Method of Test for Veterinary Drug Residues in Foods- Test of Multiresidue Analysis of β-Agonists

1. Scope

This method is applicable to the determination of 21 β-agonists (brombuterol etc. listed in the attached table) in muscle, viscera and fat of livestock products.

2. Method

After hydrolysis, extraction and purification, β-agonists are determined by liquid chromatography/tandem mass spectrometry (LC-MS/MS).

2.1. Equipment

2.1.1. Liquid chromatograph/tandem mass spectrometer.

2.1.1.1. Ion source: positive ion electrospray ionization, ESI^+.

2.1.1.2. Column: ZORBOX RRHD Eclipse Plus C18, 1.8 μm, 3.0 mm i.d. × 10 cm, or an equivalent product.

2.1.2. Homogenizer.

2.1.3. High speed dispersing device: SPEX SamplePrep 2010 GenoGrinder®, >1000 rpm, or an equivalent product.

2.1.4. Water bath: capable of controlling temperature at ± 1°C.

2.1.5. Centrifuge: centrifugal force > 10000 ×g and temperature control < 4°C.

2.1.6. Shaker.

2.1.7. pH meter.

2.1.8. Solid phase extraction vacuum manifolds.

2.1.9. Vortex mixer.

2.2. Chemicals

Methanol, HPLC grade;
Sodium acetate, AR grade;
Ammonium acetate, AR grade;
Acetic acid, AR grade;
Hydrochloric acid, AR grade;
Ammonia water (25%), AR grade;
β-Glucuronidase solution (β-glucuronidase ≥ 85000 unit/mL and sulfatase ≤ 7500 unit/mL);

Deionized water, resistivity ≥ 18 MΩ•cm (at 25°C);
Brombuterol hydrochloride, t-butylnorsynephrine (buctopamine), cimaterol, cimbuterol, clenbuterol hydrochloride, clencyclohexerol, clenisopenterol, clenpenterol hydrochloride, clenproperol, fenoterol, formoterol, isoxsuprine hydrochloride, mabuterol hydrochloride, mapenterol hydrochloride, 3-*o*-methyl-colterol, ractopamine hydrochloride, salbutamol, salmeterol, terbutaline hemisulfate, tulobuterol and zilpaterol, reference standards;
Brombuterol-d_9 hydrochloride, cimaterol-d_7, cimbuterol-d_9, clenbuterol-d_9 hydrochloride, clencyclohexerol-d_{10}, clenproperol-d_7, fenoterol-d_6 hydrobromide, formoterol-d_6, isoxsuprine-d_6 hydrochloride, mabuterol-d_9, mapenterol-d_{11} hydrochloride, 3-*o*-methyl-colterol-d_9, ractopamine-d_6, salbutamol-d_9, salmeterol-d_3, terbutaline-d_9, tulobuterol-d_9 hydrochloride and zilpaterol-d_7, isotope-labelled internal standards.

2.3. Apparatus

2.3.1. Volumetric flask: 10 mL and 50 mL.

2.3.2. Centrifuge tube: 15 mL and 50 mL, PP.

2.3.3. Solid phase extraction cartridge: Bond Elut Plexa PCX, 200 mg, 6 mL, or an equivalent product.

2.3.4. Ceramic homogenizer: Bond Elut QuEChERS P/N 5982-9313, or an equivalent product.

2.3.5. Membrane filter: 0.22-μm, Nylon.

2.4. Reagents

2.4.1. 0.2 M sodium acetate buffer solution

Dissolve 16.4 g of sodium acetate in 900 mL of deionized water, adjust pH with acetic acid to 5.2 ± 0.1, and dilute with deionized water to 1000 mL.

2.4.2. 5 mM ammonium acetate

Dissolve and dilute 0.385 g of ammonium acetate with deionized water to 1000 mL, and filter with a membrane

filter.

2.4.3. 5 mM ammonium acetate : methanol (9:1, v/v)

Mix 5 mM ammonium acetate solution and methanol at the ratio of 9:1 (v/v).

2.4.4. 0.2 N hydrochloric acid

Cautiously add 16.7 mL of hydrochloric acid to 900 mL of deionized water, then cool to room temperature, and dilute with deionized water to 1000 mL.

2.4.5. Methanol : ammonia water (95:5, v/v)

Mix methanol and ammonia water at the ratio of 95:5 (v/v).

2.5. Mobile phase

2.5.1. Solvent A

Dilute 1 mL of formic acid with deionized water to 1000 mL, and filter with a membrane filter.

2.5.2. Solvent B

Dilute 1 mL of formic acid with methanol to 1000 mL, and filter with a membrane filter.

2.6. Standard solution preparation

2.6.1. Internal standard solution

Accurately weigh equivalent 1 mg of brombuterol-d_9, cimaterol-d_7, cimbuterol-d_9, clenbuterol-d_9, clencyclohexerol-d_{10}, clenproperol-d_7, fenoterol-d_6, formoterol-d_6, isoxsuprine-d_6, mabuterol-d_9, mapenterol-d_{11}, 3-*o*-methyl-colterol-d_9, ractopamine-d_6, salbutamol-d_9, salmeterol-d_3, terbutaline-d_9, tulobuterol-d_9 and zilpaterol-d_7 isotope-labelled internal standards to each 10-mL volumetric flask, dissolve and dilute with methanol to volume as internal standard stock solutions, and then store in the freezer. When to use, mix appropriate volume of each internal standard stock solution, and dilute with methanol to 1000 ng/mL as the internal standard solution.

2.6.2. Standard solution

Accurately weigh equivalent 5 mg of brombuterol, t-butylnorsynephrine (buctopamine), cimaterol, cimbuterol,

clenbuterol, clencyclohexerol, clenisopenterol, clenpenterol, clenproperol, fenoterol, formoterol, isoxsuprine, mabuterol, mapenterol, 3-*o*-methyl-colterol, ractopamine, salbutamol, salmeterol, terbutaline, tulobuterol and zilpaterol reference standards to each 50-mL volumetric flask, dissolve and dilute with methanol to volume as standard stock solutions, and then store in the freezer. When to use, mix appropriate volume of each standard stock solution, and dilute with 5 mM ammonium acetate : methanol (9:1, v/v) to 1000 ng/mL as the standard solution.

2.7. Sample solution preparation

2.7.1. Hydrolysis and extraction

Transfer about 2 g of the homogenized sample accurately weighed into a centrifuge tube, and add 20 μL of the internal standard solution and 15 mL of 0.2 M sodium acetate buffer solution. Add one ceramic homogenizer, and shake by the high speed dispersing device at 1000 rpm for 10 min. Add 100 μL of β-glucuronidase solution, and hydrolyze in a water bath for 1 hr at 37°C. Add 2 mL of hydrochloric acid, shake for 10 min, centrifuge at 10000 ×g for 10 min at 4°C, and collect the supernatant. Centrifuge the supernatant at 5000 ×g for 10 min at 4°C, and collect the supernatant for purification.

2.7.2. Purification

Transfer the supernatant for purification from section 2.7.1. into a solid phase extraction cartridge pre-rinsed with 6 mL of methanol and 6 mL of deionized water, and discard the eluent. Wash the cartridge with 12 mL of 0.2 N hydrochloric acid solution, 12 mL of deionized water and 12 mL of methanol in order, and discard the eluents. Add 12 mL of methanol : ammonia water (95:5, v/v) to the cartridge, and collect the eluent. Evaporate the eluent to dryness by gently flushing with a stream of nitrogen at 65°C. Disslove the

residue with 1 mL of 5 mM ammonium acetate : methanol (9:1,v/v), mix by a vortex mixer, and centrifuge at 5000 ×g for 5 min. Take the supernatant as the sample stock solution. Dilute 500 μL (a) of the sample stock solution with 5 mM ammonium acetate : methanol (9:1,v/v) to 1000 μL (b), and filter with a membrane filter. Take the filtrate as the sample solution.

2.8. Matrix-matched calibration curve

Take a blank sample without adding the internal standard, and follow the procedure described in section 2.7. to obtain the blank stock solution. Add 1-50 μL of the standard solution and 10 μL of the internal standard solution to each 500 μL (a) of the blank stock solution, dilute with appropriate volume of 5 mM ammonium acetate : methanol (9:1, v/v) to 1000 μL (b) as the matrix-matched standard solutions. Operate LC-MS/MS according to the following conditions. Establish the matrix-matched calibration curve of each β-agonists by the added concertation in the range of 1-50 ng/mL vs. the ratio of peak area of each β-agonists to that of the internal standard.

LC-MS/MS operating conditions (note):

Column: ZORBOX RRHD Eclipse Plus C18, 1.8 μm, 3.0 mm i.d. × 10 cm.

Mobile phase: a gradient program of solvent A and solvent B is as follows.

Time (min)	A (%)	B (%)
0.0 → 1.0	98 → 98	2 → 2
1.0 → 5.0	98 → 90	2 → 10
5.0 → 8.0	90 → 80	10 → 20
8.0 → 10.0	80 → 70	20 → 30
10.0 → 11.0	70 → 60	30 → 40
11.0 → 12.0	60 → 60	40 → 40
12.0 → 15.0	60 → 10	40 → 90
15.0 → 19.0	10 → 10	90 → 90
19.0 → 19.1	10 → 98	90 → 2

19.1 → 23.0	98 → 98	2 → 2

Flow rate: 0.3 mL/min.

Injection volume: 10 μL.

Capillary voltage: 2.2 kV.

Ion source temperature: 120°C.

Ion source: ESI^+.

Desolvation temperature:400°C.

Cone gas flow rate: 50 L/hr.

Desolvation flow rate: 850 L/hr.

Detection mode: multiple reaction monitoring (MRM). Detection ion pair, cone voltage and collision energy are shown in the attached table.

Note: All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

2.9. Identification and quantification

Accurately inject 10 μL of the sample solution and the matrix-matched standard solutions into LC-MS/MS separately and operate according to the conditions in section 2.8. Identify each β-agonist based on the retention time and the relative ion intensities. Calculate the amount of each β-agonist in the sample by the following formula.

The amount of each β-agonist in the sample (ppm)

$$= \frac{C \times V \times F}{M \times 1000}$$

Where

C: the concentration of each β-agonist in the sample solution calculated by the matrix-matched calibration curve (ng/mL)

V: the make up volume of sample (mL)

M: the weight of sample (g)

F: the dilution factor, b/a

Note: Relative ion intensities are calculated by peaks areas of qualitation ions divided by peak areas of quantitation ions. Maximum permitted tolerances of relative ion intensities by LC-MS/MS are as follows:

Relative ion intensity (% of base peak)	Tolerance (%)
> 50	± 20
> 20～50	± 25
> 10～20	± 30
≤ 10	± 50

Remark

1. The limit of quantitation (LOQ) for each β-agonist is 0.001 ppm in muscle, and 0.005 ppm in viscera and fat.
2. Further validation shall be done when interference compounds appear in samples.

Reference

1. Shao, B., Jia, X., Zhang, J., Meng J., Wu, Y., Duan, H. and Tu, X. 2009. Multi-residual analysis of 16 β-agonists in pig liver, kidney and muscle by ultra performance liquid chromatography tandem mass spectrometry. Food Chem. 114: 1115-1121.
2. Lin, Y. T., Ting, Y., Shen, Y. R., Huang, C. N., Peng, G. J., Liao, C. D., Kao, Y. M., Wang, D. Y. and Chen, H. F. 2018. Improvement of a Multi-residue Analysis Method for the Determination of β-agonists in Poultry and Livestock Tissues. Annual Report of Food and Drug Research. 9: 52-65.

Reference chromatogram

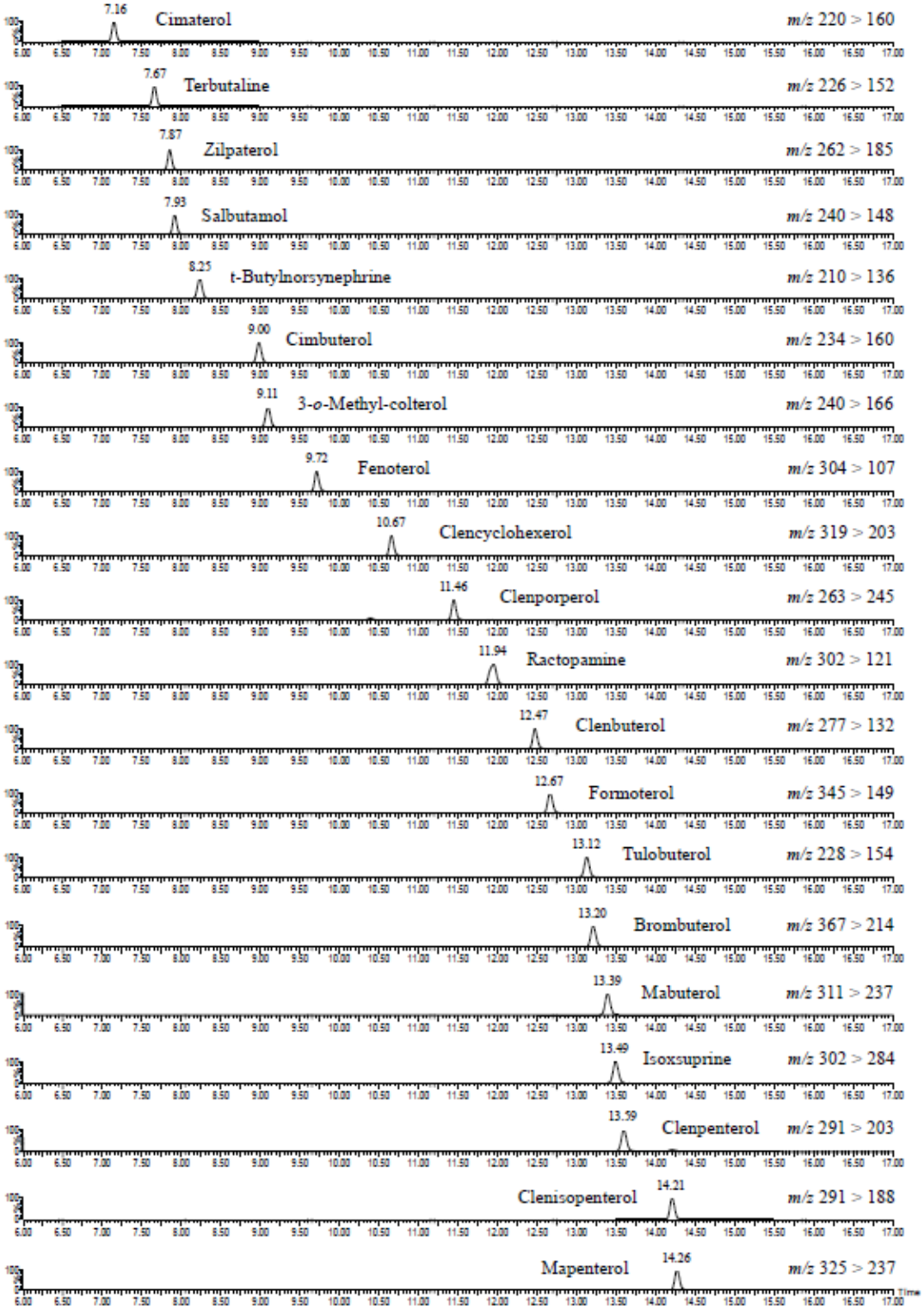

Figure. MRM chromatograms of 21 β-agonists standards and 18 isotope-labelled internal standards analyzed by LC-MS/MS.

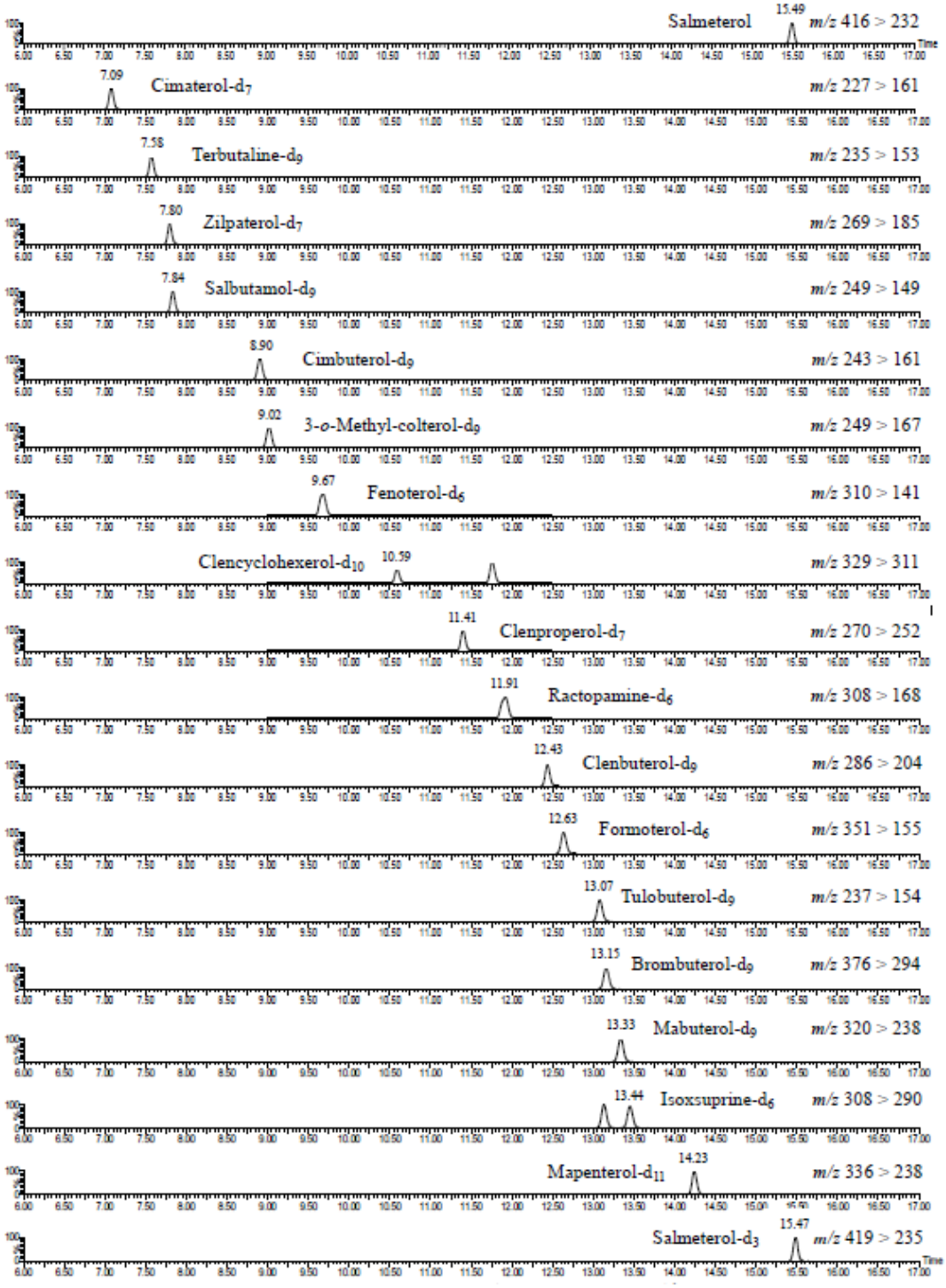

Figure. MRM chromatograms of 21 β-agonists standards and 18 isotope-labelled internal standards analyzed by LC-MS/MS (continued).

Table. The multiple reaction monitoring (MRM) parameters of 21 β-agonists and internal standards

No.	Analyte	Precursor ion (*m/z*) > product ion (*m/z*)	Declustering potential (V)	Collision energy (eV)	Internal standard
1	Brombuterol	367 > 214* 367 > 212 367 > 293	25	27 27 18	Brombuterol-d_9
2	t-Butylnorsynephrine (buctopamine)	210 > 136* 210 > 192	8	13 8	Zilpaterol-d_7
3	Cimaterol	220 > 160* 220 > 202 220 > 143	15	15 10 20	Cimaterol-d_7
4	Cimbuterol	234 > 160* 234 > 216	16	18 11	Cimbuterol-d_9
5	Clenbuterol	277 > 132* 277 > 203 277 > 259	20	30 20 10	Clenbuterol-d_9
6	Clencyclohexerol	319 > 203* 319 > 301 319 > 168	22	20 13 32	Clencyclohexerol-d_{10}
7	Clenisopenterol	291 > 188* 291 > 273 291 > 217	13	23 12 18	Mapenterol-d_{11}
8	Clenpenterol	291 > 203* 291 > 132 291 > 168	16	21 35 39	Brombuterol-d_9
9	Clenproperol	263 > 245* 263 > 203 263 > 132	15	12 18 26	Clenproperol-d_7
10	Fenoterol	304 > 107* 304 > 135	25	29 16	Fenoterol-d_6
11	Formoterol	345 > 149* 345 > 121	25	18 35	Formoterol-d_6
12	Isoxsuprine	302 > 284* 302 > 107 302 > 150	19	14 28 22	Isoxsuprine-d_6
13	Mabuterol	311 > 237* 311 > 217 311 > 202	18	20 30 35	Mabuterol-d_9
14	Mapenterol	325 > 237* 325 > 217 325 > 202	24	17 27 33	Mapenterol-d_{11}
15	3-o-Methyl-colterol	240 > 166* 240 > 134 240 > 121	18	16 28 28	3-o-Methyl-colterol-d_9

Table. The multiple reaction monitoring (MRM) parameters of 21 β-agonists and internal standards (continued)

No.	Analyte	Precursor ion (*m/z*) > product ion (*m/z*)	Declustering potential (V)	Collision energy (eV)	Internal standard
16	Ractopamine	302 > 121* 302 > 107 302 > 284	20	20 20 15	Ractopamine-d_6
17	Salbutamol	240 > 148* 240 > 222 240 > 166	20	15 15 20	Salbutamol-d_9
18	Salmeterol	416 > 232* 416 > 91 416 > 398	30	20 24 14	Salmeterol-d_3
19	Terbutaline	226 > 152* 226 > 107 226 > 125	27	16 30 25	Terbutaline-d_9
20	Tulobuterol	228 > 154* 228 > 118	20	20 20	Tulobuterol-d_9
21	Zilpaterol	262 > 185* 262 > 202 262 > 244	26	23 20 13	Zilpaterol-d_7
I.S.	Brombuterol-d_9	376 > 294	15	17	—
I.S.	Cimaterol-d_7	227 > 161	14	19	—
I.S.	Cimbuterol-d_9	243 > 161	8	14	—
I.S.	Clenbuterol-d_9	286 > 204	20	20	—
I.S.	Clencyclohexerol-d_{10}	329 > 311	10	13	—
I.S.	Clenproperol-d_7	270 > 252	8	10	—
I.S.	Fenoterol-d_6	310 > 141	27	18	—
I.S.	Formoterol-d_6	351 > 155	20	18	—
I.S.	Isoxsuprine-d_6	308 > 290	12	13	—
I.S.	Mabuterol-d_9	320 > 238	15	16	—
I.S.	Mapenterol-d_{11}	336 > 238	10	16	—
I.S.	3-o-Methyl-colterol-d_9	249 > 167	19	15	—
I.S.	Ractopamine-d_6	308 > 168	20	15	—
I.S.	Salbutamol-d_9	249 > 149	20	15	—
I.S.	Salmeterol-d_3	419 > 235	25	20	—
I.S.	Terbutaline-d_9	235 > 153	26	16	—
I.S.	Tulobuterol-d_9	237 > 154	35	20	—

No.	Analyte	Precursor ion (*m/z*) > product ion (*m/z*)	Declustering potential (V)	Collision energy (eV)	Internal standard
I.S.	Zilpaterol-d_7	269 > 185	20	33	—

Note:

1.*The quantitation ion, and the qualitation ions can be selected based on the matrix condition.

2. Internal standards can be used with different number of deuterium isotopes and their multiple reaction monitoring (MRM) parameters can be adjusted.