018 and 2019, exports decreased by 0.085%, from $5.24B to $5.23B. These products represent 0.029% of total world trade.[7,8]

Accurate food labeling is important to ensure food safety, quality management along the supply chain, and support consumer choice. Substitute species, whether trace adventitious contaminants or products of economically motivated adulteration (EMA), can introduce toxins, pathogens, veterinary residues, or allergens into products. The raw material used in sausage production is usually obtained from registered slaughterhouses where animals are examined by veterinarians.[9] Only raw material from animals whose tissues have been approved for human consumption can be used to elaborate meat products.

The Food and Agricultural Organization of the United Nations (FAO) stipulates that the maximum residue limits (MRLs) for clenbuterol in muscle and fat is 0.2 μg kg^{-1}, whereas for liver and kidney, it is 0.6 μg kg^{-1},[10,11] and must be observed especially in countries that have registered clenbuterol as veterinary drug. These tissues must also be free of prion diseases (e.g., bovine spongiform encephalopathy [BSE]) that can affect animal and human health.[12] The hormones, antibiotics, and veterinary drugs present in meat must be evaluated[13–16] before consumption.

Clenbuterol ((4-amino-α-[(*tert*-butyl amino)methyl]-3,-4-dichlorobenzylalcohol; Clb) is used as a racemic mixture *R*-(−) and as *S*-(+)-clenbuterol. It is authorized as a bronchodilator and decongestant in clinical management in human and veterinary therapeutics. The World Anti-Doping Agency's (WADA) included the clenbuterol in the list of prohibited substances in sport.[17,18] In the latter, it is used with equines and companion animals, and as a tocolytic agent in bovines.[17–21] It has, however, also been used illegally to promote animal growth because it causes a significant increase in muscle mass and, simultaneously, decreases fat deposits.[22,23] For these reasons, its use is strictly prohibited in the European Union (since 1996), the United States (1991),[24] Mexico (2007),[25,26] and many other countries.[27] Because the *R*-(−) enantiomer stimulates β2-receptors, it is recognized as a eutomer.[28] The *S*-(+) enantiomer blocks the effect of β1-receptors,[29] can accumulate in various areas of an animal's body,[20,29] and can be transmitted to humans through contaminated foods. It has the potential to cause unintentional intoxication with clenbuterol.[22,23,30]

Several authors have reported that *S*-(+)-clenbuterol is retained in edible tissues of swine and poultry to a greater extent than levorotatory stereoisomers. A similar phenomenon is observed in broiled liver, as Smith[31] and Nielen et al[32] noted that *S*-(+)-clenbuterol was retained more than *R*-(−)-clenbuterol in pork, though the proportions were reversed in beef and lamb. Clenbuterol shows stability in boiling water at 100°C, but at a much higher temperature (260°C) loses this stability.[33,34]

The aims of this study were to (1) develop a rapid, sensitive, accurate method for identifying and quantifying clenbuterol in beef sausages, possibly due to the use of contaminated animal meat or gelatin; (2) demonstrate that the method is specific for the analysis of enantiomeric clenbuterol in samples of sausage; and (3) evaluate if the proportion of the clenbuterol enantiomers in samples of processed beef such as sausages remains as in unprocessed meat even after having gone through a cooking process.

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

Racemic clenbuterol was obtained from USP (Rockville MD, USA). The pure enantiomers *R*-(−)-clenbuterol (enantiomeric excess [*ee*] = 95.2%) and *S*-(+)-clenbuterol (*ee* = 99.4%), and $^{2}H_{9}$-clenbuterol were purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada) and used as internal standards (*IStd*) for UHPLC–MS/MS analysis. Methanol, acetonitrile, *tert*-butyl methyl ether, and formic acid were supplied by JT Baker (Palo Alto, CA, USA). All reagents used were of analytical grade. The solvents utilized were of HPLC-MS grade. Ultra-pure deionized water prepared using the Millipore® Direct Q3 water purification system (Merck-Millipore, Molsheim, France) was utilized throughout the experiment. A batch of organic beef sausages free of traces of clenbuterol was used as a reference.

2.2 | Test materials

All samples were purchased in 2019. For this study, 15 samples of beef sausage of different brands were purchased in national grocery store chains, and in regional and local grocery stores in different localities in Mexico. Approximately 200–300 g per sample was acquired. The purchasing strategy considered sausages labeled as containing beef only and products whose list of ingredients mentioned only one source animal species. After collection, all samples were transported to the laboratory under refrigeration conditions (<4°C).

2.3 | Preparation of standard solutions

A series of standard solutions were prepared by progressively eluting the stock solutions with methanol. Stock solutions of clenbuterol and $^{2}H_{9}$-clenbuterol were prepared in methanol at a concentration of 100 μg ml^{-1} and stored in amber, screw-cap glass vials at 2°C–8°C.

2.4 | UHPLC–MS/MS conditions

Clenbuterol was identified, quantified, and separated chromatographically in a TQMS XEVO (WATERS UHPLC–MS/MS) via electrospray ionization (ESI+) operated in positive mode and using an analytical column (Acquity UPLC BEH C18, 2.1 × 100 mm, 1.7 μm, WATERS, Milford, MA, USA). The chromatographic flow rate was 0.6 ml min^{-1}. The mobile phases were 0.01% formic acid in water as mobile phase A and mobile phase B was prepared with acetonitrile and 0.01% of formic acid. A gradient elution program was begun at 95% A for 0.6 min, followed by 10% A (0.6 ml min^{-1}) for 7.8 min. The phase was then set to initial conditions for 4.5–9.0 min. Ten microliters of extract was injected into the system at a run time of 5 min, and the temperature of the analytical column and autosampler was maintained at 40°C and 10°C, respectively. The spectrometric parameters were as follows: capillary voltage set at 1.2 kV for ESI+, a cone voltage of 18 V, and a collision energy (CE) setting of 16 eV. The ion source temperature was 150°C, and the desolvation temperature was 600°C. Diagnostic ions

were optimized by injecting the stock solutions for clenbuterol diluted in methanol. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. Data were acquired and processed using MassLink 4.1 (WATERS, Milford MA, USA). Clenbuterol's structure includes two chlorine atoms. The precursor ion, $[M + H]^+$ *m/z* 279, corresponds to a ^{37}Cl-^{35}Cl combination with an isotopic distribution pattern and relative intensities 9:6:1 *m/z* 277–279–281, consistent for two chlorine atoms. The diagnostic ions were *m/z* 279 → 205, 279 → 170, 279 → 132, 277 → 168, 277 → 140, and 277 → 132 for clenbuterol, and *m/z* 286 → 204 for $^2H_9$-clenbuterol.

2.5 | Sample preparation

Prior to the analysis, the packaging material was removed and discarded. The edible product was ground in a mortar to obtain a homogeneous mass. The samples were stored in clean polyethylene containers at −18°C until analysis. Previously, a set of solvent were evaluated, the optimum solvent for the clenbuterol extraction was the one that selectively extracted clenbuterol and did not extracted other interfering substances with this β-agonist, and all assays were performed in triplicate. In brief, 15 g of sausage were weighed in a 50-ml Falcon tube, and then, 50 μl of $^2H_9$-clenbuterol (50 ng ml^{-1}) as *IStd* and hexane (20 ml) were added. The mixture was shaken for 20 min at 100 rpm and centrifuged at 1459*g* (4500 rpm) (Rotina 38R, Hettich Zentrifugen) at 20°C for 10 min. The tube was collocated at −45°C for 15 min, and the organic layer was separated and discarded. At that point, 20 ml of HCl 1 M were added to the aqueous layer, shaken for 20 min at 100 rpm, and centrifuged at 1459*g* for 10 min. The aqueous layer was decanted in a new 50-ml Falcon tube and adjusted to pH 12 with potassium hydroxide (5 M). It was extracted with *tert*-butyl methyl ether (20 ml) by shaking gently for 20 min at 100 rpm and then centrifuged at 1459*g* (4500 rpm) for 10 min. The tube was collocated at −45°C for 10 min, and the aqueous layer was separated and discarded. The organic layer was transferred to a clean tube and evaporated to dryness under an N_2 gas stream at 38°C. For the analysis in UHPLC–MS/MS system, 100 μl of the water:acetonitrile (1:1 v/v) mobile phase was added for redissolution. Dilution was required for the batches of sausage with Clb concentrations outside the calibration limits.

2.6 | Enantiomeric analyses using UHPLC–MS/MS

The enantiomeric separation of *R*-(−) and *S*-(+)-Clb detected in the sausage samples by UHPLC–MS/MS was conducted using a HP1200 System (Agilent Technologies, Waldbronn, Germany) chromatograph equipped with a binary gradient pump and a Lux Cellulose-3 analytical column (250 × 4.6 mm, 5-μm particles) from Phenomenex (Torrance, CA, USA). This step was performed at 30°C, coupled to an MS/MS by ESI using a 6410 Triple Quad. Separation was achieved using water containing 10-mM ammonium formate (pH 10.3) (mobile phase A) and methanol containing 10-mM ammonium formate (mobile phase B). The flow rate was set at 550 μl min^{-1} under isocratic conditions (37.5% of A & 62.5% of B). The total run time was 16 min. The ion source was operated in ESI (+) mode at 350°C using an ionization voltage of 4000 V, a gas flow of 12 L min^{-1}, and a nebulizer at 35 psi. The fragmentor and collision voltages were optimized by infusing racemic clenbuterol standard solutions at a concentration of 1 μg ml^{-1} using the MS2 Scan and product ion scan, respectively. All analyses were performed in the MRM acquisition mode: *m/z* $[278 + H]^+$ for *R*-(−) and *S*-(+)-Clb, in the same way the transition *m/z* 286→204 was used for $^2H_9$-clenbuterol. For the latter, the typical isotopic distribution of ^{35}Cl (*m/z* 276) and ^{37}Cl (*m/z* 278) $[278 + H]^+$ of the one chlorine atom in the molecule structure was considered the diagnostic ion.

2.7 | Validation assay

The analytical method was validated according to Eurachem[35] that determines the limit of detection (LOD), the lower limit of quantification (LLOQ), accuracy, matrix effects (MEs), linearity, carry-over, repeatability, and specificity. Specificity was evaluated using a set of 10 different blank beef sausage samples to identify the absence of potential interfering species due biological matrix that might coelute close to the expected retention time of clenbuterol. Specificity was considered acceptable when the retention time of the target analyte showed that no interfering components were detected. Linearity was evaluated using a fresh calibration curve by spiking samples of organic beef sausages samples with *IStd* clenbuterol in quadruplicate at five working concentrations (20, 50, 100, 200, & 500 pg g^{-1}). The peak area ratio versus the final concentration of the analyte was used. The ME parameter was evaluated by comparing the peak area of the unextracted sample to that of the aqueous standards. Six beef sausage samples were fortified with 20, 100, and 500 pg g^{-1} of clenbuterol and spiked with $^2H_9$-Clb as the internal standard. The samples were prepared as described previously. ME was calculated to distinguish extraction efficiency from matrix-induced signal suppression/enhancement. The percentage of ME was calculated as: (peak area in urine-peak area in water)/peak area in water × 100.

Extraction recovery was evaluated in one set of target sausage samples fortified with clenbuterol *IStd* at 20, 100, and 500 pg g^{-1} ($n = 4$). Estimates of the recovery for the target analyte were made by comparing the peak area ratios of the fortified samples and the *IStd*, prior to extraction with samples in which the analyte had been added post-extraction. The *IStd* was added to both sets of samples post-extraction. LOD was estimated as the lowest concentration that resulted in a signal-to-noise ratio greater than 3:1, while LLOQ was the concentration of the analyte with a signal-to-noise ratio of 10:1. The precision and accuracy of this method were evaluated using a set of organic beef sausage reference samples spiked with 20, 100, and 500 pg g^{-1} of clenbuterol ($n = 4$).

Intra-assay precision and accuracy were calculated using four data points for each concentration obtained on the same day, whereas interassay precision and accuracy were calculated using the data obtained from three consecutive days for each clenbuterol concentration. Accuracy is expressed as the percentage of the measured

concentration to the nominal concentration. Precision is shown as a percentage of the relative standard deviation (RSD%). The carry-over effect was assessed by injecting three extracts of organic beef sausage reference samples successively after injection of a sample extract spiked with 500 pg g^{-1} of clenbuterol. This parameter was considered acceptable when it was ≤2.5% of the signal obtained at LLOQ.

3 | RESULTS AND DISCUSSION

3.1 | Assay characterization

The method employed for a quantitative determination of clenbuterol in beef sausage samples was comprehensively characterized and the results are summarized in Tables 1 and 2. A MRM method was used to analyze 10 samples of organic beef sausages by UHPLC–MS/MS. Standard solutions (1.0 μg ml^{-1}) of clenbuterol were infused directly into the mass spectrometer to obtain their precursor ions and characteristic product ions and to determine CE, collision gas (CAD), and collision exit potential (CXP). The two most abundant transitions were optimized in positive mode. In positive ion mode, the analyte produced the protonated molecule $[M + H]^+$, which was characterized in terms of its retention time and ion transitions. The most intense product ion, $[M + H]^+$ *m/z* 279, was used for quantification; the other was considered a confirmation ion.

3.1.1 | Linearity and specificity

The calibration curve was prepared in the working range of 20–500 pg g^{-1} and evaluated using UHPLC–MS/MS. Responses were considered linear when the correlation coefficient was ≥0.99. The linear equation was established as: $y = 0.0037x + 0.0151$ with $R^2 = 0.9998$ (Figure 1). These data suggest a linear relation with respect to concentration; a lack-of-fit test was performed. The result of this statistical test confirmed that linearity was within the range studied. Statistical data analyses are available in the supporting information.

Specificity was evaluated by comparing the retention times of the target analyte and $^2H_9$-clenbuterol (*IStd*). In a typical chromatogram, the retention time of clenbuterol in the sample was 2.08 min (Figure 2), and no interfering peaks were observed around the retention time in the 10 blank beef sausage samples tested. Results show that the method had high selectivity and can be utilized successfully to analyze the matrices in question.

3.1.2 | Precision and accuracy

Precision and accuracy were evaluated by intraday and interday analyses using samples of organic beef sausage at three concentrations fortified with clenbuterol (20, 100, and 500 pg g^{-1}). This procedure was replicated four times. The low RSD values of ≤2.5% show the precision of the method. The results of the relative percentage of standard deviations (RSD%) for the intraday and interday assays are shown in Table 1. In all cases, values were below 2.3%, demonstrating that the method developed for detecting clenbuterol in beef sausage by UHPLC–MS/MS provides good accuracy and precision, as well as reproducibility.

3.1.3 | Recovery

Extraction recovery was evaluated by comparing the peak areas derived from the corresponding subtraction using both sets of samples, with spiking after extraction. The recovery for all clenbuterol concentrations ranged from 95.70% to 100.04% (Table 2). These results are considered satisfactory. The clenbuterol extraction procedure did not require an extra sample clean-up step, so we were able to reduce sample preparation time and costs.

3.1.4 | Matrix effect

Because the presence of a co-extractive from the matrix could have influenced the ionization of the target analyte in the ESI mode, the ME sample was evaluated using an MS/MS (MRM mode) detector by comparing a standard in an organic solution to the matrix spiked with the standard (see the reference material). No significant enhancement or suppression differences were observed. Table 2 shows the slope ratios (ME) and ME% observed in the sausage matrices.

TABLE 2 Recovery % and the matrix effect observed in sausages spiked with clenbuterol

		Recovery %			ME
Nominal concentration (pg g^{-1})		Mean	SD	RSD (%)	%
Low	20	95.70	1.68	1.75	4.79
Middle	200	100.58	1.75	1.76	3.65
High	500	100.04	0.28	0.28	1.10

TABLE 1 Accuracy and intraday and interday precision for clenbuterol in the validation procedure

Nominal concentration (pg g^{-1})		Within-run (*intraday* assay $n = 4$)				Between-run (*interday* assay $n = 12$)			
		Mean (pg g^{-1})	SD	Accuracy (%)	Precision (RSD %)	Mean (pg g^{-1})	SD	Accuracy (%)	Precision (RSD %)
Low	20	19.67	0.31	93.37	2.10	18.852	0.44	94.26	2.34
Middle	100	101.48	1.31	101.48	1.29	100.15	1.42	100.15	1.42
High	500	498.19	4.91	99.64	0.99	499.00	2.71	99.80	0.54

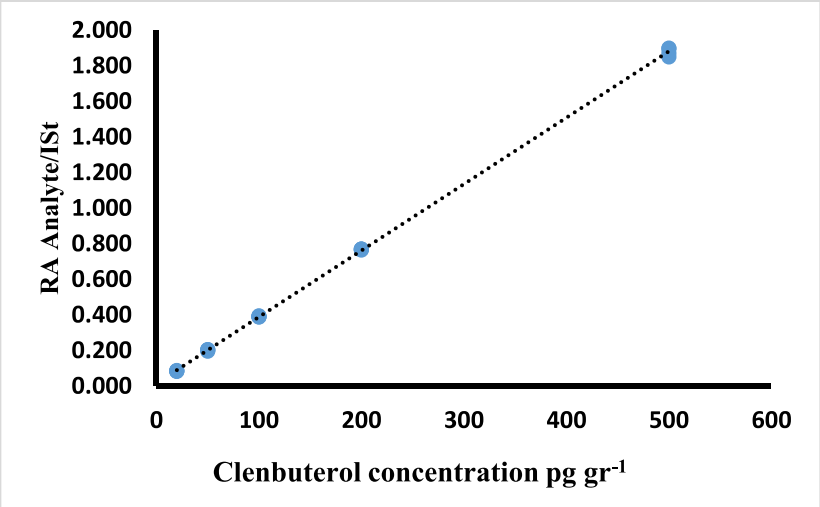

FIGURE 1 Calibration curve prepared by spiking samples of organic meat sausage with clenbuterol in quadruplicate at five concentrations: 20, 50, 100, 200, and 500 pg g^{-1} [Colour figure can be viewed at wileyonlinelibrary.com]

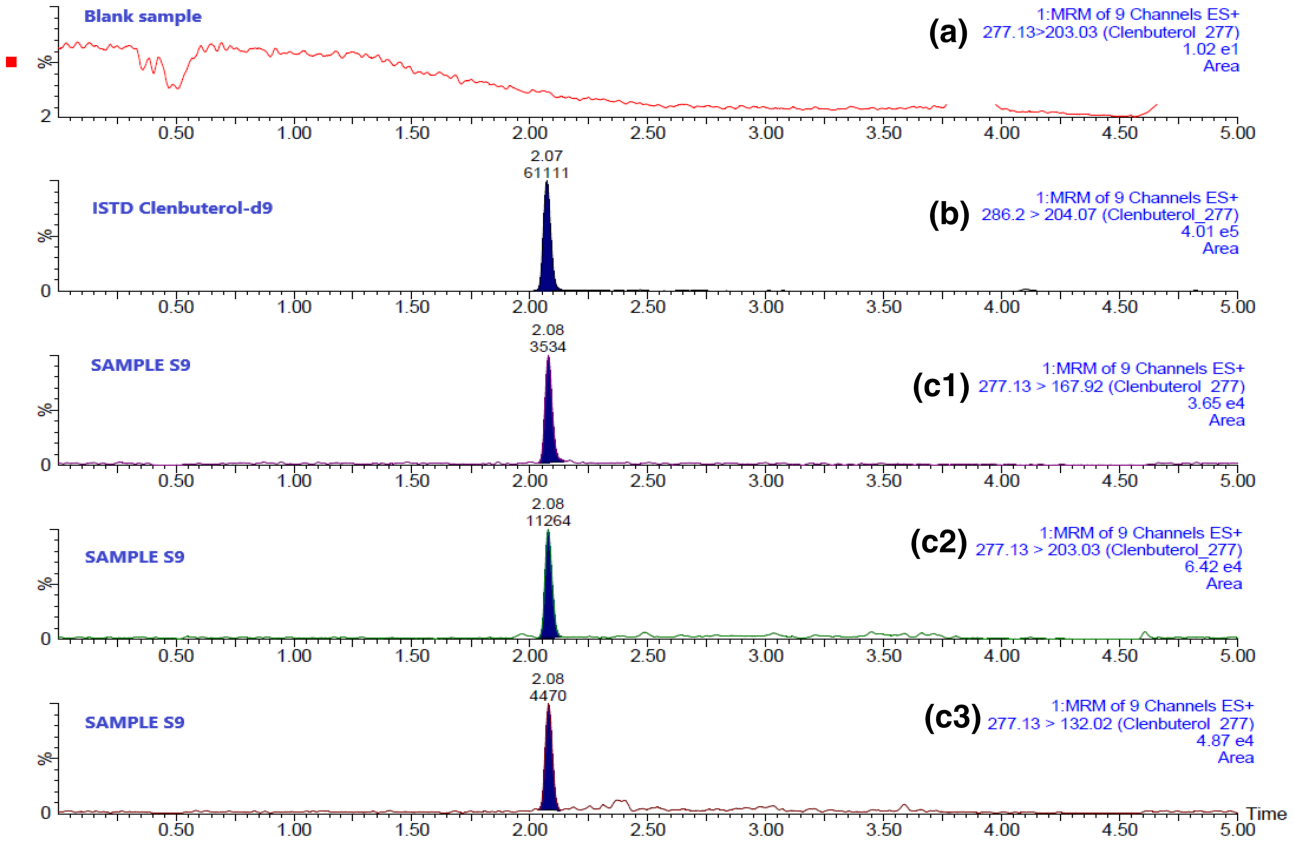

FIGURE 2 Extracted product ion mass chromatogram of sample S9. (**A**) Mass chromatogram of a target sausage sample, no endogenous interferences were detected; (**B**) a target sausage sample spiked with 0.10 pg ml^{-1} of $^{2}H_{9}$-clenbuterol; and (**C1**, **C2**, & **C3**) ion mass chromatograms for specific transitions observed in sample S9 (471 pg g^{-1} of clenbuterol were detected in this sample). No interference at retention times of clenbuterol enantiomers was observed [Colour figure can be viewed at wileyonlinelibrary.com]

3.1.5 | LOD and LLOQ

LOD and LLOQ were estimated using signal-to-noise ratios of 3:1 and 10:1, respectively. The experimental LOD for the method employed was defined as the lowest concentration of Clb spiked to the beef sausage reference. The experimental LOD (5 pg g^{-1}) showed a peak signal intensity threefold higher than the matrix without the target analyte. In the case of LLOQ (10 pg g^{-1}), we considered a peak signal intensity 10-fold higher than that of the target matrix.

3.1.6 | Carry-over

The chromatograms for the target samples assessed showed no noticeable carry-over (<0.1%), calculated as the ratio between the peak areas.

3.2 | Detection of clenbuterol in beef sausage samples

To verify the applicability of the method developed, 15 batches of sausage were prepared and tested following that procedure. Clenbuterol concentrations were evaluated, and an enantiomeric analysis was performed (*R*-(−)-Clb, *S*-(+)-Clb and *R*/*S* ratios). Data are shown in Table 3. The *R*/*S* ratio values indicated the existence of the accumulation effect of *R*-(−)-clenbuterol in beef muscle. Results showed that 73% of the samples tested contained racemic clenbuterol in concentrations of 30–471 pg g^{-1}. Although there are previous reports[36] regarding the analysis of beef sausages in order to detect the presence of clenbuterol, no results were obtained from samples with the presence from clenbuterol. This may possibly be due to the working range that was from 0.05 to 2 μg kg^{-1}. In our method, the LLOQ was 10 pg g^{-1}, which allows clenbuterol to be detected at trace levels. Only four of our sausage samples had brand names, and the other 11 had no trademark because they were drawn from bulk materials. Of the 15 samples, four (26%) showed clenbuterol concentrations of 30–90 pg g^{-1}, and seven (46%) had clenbuterol concentrations of 114–471 pg g^{-1}. No traces of clenbuterol were detected in four samples. In four different sausage brands, clenbuterol was identified at concentrations ranging from 90 to 471 pg g^{-1}. Clenbuterol was not detected in only in two of the sausage brands tested. Table 3 provides a detailed list of all the samples tested. The lowest amount was found in unbranded samples. This variation can be explained by the different batches of beef and raw material used in the manufacturing process of the sausages tested. It is important to note that the clenbuterol molecules showed stability during the cooking process (at 70°C), as some authors had shown previously.[33]

In Figure 2, the chromatograms for the specific transitions *m*/*z* 277 → 167, 277 → 203, and 277 → 132 detected by mass spectrometry (C1, C2, and C3) are shown. The molecular ion mass for regular clenbuterol was *m*/*z* 277, retention time 2.08. The regular clenbuterol detected are shown in C1, C2, and C3, as well as the characteristic transitions. The method developed herein is specific for detecting clenbuterol. Other ingredients present in the sausages were removed during extraction. This procedure helped obtain clear chromatograms free of interferences.

The clenbuterol concentrations detected were comparable with those described for beef muscle by Wang et al, and Parr et al, who found concentrations of 0.2–45.3 μg kg^{-1}. The FAO recommends an MRL of 0.2 μg kg^{-1} for muscle and fat.[10]

In several countries, including Mexico,[20] China,[27,30] Ecuador,[9] and Italy,[22] beef contaminated with clenbuterol in local markets has been detected, so parts of those contaminated animals may have been used in sausage production. The World Health Organization[11] and European Commission[37] define contaminants as any substance not intentionally added to food or feed for food-producing animals. As the present study shows, it is possible to detect residues of veterinary drugs, feed additives, and manufacturing, processing, and preparation operations that could be considered toxicologically significant, as in the case of clenbuterol.

TABLE 3 List of sausages samples tested clenbuterol average concentrations detected and enantiomeric analysis

			Enantiomeric analysis		
Sample code	Clenbuterol (pg g^{-1})	Place[a]	*R*-(−) %	*S*-(+) %	*R*/*S* ratio
S1	ND	Mexico State	—	—	—
S2	31	Mexico State	59.71	40.29	1.48
S3	60	Jalisco State	60.28	39.72	1.51
S4	62	Jalisco State	58.84	41.16	1.45
S5	80	Mexico City	61.81	38.19	1.61
S6	313	Mexico City	62.77	37.23	1.68
S7	ND	Guanajuato State	—	—	—
S8	114	Mexico State	60.34	39.66	1.52
S9	471	Mexico City	59.38	40.62	1.46
S10	44	Mexico State	61.39	38.61	1.59
S11	ND	Mexico City	—	—	—
S12	90	Guanajuato State	60.77	39.23	1.54
S13	120	Mexico State	58.60	41.40	1.41
S14	ND	Mexico City	—	—	—
S15	250	Mexico City	60.16	39.84	1.51

Abbreviation: ND, not detected.
[a]Estate of the Mexican Republic.

The results of our study reveal the presence of clenbuterol, a substance that could represent a health risk. In fact, the amount of clenbuterol found in some samples exceeds the maximum acceptable daily intake of 0–0.004 μg kg^{-1} of body weight for children.[10] In the case of a child weighing 15 kg, consuming 100 g of sausage containing a clenbuterol concentration of 471 pg g^{-1} could exceed the recommended acceptable daily intake of 0–0.004 μg kg^{-1} of body weight. Therefore, it could be toxic.[38]

To the best of our knowledge, no international regulations describe the potential risk of consuming clenbuterol in sausages. It is important to verify that all animals and animal products destined for human consumption are free of these substances. Some countries regulate the maximum amount of this β-agonist that beef may contain. The issues related to veterinary drug residues mentioned above originate from food-producing animals that received drug treatment due to illness or during feeding. After slaughter, detection of residual amounts of drugs or toxicologically active metabolites in the edible tissues (meat, organs) or other products (milk, eggs) of these food-producing animals is referred to as veterinary drug residue.[11]

Clenbuterol enantiomers have similar physicochemical properties but exhibit distinct biological behaviors, pharmacokinetic, pharmacodynamic, and toxicological activity, and biodegradation characteristics, as well as varied accumulation patterns in beef muscle.[17,30–32] In this regard, 11 samples of sausage with predetermined clenbuterol concentrations were included to determine their enantiomeric composition using the methodology described above. Though the time required for the enantiomeric analyses to identify these enantiomers was longer (16 min) (Figure 3), the resolution between peaks was better than previously reported by other authors at shorter times (5 min), and similar to that reported by Gausepohl and Blaschke at 12.5 min.[39]

Eleven meat sausages samples with predetermined clenbuterol concentrations were included for the determination of enantiomeric composition, using this methodology. Evaluation of the (*R*)- and (*S*)-

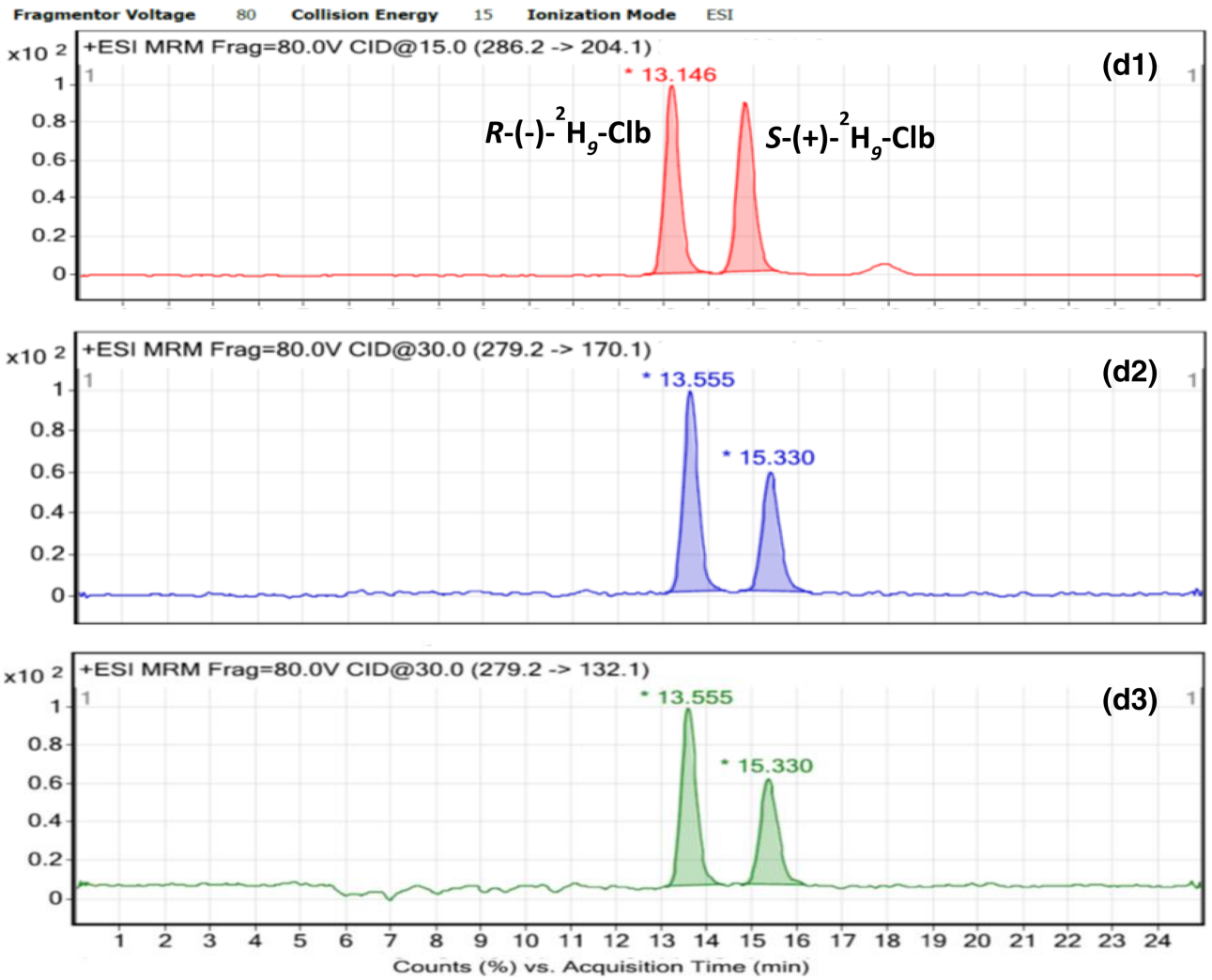

FIGURE 3 Representative enantioseparation ion chromatograms of the sausage tested. (**D1**) UHPLC–MS/MS transition *m/z* 286 → 204 and retention time for *R*-(−)-$^{2}H_{9}$-clenbuterol and *S*-(+)-$^{2}H_{9}$-clenbuterol (spiked at 0.5 ng ml^{-1} R_s = 1.3) and (**D2**) and (**D3**) UHPLC–MS/MS transitions *m/z* 279 → 170 and 279 → 132 observed for clenbuterol enantiomers in sample S9 [Colour figure can be viewed at wileyonlinelibrary.com]

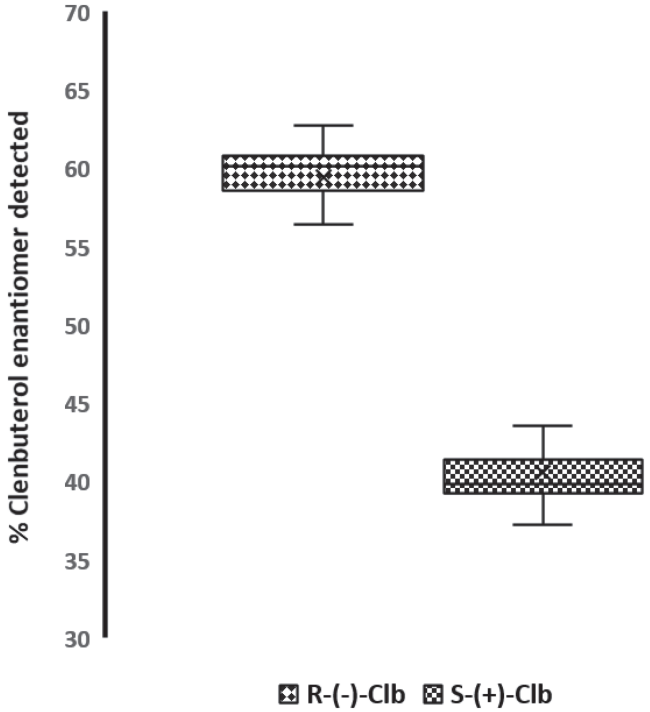

FIGURE 4 Boxplot of % *R*-(−) and *S*-(+)-clenbuterol in 11 sausage samples analyzed by LCMS/MS. A significant difference was observed for *R*-(−)-Clb and *S*-(+)-Clb (Wilcoxon test $P < 0.05$)

clenbuterol percentages and their *R*/*S* ratios showed them to be near 1.5 ($n = 11$) (Table 3). A nonparametric test assessed whether a significant statistical difference existed among the enantiomer percentages in these samples (Figure 4). A Wilcoxon test showed statistical significance, as the levogyre enantiomer was present at a higher percentage (59%) than the *S*-(+) enantiomer (41%). The statistical data analyses are available in the supporting information (Wilcoxon signed-rank test). Wang and cols identified that in unprocessed beef samples, the *R*-(−) enantiomer is found in greater proportion (56%). The clenbuterol is stable in boiling water at 100°C but degrades above 260°C. In this sense, we observed that enantiomeric proportion determination in beef sausages samples (Table 3) is similar to the reports of other authors for beef muscle. This proportion is very similar even after going through a cooking process during manufacturing.

Clenbuterol hydrochloride has a marked potency, an extended half-life of up to 40 h, and good absorption from the gastrointestinal tract with a bioavailability of 70%–80%.[24,40] This drug is approved for treating asthma in humans, available in 0.01- or 0.02-mg tablets, and liquid preparations with a daily regimen of 0.02–0.03 mg twice a day. Doses used by bodybuilders may range from 20 to 200 μg once or three times a day. In pediatric cases involving the intentional or unintentional ingestion of preparations with clenbuterol, hospitalization has been required.[39]

In summary, many of the sausage batches tested did contain clenbuterol, likely due to the low quality of the raw material used (bovine meat or contaminated gelatin) and/or deficient industrial quality processes during the elaboration of the sausages.

4 | CONCLUSIONS

In this study, a straightforward form of sample preparation based on liquid–liquid extraction combined with UHPLC–MS/MS was validated for detecting and quantifying clenbuterol in beef sausage. Chromatographic identification was achieved with a run time <5 min. Satisfactory validation was also obtained for specificity, linearity, recovery, precision, and the ME. The method developed was applied successfully to detect clenbuterol in sausage samples; additionally, the enantiomeric analysis showed that the *R*-(−)-Clb enantiomer is observed in a high percent than the *S*-(+)-Clb. For elite athletes, the presence of clenbuterol in beef sausages could represent a problem in anti-doping controls. An adverse analytical finding due to the presence of clenbuterol could be due to ingestion of contaminated meat products, gelatin, or sausages.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Udomkun P, Ilukor J, Mockshell J, et al. What are the key factors influencing consumers' preference and willingness to pay for meat products in Eastern DRC? *Food Sci Nutr*. 2018;6(8):2321-2336. doi:10.1002/fsn3.813
2. Domínguez R, Pateiro M, Agregán R, Lorenzo JM. Effect of the partial replacement of pork backfat by microencapsulated fish oil or mixed fish and olive oil on the quality of frankfurter type sausage. *J Food Sci Technol*. 2017;54(1):26-37. doi:10.1007/s13197-016-2405-7
3. Wu JHY, Micha R, Mozaffarian D. Dietary fats and cardiometabolic disease: mechanisms and effects on risk factors and outcomes. *Nat Rev Cardiol*. 2019;16(10):581-601. doi:10.1038/s41569-019-0206-1
4. Botez E, Nistor OV, Andronoiu DG, Mocanu GD, Ghinea IO. Meat products reformulation: nutritional benefits and effects on human health. In M. Chavarri-Hueda, ed. *Functional Food—Improve Health through Adequate Food*. 1st ed. Intech Open; 2017;167–184. doi:10.5772/intechopen.69118
5. National Heart Foundation of Australia (NHFA) A review of the relationship between dietary fat and overweight/obesity. 2003. Accessed August 18, 2021. https://resources.heartfoundation.org.au/images/uploads/publications/Dietary-fat-ovob-Review.pdf
6. Sacks FM, Lichtenstein AH, Wu JHY, et al. Dietary fats and cardiovascular disease: a presidential advisory from The American Heart Association. *Circulation*. 2017;136(3):1-23. doi:10.1161/CIR.0000000000000510

7. The Observatory of Economic Complexity (OEC) Sausages (HS: 1601) product trade, exporters, and importers 2021. Accessed May 18, 2021. https://oec.world/en/profile/hs92/sausages
8. Reportlinker Global bakery and sausage industry 2019. Accessed May 18, 2021. https://www.reportlinker.com/report/search?keywords=Sausage
9. Espinoza W, Vargas JP, Gualpa F, et al. Survey of clenbuterol in bovine muscle and liver in Ecuador. *Food Add Contam Part B*. 2020; 13(2):107-114. doi:10.1080/19393210.2020.1735534
10. Food and Agriculture Organizations of the United Nations-World Health Organizations, Codex Alimentarius Maximum residue limits (mrls) and risk management recommendations (RMRs) for residues of veterinary drugs in foods. FAO-CX/MRL 2–2018. Accessed September 29, 2021. http://www.fao.org/fao-who-codexalimentarius/codex-texts/maximum-residue-limits/en/
11. Food and Agriculture Organizations of the United Nations-World Health Organizations, Codex Alimentarius General standard for contaminants and toxins in food and feed. CXS-193-1995. Accessed September 29, 2021. http://www.fao.org/fao-who-codexalimentarius/codex-texts/list-standards/en/
12. United States Department of Agriculture, National Organic Standards Board (USDA-NOSB) Gelatin processing. Accessed June 20, 2021. https://www.ams.usda.gov/sites/default/files/media/Gelatin%20Fish%20TR%20Review.pdf
13. Food and Agriculture Organization of the United Nations Food safety and quality: detail edible gelatin monograph 1, additive-171-FAO. Accessed September 29, 2021. http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/detail/es/c/83/
14. Ramos F, Castilho MC, Silveira MIN. Estudo dos efeitos do processamento culinário sobre os resíduos de clembuterol e salbutamol em fígado de vitela. *Rev Port Farm*. 2000;3:117-123.
15. Heitzman RJ. Veterinary drug residues—residues in food producing animals and their products—reference materials and methods. In: CEC Report EUR 15127-EN, ed. *Commission of the European Communities*. 2nd ed. Blackwell Scientific Publications; 1994.
16. Md Shohel R, Seung YL, Hae JK, Sun JH. Reducing veterinary drug residues in animal products: a review. *Food Sci Anim Resour*. 2019; 39(5):687-703. doi:10.5851/kosfa.2019.e65
17. Parr MK, Blokland MH, Liebetrau F, et al. Distiction of clenbuterol intake from drug or contaminated food of animal origin in a controlled administration trial the potential of enantiomeric separation for doping control analysis. *Food Addit Contam Part A*. 2017;34(4):525-535. doi:10.1080/19440049.2016.1242169
18. Hernández MD, Villaseñor A, Sofia-Piña S, et al. Evaluation of R-(−) and S-(+) clenbuterol enantiomers during a doping cycle or continuous ingestion of contaminated meat using chiral liquid chromatogaphy by LC-TQ-MS. *Drug Test Anal*. 2019;11(8):1238-1247. doi:10.1002/dta.2612
19. Velasco-Bejarano B, Bautista J, Noguez MO, Camacho E, Rodríguez ME, Rodríguez L. Resolution of R-(−) and S-(+)-enantiomers of clenbuterol in pharmaceutical preparations and black-marked products using liquid chromatography-tandem mass spectrometry. *Drug Test Anal*. 2017;9(11-12):1738-1743. doi:10.1002/dta.2294
20. Velasco-Bejarano B, Bautista J, Rodríguez ME, López-Arellano R, Arreguín-Espinosa R, Velasco-Carrillo R. Quantification and stereochemical composition of *R*-(−) and *S*-(+) clenbuterol enantiomers in bovine urine by liquid chromatography-tandem mass spectrometry. *J Anal Toxicol*. 2020;44(3):237-244. doi:10.1093/jat/bkz087
21. Cristino A, Ramos F, Noronha da Silveira MI. Control of the illegal use of clenbuterol in bovine production. *J Pharm Biomed Anal*. 2003;32(2): 311-316. doi:10.1016/S0731-7085(03)00073-6
22. Sporano V, Grasso L, Esposito M, Oliviero G. Clenbuterol residues in non-liver containing meat as a cause of collective food poisoning. *Vet Hum Toxicol*. 1998;40(3):141-143.
23. Martínez-Navarro JT. Food poisoning related to consumption of illicit b-agonist in liver. *The Lancet*. 1990;336(8726):1311. doi:10.1016/0140-6736(90)92990-y
24. Brett J, Dawson AH, Brown JA. Clenbuterol toxicity: a NSW Poisons Information Centre experience. *Med J Aus*. 2014;200(4):219-221. doi:10.5694/mja13.10982
25. Norma Oficial Mexicana de Emergencia Especificaciones técnicas para el control del uso de beta-agonistas en los animales. Diario Oficial de la Federación. 01 de marzo de 2002, NOM-EM-015-ZOO-2002. Accessed June 13, 2021. http://dof.gob.mx/nota_detalle.php?codigo=720442&fecha=02/09/2002
26. Marban-Arcos SE, López-Jiménez FA. Intoxicación alimentaria por clenbuterol. *Rev Sanidad Militar México*. 2011;65:121-124.
27. Wang ZL, Zhang JL, Zhang N, Zhao Y. Mass spectrometric analysis of residual clenbuterol enantiomers in swine, beef and lamb meat by liquid chromatography tandem mass spectrometry. *Anal Methods*. 2016;8(20):4127-4133. doi:10.1039/C6AY00606J
28. Waldeck B, Widmark E. Steric aspects of agonism and antagonism at beta-adrenoceptors-experiments with the enantiomers of clenbuterol. *Acta Pharmacol Toxicol*. 1985;56(3):221-227. doi:10.1111/j.1600-0773.1985.tb01279.x
29. Ruffolo RR. Chirality in α- and β-adrenoreceptor agonists and antagonist. *Tetrahedron*. 1991;47(48):9953-9980. doi:10.1016/S0040-4020(01)96045-4
30. Pulce C, Lamaison D, Keck G, Bostvironnois C, Nicolas J, Descotes J. Collective human food poisonings by clenbuterol residues in veal liver. *Vet Hum Toxicol*. 1991;33(5):480-481.
31. Smith DJ. Total radioactive residues and clenbuterol residues in edible tissues and the stereochemical composition of clenbuterol in livers of broilers after exposure to three levels of dietary [^{14}C] clenbuterol HCl and three preslaughter withdrawal periods. *J Anim Sci*. 1998;76(12):3043-3053. doi:10.2527/1998.76123043x
32. Nielen MWF, Lasaroms JJP, Essers ML, et al. Multiresidue analysis of beta-agonist in bovine and porcine urine, feed and hair using liquid chromatography electrospray ionization tandem mass spectrometry. *Anal Bioanal Chem*. 2008;391(1):199-210. doi:10.1007/s00216-007-1760-7
33. Rose MD, Shearer G, Farrington WHH. The effect of cooking on veterinary drug residues in food: clenbuterol. *Food Addit Contam*. 1995;12(1):67-76. doi:10.1080/02652039509374280
34. Pinheiro I, Jesuino B, Barbosa J, Ferreira H, Ramos F, Matos MS. Clenbuterol storage stability in the bovine urine and liver samples used for European official control in the Azores islands (Portugal). *J Agric Food Chem*. 2009;57(3):910-914. doi:10.1021/jf802995e
35. Eurachem. The fitness for purpose of analytical methods. *A Laboratory Guide to Method Validation and Related Topics*, 2 Ed., Eurachem; 2014. Accessed June 18, 2021. https://www.eurachem.org/images/stories/Guides/pdf/MV_guide_2nd_ed_EN.pdf
36. Jiebo M, Shujing L, Hong X, Wei L, Sun T. Rapid analysis of three b-agonist residues in food of animal origin by automate on-line solid-phase extraction coupled to liquid chromatography and tandem centermass spectrometry. *J Sep Sci*. 2014;37:2431-2438. doi:10.1002/jssc.201400442
37. Official Journal of the European Union COMMISSION REGULATION (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Accessed September 29, 2021. https://ec.europa.eu/health/sites/default/files/files/eudralex/vol-5/reg_2010_37/reg_2010_37_en.pdf

38. Bonney CF, Hatten B, Wang GS. Toxicity from unintentional pediatric ingestion of a performance-enhancing drug: a case report with review of clenbuterol toxicity and treatment. *J Emerg Med*. 2019;9(4):105-108. doi:10.1016/j.jemermed.2019.06.016
39. Gausepohl C, Blaschke G. Stereoselective determination of clenbuterol in human urine by capillary electrophoresis. *J Chromatogr B*. 1998;713(2):443-446. doi:10.1016/S0378-4347(98)00178-9
40. Woolum J, Mancuso N, Rutter PW, Baum RA, Akpunonu P. Chomping at the bit: a descriptive report on pediatric clenbuterol ingestion. *J Pharm Pract*. 2019;33(3):386-389. doi:10.1177/0897190018823114

SUPPORTING INFORMATION

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