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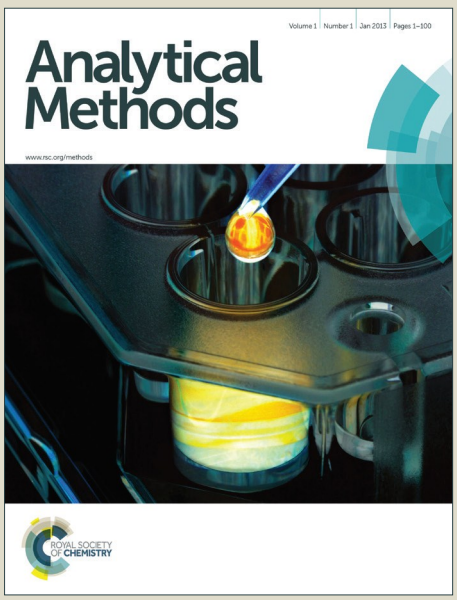

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ARTICLE

Mass Spectrometric Analysis of Residual Clenbuterol Enantiomers in Swine, Beef and Lamb meat by Liquid Chromatography Tandem Mass Spectrometry

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A method for determining the ratio of clenbuterol enantiomers (R/S ratio) in edible meat by High performance liquid chromatography-mass spectrometry has been developed and validated, and clenbuterol chiral determination in beef and lamb meat were first reported. The practical procedure involves acid extraction followed by one solid-phase clean-up step with weak cation-exchange resins. $^{2}H_{9}$-(+/-)-clenbuterol was used as an internal standard. R-(-)-clenbuterol and S-(+)-clenbuterol were completely baseline separated and detected by HPLC-MS/MS. The limit of detection (LOD) for enantiomers of clenbuterol was 0.1μg/kg and the limit of quantification (LOQ) was 0.2μg/kg. The spiked R-(-)-clenbuterol or S-(+)-clenbuterol in the blank sample was stable and the conversion between R-(-)-clenbuterol and S-(+)-clenbuterol was not detected in the sample procedure. The R/S ratio of racemicclenbuterol spiked in the blank sample ranged between 0.92 to 1.06 at high and middle and the average value of LOQ concentration was 1.00. The R/S ratio in a total of 94 clenbuterol residues in edible meat samples collected from the market was calculated using the current method, and the R/S ratio in swine meat (5 samples) ranged from 0.65 to 0.77; the R/S ratio in beef meat (73 samples) ranged from 0.89 to 2.42; the R/S ratio in lamb meat (16 samples) ranged from 1.45 to 2.82. This is the first time that the different R/S ratios in farm animal meat have been calculated and reported. The effect of R/S ratio differences in food safety, bioactivity and doping control test is still unknown but is an area of future investigation. This technique can be considered as a starting point for the distribution and pharmacokinetics of clenbuterol enantiomers in livestock.

1 Introduction

The sympathomimetic agent, clenbuterol (4-amino-a-[(tertbutylamino) methyl]-3,5-dichlorobenzyl alcohol) which exhibits β_2-agonist activities is applied as a bronchodilatory, tocolytic, and mucolytic agent in the symptomatic treatment of respiratory diseases in both humans and animals.[1-4]It also promotes the growth of muscular tissue and the reduction of body fat; hence, it is illicitly used as a nutrient-repartitioning agent in meat-producing animals. Because of the anabolic effects, clenbuterol is prohibited by the World Anti-Doping Agency. [5] The use of clenbuterol as a growth promoter and its accumulation in foodstuffs can have adverse effects on public health, such as accidental human poisoning associated with consumption of meat products and liver contaminated with clenbuterol residues.[6-8] However, although clenbuterol is banned for growth promotion in food-producing animals in the European Union and most other countries such as China, Canada, and the USA, some feed manufactures and farmers still illegally use this drug as a feed additive to increase profits.[9] Thus, it may be added to feed in dosages 5-10 times higher than the therapeutic dose for economic benefit.[10-11] Clenbuterol is a potent chiral β_2-adrenergic agonist. While R-(-)-clenbuterol form is active at the β_2-adrenergic receptor, the S-(+)-clenbuterol form is inactive. The accumulation of clenbuterol in edible tissues of swine was found to be enantiomerically enriched towards the dextrorotatory (+)-clenbuterol in a time-dependent manner, that is with longer drug cessation periods, the S-(+)-clenbuterol is enriched in tissues while R-(-)-clenbuterol is depleted.[12-13]Additionally, the broilers were the same as the swine. Up to now, there is no published data with regard to the R/S ratio in beef and lamb meat. Based on our market data in 2012-2014, clenbuterol residues were found at a higher rate in beef and lamb than that in swine. Therefore, it is important to investigate the distribution and residues of R-(-)-clenbuterol and S-(+)-clenbuterol in beef and lamb meat.

To date, several analytical methods have been reported for analysis of clenbuterol enantiomers. For example, liquid chromatography-mass spectrometry has been used to separate clenbuterol enantiomers in human urine during urine sample analysis. [14-15] Capillary zone electrophoresis has also been widely used to separate clenbuterol enantiomers, [16-19] but the quantitative accuracy and sensitivity in these

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publications are not suitable for clenbuterol residues in meat. Gas chromatography (GC)-MS methods have been also used for the analysis of chiral clenbuterol residues in swine; [12] however, GC methods for β-agonists requires sample derivatization due to their high polarity and low volatility, which is a time-consuming (up to 50 mins when clenbuterol enantiomers were nearly baseline separated), tedious, laborious, and an expensive process. Another reason for the difficulty in analysis of clenbuterol enantiomers in meat is the stability of R-(-)-clenbuterol and S-(+)-clenbuterol during the sample procedure. Therefore, development of specific and sensitive analytical methods, including both sample preparation and determination would be critical to regulate the occurrence of the enantiomers and levels of recognized chiral contaminants in animal products for risk assessment and assurance of consumer protection.

In this study, the aim was to develop an innovative, simple, rapid, accurate, and sensitive direct chiral LC-MS/MS method for determining the R/S ratio in animal meat. To the best of our knowledge, clenbuterol chiral determination in beef and lamb meat has not been previously reported.

2 Experimental

2.1 Chemicals and reagents

All solvents were of HPLC grade obtained from Dima Tech, Inc. (Richmond Hill, CA, USA). Sodium hydroxide, ammonia and perchloric acid were purchased from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). Ammonium formate was purchased from Sigma (Beijing, China). R-(-)-clenbuterol and S-(+)-clenbuterol (p.a.) were purchased from Toronto Research Chemicals Inc. (North York, Canada). Clenbuterol (p.a.) was purchased from Shanghai ANPL Scientific Instrument Co., Ltd (Shanghai, China) and the deuterated internal standard, $^{2}H_{9}$-(+/-)-clenbuterol (p.a.) was from the National Measurement Institute of Australia (Sydney, Australia). The standard stock solutions of these compounds were prepared in methanol (1mg/mL of their free bases). Oasis-MCX (150 mg, 6cc) solid-phase extraction (SPE) cartridges were provided by Waters (Shanghai, China). Water was of MilliQ grade.

2.2 Sample preparation

Liver (swine) and muscle tissues (swine, beef and lamb) were thawed at 4℃ and a portion was diced into small pieces. In a 50ml plastic centrifuge tube, 10g tissue sample was spiked with 20ng of the internal standard (ISTD) $^{2}H_{9}$-clenbuterol by adding 0.2mL of a working solution containing 0.1ng/μL $^{2}H_{9}$-clenbuterol before the addition of 10mL of 20% methanol aqueous solution. The mixture was homogenized at 12000rpm using the IKA T18 homogenizer (Germany). Purified water (4mL) was used to clean the dispersion tools (S18N-19G) 2 times. Two mL perchloric acid was added to the sample tube and the sample was shaken for 30s and then centrifuged at 10000rpm at 10℃. The separated aqueous layer was transferred to a fresh test tube. After adding 1.4mL of 12mol/L NaOH, the pH value of aqueous solution was adjusted between 4 and 5. The aqueous layer was transferred to a MCX solid phase extraction cartridge, preconditioned with 6mL of methanolic ammonia (5%), 6mL of methanol, and 6mL of water. After the aqueous layer had passed the adsorber resin by gravity flow, 6mL of methanol was used to clean the adsorber resin and elution was accomplished with 6mL of methanolic ammonia (5%). The elution was then collected in a glass tube and evaporated to dryness under 65℃. Subsequently, the dry residue was reconstituted in 0.2mL of methanol and passed through a regenerated cellulose syringe filter (0.22-μm poresize). 10μL of this reconstituted residue was injected into the LC-MS/MS system. The samples used for experiments were obtained from the supermarkets and farmers markets around the major cities of china during 2012-2014. We collected the samples from different markets (at least 1 supermarket and 1 farmers market) in each city. Most of the positive cases were from the farmers markets.

2.3 Instrument parameters

All analyses were performed on an Agilent triple-quadrupole 6410 LC-MSD equipped with mass spectrometry detector of electrospray interface. The LC was equipped with a Chirobiotic T analytical column (2.1×150 mm, particle size 5 mm; Sigma, Taufkirchen, Germany) and the separation was carried out at constant temperature (30℃). The mobile phase was 10 mM/L methanol ammonium formate. The flow rate was set at 0.4mL/min. Multiple reaction monitoring (MRM) mode was used to detect the 2 analytes in positive ionization mode. The spray voltage was set at 3.5 kV and the ion source was operated at 300℃. Nitrogen was also used as nebulizing and drying gas and the pressure was set at 40psi.

2.4. Method validation

2.4.1 Optimal separation

The parameters used to optimize the separation were analyzed at buffer concentration: 5, 10, 15, 20mMol/L, flow rate: 0.3, 0.4, 0.5ml/min and column temperature: 10, 20, 30, 40℃.

2.4.2 LOD and LOQ

The limits of detection (LODs) were defined as the lowest level that could be calculated with the diagnostic ions present at a signal-to-noise (S/N) ratio greater than 3 and the R/S ratio of clenbuterol enantiomers equal to nearly 1. The limits of quantitation (LOQs) were defined as the lowest level that could be calculated with the diagnostic ions present at a S/N ratio greater than 10 and the R/S ratio of clenbuterol enantiomers equal to 1.

2.4.3 Linearity and stability

Ten blank tissue samples were spiked with R-(-)-clenbuterol and S-(+)-clenbuterol at five different concentrations of 0.2, 0.4, 0.6, 1, 4μg/kg. Eight blank tissue samples were spiked with the racemic clenbuterol at eight different concentrations of 0.2, 0.4, 1, 5, 10, 15, 25, 50μg/kg.

2.4.4 Recoveries

The recoveries of R-(-)-clenbuterol and S-(+)-clenbuterol were determined at a concentration of 0.4, 2 and 20μg/kg

(racemic clenbuterol spiked in blank sample). Six blank tissue specimens were spiked with racemic clenbuterol prior to sample preparation and the other 6 blank tissue samples were extracted according to the method described above, followed by the addition of the target substances into the final extracts. The ISTD solution was spiked into the extract in both sets of samples before evaporation. Recoveries were calculated by comparison of mean peak area ratios of clenbuterol enantiomers and the ISTD spiked samples before and after extraction. Three different tissue specimens, swine, beef and lamb were declared negative after routine analysis and were prepared as described above in order to study the interfering peaks in the selected ion or transition chromatograms at the expected retention times of the analytes.

2.5 R/S ratios in swine beef and lamb

Six different clenbuterol residues specimens from swine, beef and lamb that were declared positive after routine analysis were prepared as described above, in order to study the typical R/S ratio in different animals. The reproducibility and within-laboratory reproducibility were measured on the same 18 fortified blank tissue samples (n=6 replicates per concentration level and analyzed in three independent analytical runs) and expressed by the variation at concentrations of 0.4, 2 and 20μg/kg. Two replicates of 94 clenbuterol residues specimens (5 from swine, 73 from beef and 16 from lamb) were prepared as described above, in order to study the distribution of R/S ratios in real meat samples.

3 Results and discussion

Nowadays, the detection of clenbuterol residues in animal tissue samples is straightforward and sensitive. Compared with the flow rate and column temperature, the buffer concentration has a great impact on the peak resolution. By decreasing the buffer concentration from 20 to 5mMol/L, the separation of enantiomers was improved significantly, but the ionic strength decreased rapidly at 5mMol/L. Furthermore, the buffer concentration and flow rate determining the retention time and the column temperature can also affect the peak resolution, but it is not an important factor in separation. Optimal separation was obtained using 10mMol/L of ammonium formate at 0.4ml/min and 30℃.

Method performance was evaluated in terms of limit of detection, limit of quantification, linearity, precision, and recovery. Applying peak area ratios of both enantiomers and their corresponding ISTD, a valid comparison of corrected peak areas of the R-(-)-clenbuterol and S-(+)-clenbuterol was used to determine the R/S ratio.

3.1. Validation

By means of chiral HPLC, the enantiomers of clenbuterol were baseline separated under ESI-compatible conditions using methanol/ammonium formate and the analytes were detected with tandem mass spectrometry. Using 9-fold deuterated clenbuterol, adequate sample preparation and analysis were strictly controlled and ion suppression/enhancement effects were eliminated to a great extent. The specificity for the R-(-)-clenbuterol and S-(+)-clenbuterol was also satisfactory as no interfering substances were found at the appropriate retention times (Fig.1).

3.1.1 The LOD and LOQ

The LOD of the method was estimated with 100pg/g via signal-to-noise ratio (>3). The LOQ of the method was estimated with 200pg/g via signal-to-noise ratio (>10). The stability of R-(-)-clenbuterol or S-(+)-clenbuterol in the sample procedure was evaluated ranging from 0.2 to 4 and there was no conversion between the R-(-)-clenbuterol or S-(+)-clenbuterol in the sample preparation.

3.1.2 The linearity and stability of R-(-)-clenbuterol and S-(+)-clenbuterol

The calibration graphs of R-(-)-clenbuterol, S-(+)-clenbuterol and racemic clenbuterol were obtained by plotting the peak area ratio of the quantitative ion pair of each standard to internal standard versus clenbuterol concentration of 0.2–4μg/kg (R-(-)-clenbuterol), 0.2-4 μg/kg (S-(+)-clenbuterol) and 0.2-50 μg/kg (racemic clenbuterol). The results of the linearity were shown in Table 1. The correlation coefficients (r^2) of the calibration curves were above 0.9958.

With regard to the quantitative linearity, the slope of the R-(-)-clenbuterol and S-(+)-clenbuterol in the racemic clenbuterol was almost equal. In contrast, the R-(-)-clenbuterol or S-(+)-clenbuterol spiked separately in tissue blanks and the slope of the R-(-)-clenbuterol was clearly different with the S-(+)-clenbuterol(Table 1). As R-(-)-clenbuterol and S-(+)-clenbuterol were both present together in tissue samples, applying the racemic clenbuterol to quantify the R-(-)-clenbuterol or S-(+)-clenbuterol was demonstrated as a valid method of quantification. There was very little change in the ratio of $^2H_9$-R-(-)-clenbuterol and $^2H_9$-S-(+)-clenbuterol in the sample operation; however, the R/S ratio was stable and the average value was 1.00 (concentration of racemic clenbuterol was between 0.2-50μg/kg). Both R-(-)-clenbuterol with the $^2H_9$-R-(-)- clenbuterol decreased or increased together compared with S-(+)-clenbuterol. The same phenomenon was also observed with S-(+)-clenbuterol.

3.1.3 Recovery

Recoveries of clenbuterol enantiomers were tested with blank tissue samples spiked with three different concentrations: 0.4, 2 and 20μg/kg. The results were shown in Table 2. The mean recoveries and reproducibility varied from 60.4 to 92.2% for R-(-)-clenbuterol and 59.0 to 91.5% for S-(+)-clenbuterol. The R/S ratio was between 0.95 to 1.04 (intraday) and 0.92 to 1.06 (interday) for R-(-)-clenbuterol and S-(+)-clenbuterol, respectively. The recoveries and coefficients of variation for clenbuterol enantiomers indicated that they were improved compared with the previously developed methods for swine samples by GC-MS, [12] possibly due to the use of a more sensitive instrument for quantification in the present study.

It was important to notice the column robustness especially in sequence injections. When over more than 300 injections, the separation of clenbuterol enantiomers may be not completely and the column should be cleaned in order to achieve the perfect separation and accuracy of quantification.

3.2 R/S ratio in swine tissue

One liver and 4 muscle tissue samples were tested by the present method (the concentration of racemic clenbuterol ranged from 40.2 to 7.1μg/kg). The results shown in Fig.2 demonstrate that the R/S ratios were consistently less than 1. The value of liver was 0.73 while the rest were below 0.70 with a minimum value of 0.65 and an average value of 0.68. In Fig.2, the data were arranged in accordance with the concentration of clenbuterol residues from high to low, the R/S ratio decreased slowly along with the degressive concentration, which indicated the existence of the accumulation effect of S-(+)-clenbuterol in swine muscle. Similar results were generated in the pharmacokinetics and metabolism of clenbuterol in swine.[12] However, the average value (0.68, R/S ratio=2:3) was significantly different compared with the reported data (R/S ratio=1:3). [12] In this study, the oxizolidin-3-one derivative of clenbuterol formed by derivatization with phosgene was analyzed by gas chromatography-mass spectrometry in the selected-ion mode, This was an indirect method of quantification in comparison with the present method and the separation of R-(-)-clenbuterol or S-(+)-clenbuterol were baseline separated. Additionally, the stereochemical composition ratio and concentration ratio of R-(-)-clenbuterol and S-(+)-clenbuterol were unique in this study. As shown in earlier studies, therapeutic clenbuterol administration results in the urinary elimination of both optical isomers in approximately equal amounts within a period of 18 h. [16] Therefore, the R/S ratios in swine muscle may provide an alternative approach to demonstrate that the clenbuterol-positive in doping control test was probably due to swine meat contamination when the R-(-)-/S-(+)-clenbuterol ratio was consistently less than 1.

3.3 R/S ratio in beef and lamb muscle

In total, 73 beef muscle samples were analyzed by the current method (the concentration of racemic clenbuterol ranged from 45.3 to 0.2μg/kg) as shown in Fig. 2. The R/S ratios consistently varied from 0.89 to 2.42 with an average value of 1.33. The progressive increase of R/S ratio indicated the existence of the accumulation effect of R-(-)-clenbuterol in beef muscle. Fig.2 indicated the analytical results of 16 lamb muscle samples using the current method (the concentration of racemic clenbuterol ranged from 33.8 to 0.3μg/kg). The R/S ratios varied from 1.45 to 2.82 with an average value of 2.13. In Fig.2, the R/S ratios progressively increased with decreasing concentration, which indicated the existence of the accumulation effect of R-(-)-clenbuterol in lamb muscle. This result of lamb and beef muscle was significantly different from that of swine muscle.

There have been several well-known toxic events caused by the ingestion of liver and meat containing clenbuterol residues in Spain (Asturias, León, Palencia, and Catalonia), [20-21] France (Lyon), [22] Italy (Caserta and Assisi), [23-24] and Portugal (Ourém,Lousã, Ovar and Peso da Régua). [6] Amongst these incidents, one resulted from lamb consumption and other 9 originated from beef consumption. In addition, the therapeutic activity of clenbuterol has been shown to be associated with the R-(–) enantiomer with little or no adrenoceptor stimulation attributed to the S-(+) enantiomer. Enantiomers have similar physicochemical properties but exhibit different biological behavior, pharmacodynamic, pharmacokinetic, toxicological activity, persistence, and biodegradation characteristics. As a consequence of this stereoselectivity in the accumulation of beef and lamb muscle, consumers poisoned after the consumption of clenbuterol-contaminated beef and lamb muscle had to have eaten at most a therapeutic dose of clenbuterol. Nevertheless, animals receiving illegal clenbuterol have been administered much large amounts of clenbuterol than those in experimental or oral therapeutic dose. Data from animals treated illegally with clenbuterol indicated that edible tissues may contain fairly high tissue concentrations of parent clenbuterol. Therefore, the clenbutreol residues in beef and lamb muscle may induce more documented clenbuterol toxic occurrences in humans after consumption of contaminated livestock.

The pharmacokinetics and metabolism of clenbuterol have been investigated in a number of species including man, rat, dog, and rabbit. Therefore, by applying the current method, the tissue distribution and residues of R-(-)-clenbuterol and S-(+)-clenbuterol in livestock will be an area of further investigation.

3.4 Typical animal tissue samples analysis

The mean reproducibility of swine liver, beef muscle sample (n = 1) and lamb muscle sample (n = 1) were calculated by each six replicates, as shown in Table 3. The average R/S ratio of swine liver was 0.73 and the value ranged from 0.68 to 0.75. The relative standard deviation (RSD) of intradays and interdays was 2.5% and 2.32%, respectively. The average R/S ratio of beef muscle was 1.26 and the value ranged from 1.13 to 1.41. The relative standard deviation (RSD) of intradays and interdays was 6.28% and 4.67%, respectively. The average R/S ratio of lamb muscle was 2.34 and the value ranged from 2.22 to 2.40. The relative standard deviations (RSDs) of intradays and interdays were 5.79% and 5.40%, respectively. The above-mentioned data showed that the real sample could be quantified by the present method when the R/S ratio was larger or less than 1. As different R/S ratio was present in different animal specimens, the enantiomeric depletion of clenbuterol was also different in animal tissue after ingestion, which was considered as a potential tool to support anti-doping authorities in deciding whether or not a suspension was required for an athlete whose doping control sample was judged as clenbuterol-positive. Otherwise, the US Food and Drug Administration and EU have established guidelines to favor the production of single enantiomer drugs. The current study could be helpful to build the standard guidelines for clenbuterol enantiomer levels in meat.

4 Conclusions

By using the chiral HPLC, the buffer concentration, flow rate and column temperature were examined and optimized, and a fast and sensitive LC/MS/MS method was also developed for

the determination of the clenbuterol enantiomers in animal tissues. The method was successfully applied to the determination of clenbuterol enantiomers in a large number of clenbuterol residue samples, and the R/S ratios in different farm animal muscles were also investigated. The current approach has thus far proved to be optimal, and the ratio of clenbuterol enantiomers in edible tissue was influenced substantially from the time course of drug administrated to the animal. The depletion of enantiomers was also significantly distinct in swine, beef and lamb. The S-(+)-clenbuterol accumulation in swine muscle and liver was time-dependent. In contrast, the R-(-)-clenbuterol accumulated in beef and lamb muscle, specifically in lamb. The value of R/S ratio in beef and lamb meat was reported and calculated for the first time in this study.

In summary, the methodology reported in this study could be considered as a starting point for the follow-up studies, which may involve the analysis of the distribution and enantiomeric clenbuterol composition in livestock. The difference of R/S ratio in swine, beef and lamb should have a positive impact on food safety and doping control tests.

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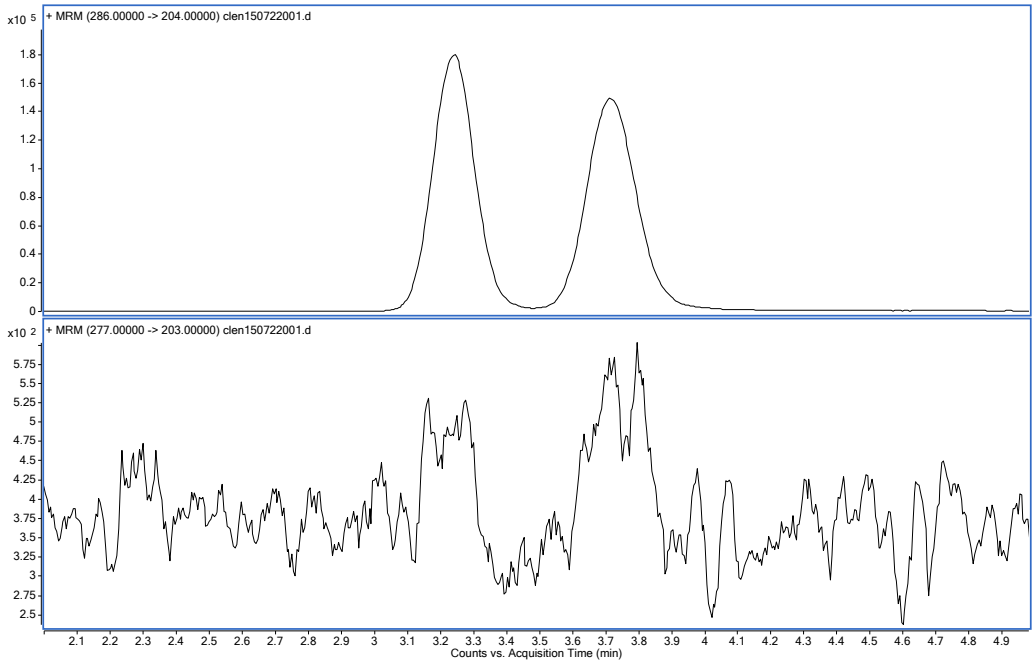
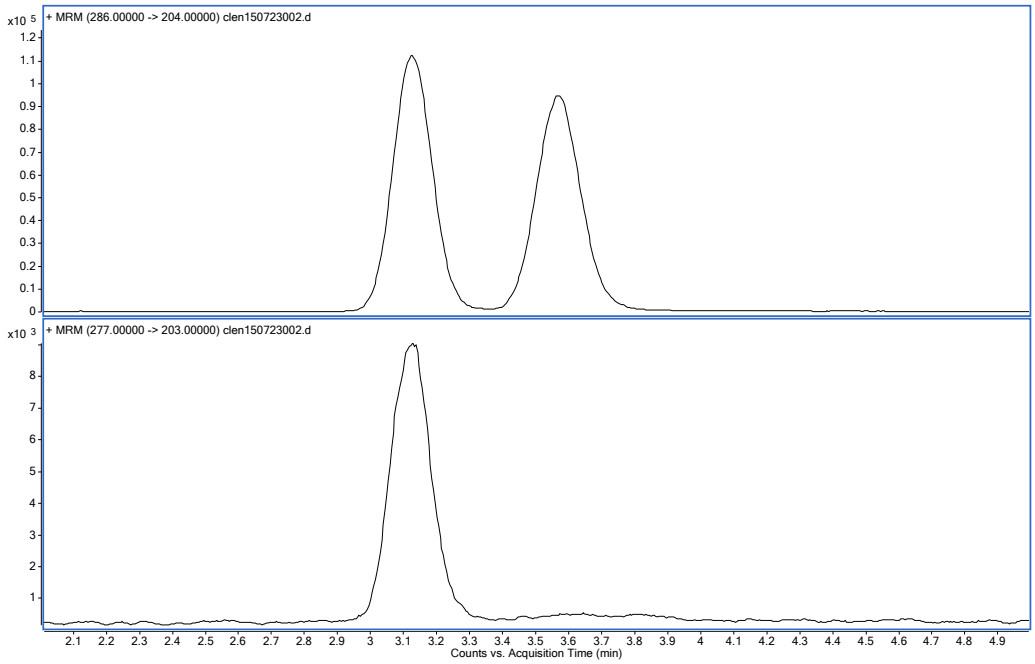
Fig.1 Extracted ion pairs chromatograms [m/z 277→203 (lower pane) and m/z 286→ 204 (top pane) for $^{2}H_{9}$-clenbuterol (ISTD)]. The peaks represent R-(-)-clenbuterol at 3.2 min and S-(+)-clenbuterol at 3.8 min.

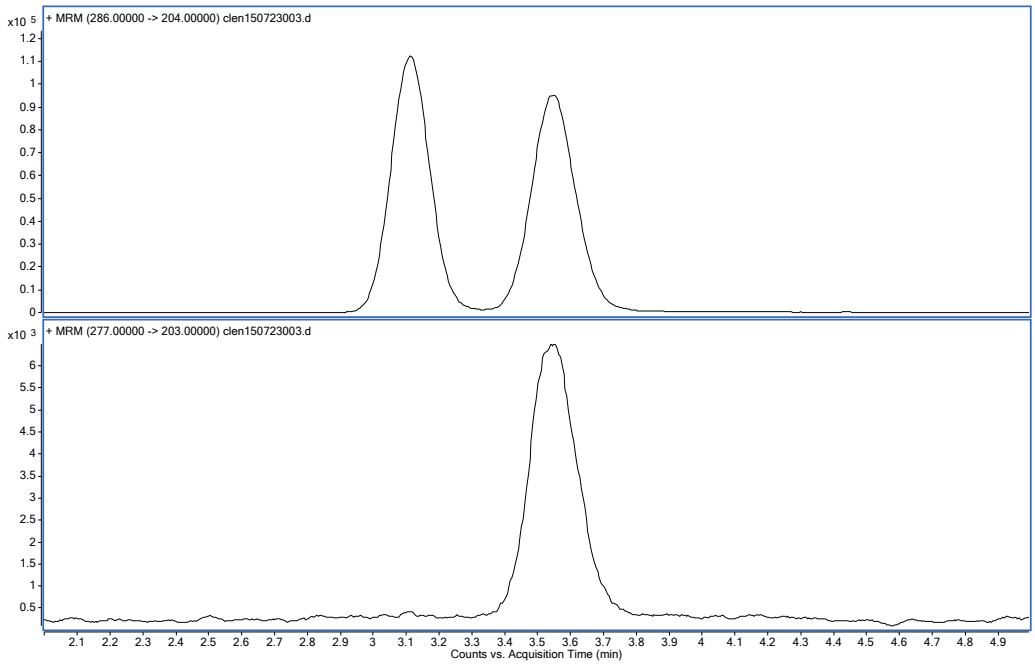
a)Blank meat sample containing the ISTD only



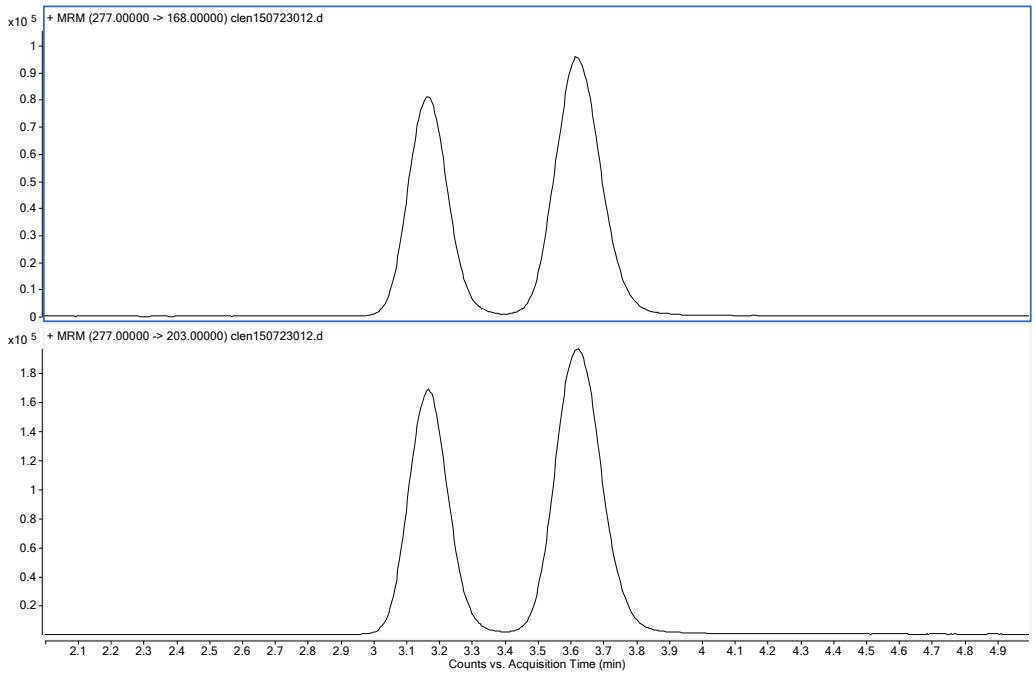
(b) meat sample spiked with 100 pg/g of R- (-)-clenbuterol (QC);



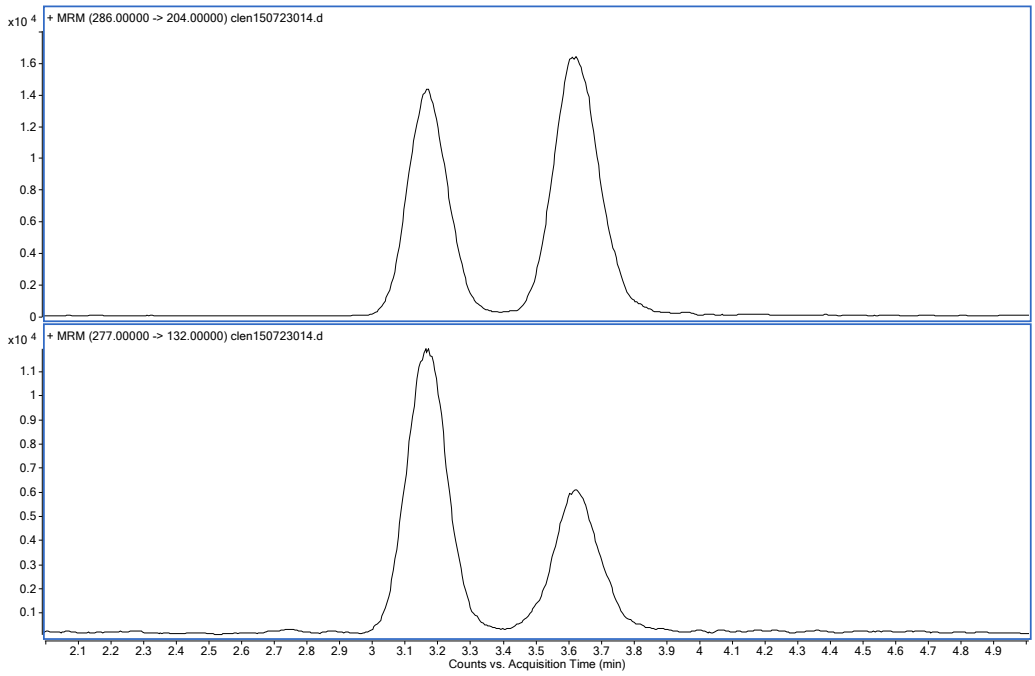
(c) meat sample spiked with 100 pg/g of S-(+)-clenbuterol (QC);



(d) chiral clenbuterol residues in swine liver



(e) chiral clenbuterol residues in beef meat



(f) chiral clenbuterol residues in lamb meat

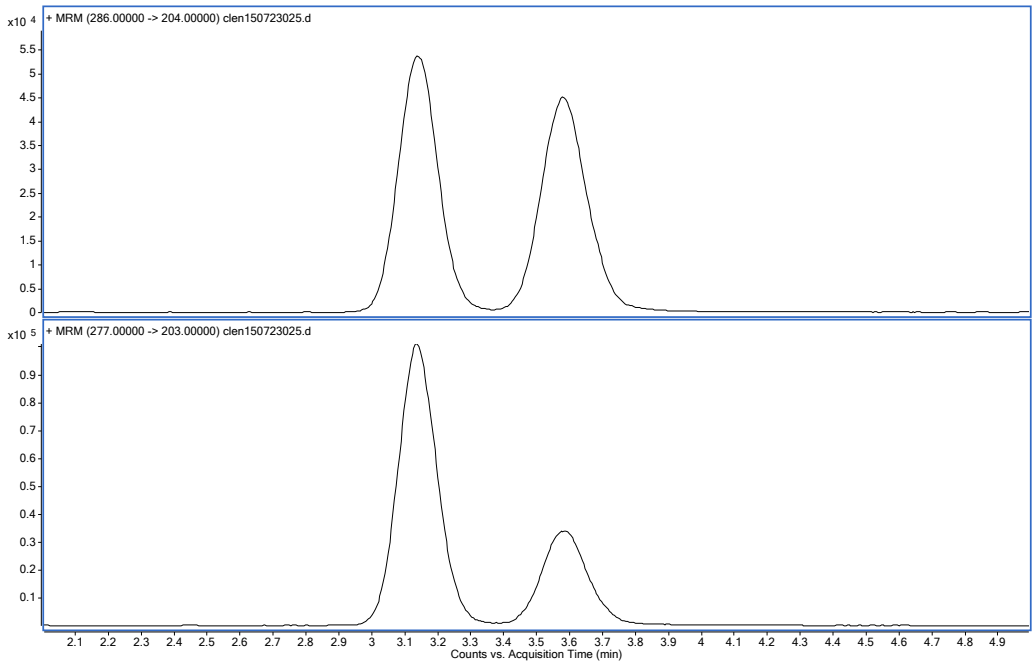
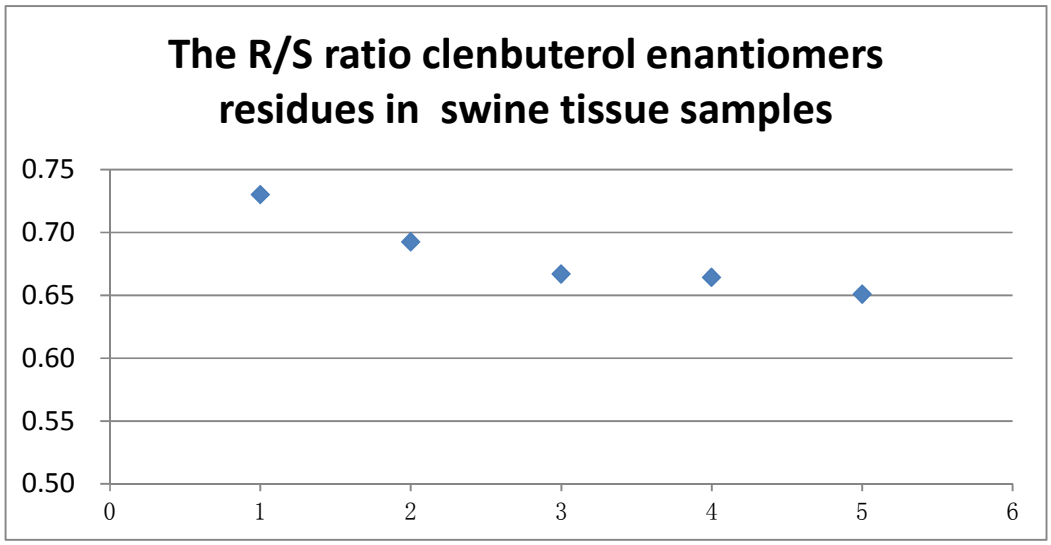
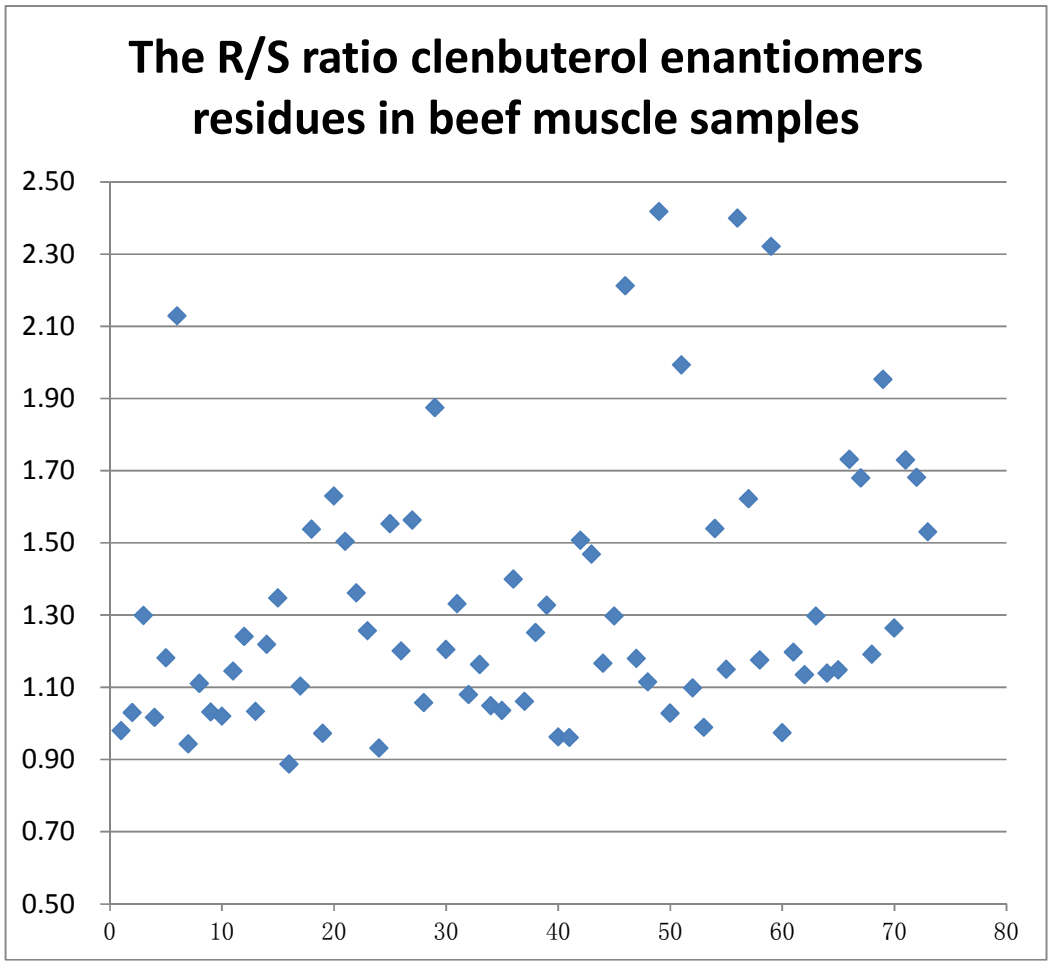


Fig.2 The R/S ratios clenbuterol enantiomers residues in swine tissue (a), beef muscle (b)and lamb muscle(c) samples.(The peak areas of R-clenbuterol (a), $^{2}H_{9}$-R-clenbuterol (b), S-clenbuterol (c) and $^{2}H_{9}$-S-clenbuterol (d) were auto-intergated. The correccted respose of R-clenbuterol = a/b (e), The correccted respose of S-clenbuterol = c/d (f), the R/S ratios clenbuterol enantiomers = e/f.)

a)



b)



c)

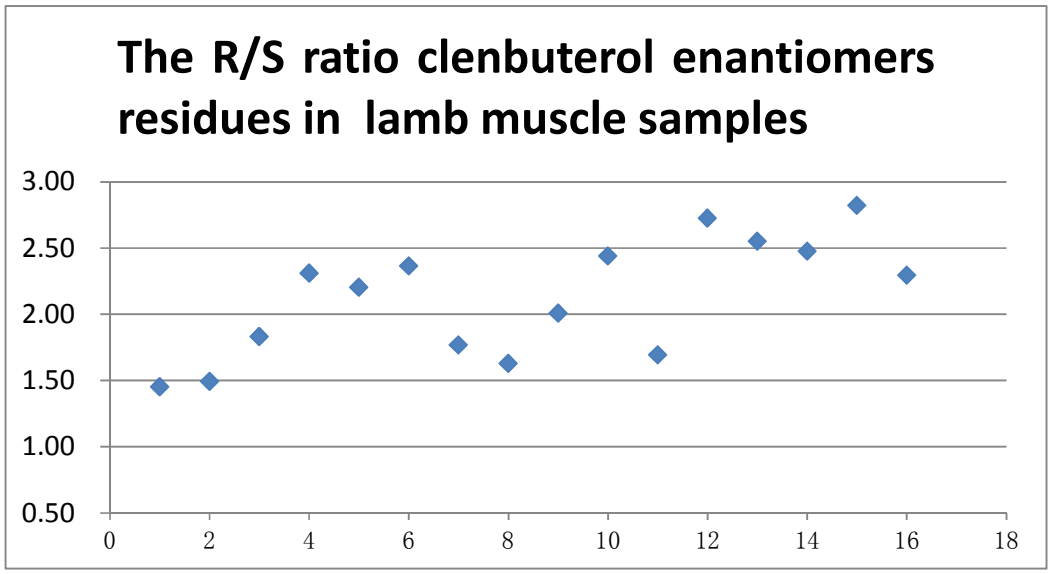


Table 1 Calibration curves, limits of detection and limits of quantification for R-(-)-Clenbuterol and S-(+)-Clenbuterol

Compound	Calibration curves	linearity range μg/kg	Correlation coefficient square	Average R/S ratio value	R/S ratio range	RSD %	LOD μg/kg	LOQ μg/kg
R-(-)-Clenbuterol*	y = 0.4772x -0.1469	0.1-25	0.9958	1.00	0.97-1.02	1.51	0.1	0.2
S-(+)-Clenbuterol*	y = 0.4684x -0.0833	0.1-25	0.9965					
R-(-)-Clenbuterol	y = 2.5942x	0.2-4	0.9908	1.13	1.03-1.40	14.1		
S-(+)-Clenbuterol	y = 2.2453x	0.2-4	0.9933					

The racemic clenbuterol to quantify the R-(-)-clenbuterol or S-(+)-clenbuterol is a valid method of quantification； * = from racemic clenbuterol.

Table 2 Average recoveries, interday and intraday precision (RSDs) for the R-(-)-Clenbuterol and S-(+)-Clenbuterol at three different spiked levels (n=6)

Sample	Spiked clenbuterol* Concentration μg/kg	Recovery %		Intraday			Interday		
		R-(-)-Clenbuterol	S-(+)-Clenbuterol	Average R/S ratio value	R/S ratio range	RSD %	Average R/S ratio value	R/S ratio range	RSD %
Blank tissue	0.4*	60.4-65.2	59.0-65.2	1.01	0.99-1.03	1.80	1.00	0.92-1.03	3.17
	2*	81.1-85.2	78.6-86.2	1.00	0.95-1.04	3.25	1.00	0.95-1.04	2.65
	20*	80.6-92.2	77.8-91.5	1.00	0.98-1.03	2.21	1.01	0.96-1.06	2.93

*=racemic clenbuterol.

Table 3 The R/S ratio, interday and intraday precision (RSD) for the real typical animals tissue samples (n=6)

Sample	Clenbuterol residues sample μ g/kg		Intraday			Interday		
	R-(-)-Clenbuterol	S-(+)-Clenbuterol	Average R/S Ratio value	R/S ratio range	RSD %	Average R/S Ratio value	R/S ratio range	RSD %
Swine	17.34	23.76	0.73	0.70-0.75	2.50	0.72	0.68-0.75	2.32
Beef	15.23	12.05	1.23	1.13-1.41	6.28	1.26	1.13-1.41	4.67
Lamb	3.30	1.52	2.34	2.26-2.40	5.79	2.32	2.22-2.40	5.40