Supporting information

Quantitative Determination of Bulk Molecular Concentrations of *β*-agonists in Pork Tissue Samples by Direct Internal Extractive Electrospray Ionization-Mass Spectrometry

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1. The analysis process of *β*-agonists in pork by GC-MS

The GC-MS experiment was carried out on 7000c (Agilent) and the process was presented as follows [1]:

1.1 Extraction

5.0 g pork was weighed carefully, followed by grinded with 20 mL 0.1 mol/L $HClO_4$ to form a homogenate. Then the homogenate was transferred to a glass tube and treated with ultrasound for 20 min, followed by heat treated at 80 ^{o}C for 30 min. After cool down to room temperature, the homogenate was centrifuged to separate the supernatant. The residue was washed with 5 mL 0.1 mol/L $HClO_4$ and centrifuged to separate the supernatant again, followed by added into the previous supernatant. Then the supernatant was adjusted to pH 9.5 with 1 mol/L NaOH. If there were precipitates produced, please centrifuge again to separate the supernatant. Then, 8 g NaCl was added into the supernatant. After the NaCl dissolved, 25 mL mixture of isopropanol and ethyl acetate (v/v=4/6) was added into the supernatant to extract the analytes with 20 minutes shake, followed by complete evaporation of the solvent to concentrate the analytes. Then, 1 mL 0.1 mol/L NaH_2PO_4 was used to dissolve the analytes, followed by filtration.

1.2 Purification

Weak cation exchange column (WCEC) was used to purify the analytes. The analytes solution prepared above was first added in the WCEC, followed by washed with 4 mL water and 4 mL ethanol successively. Then 6 mL mixture of ethanol and ammonia (v/v=98/2) was used to

extract the analytes, followed by complete evaporation of the solvent.

1.3 Derivatization

200 μL methanol was used to dissolve the purified and dried analytes. Then, the analytes solution was evaporated completely, followed by added 40 μL derivative reagent (BSTFA) and derived at 75 ^{o}C for 90 min. After derivatization, the analytes was mixed on a vortex mixer for 30 s, and then dried on a N_2-evaporater. 200 μL toluene was used to dissolve the derived analytes for GC-MS analysis. Meanwhile, standard *β*-agonists solution was derived at the same time.

1.4 GC-MS analysis

a) Gas chromatography column: DB-5MS, 30 m×0.25 mm×0.25 μm.

b) Carry gas: He, pre-column pressure: 8 psi.

c) Temperature of inlet: 240 ^{o}C.

d) Sample volume: 1 μL, splitless.

e) Procedure of column heating:

Table S-1 Procedure of column heating

Time/min	Column temperature / ^{o}C
0	70
1	70
8	200
17	245
18.5	280
23	280

f) EI source

g) Scanning mode: SIM

h) Electron bombardment energy: 70 eV.

i) Temperature of ion source: 200 ^{o}C.

j) Temperature of interface: 285 ^{o}C.

k) Solvent delay: 8 min.

l) Mass analyzer: Triple quadrupole

1.5 Determination

Inject 1 μL derived sample solution or standard solution into GC-MS.

Reference:

[1]. GB/T 5009.192-2003, *Determination of 4-amino-3,5-dichloro-a [(tert-butylamino) methy]-benzyl alcohol (clenbuterol) residues in animal foods*, Standards Press of China: Bei Jing, 2003.

2. The analysis process of *β*-agonists in pork by LC-MS

The HPLC-MS experiment was carried out on a liquid chromatograph (Shimadzu LC30 system) and a linear trap quadruple (LTQ) Mass Spectrometer (Thermo Scientific) equipped ESI source, and the process was presented as follows.[2]

2.1 Sample preparation

2.1.1 Extraction

2.0 g pork sample was put into 50 mL Centrifuge tube, then 8 mL sodium acetate buffer was added into the centrifuge tube, followed by grinded to a homogenate. 50 μL *β*-Glucuronidase/aryl sulfatase was added into the homogenate with sufficient mixing and kept at 37 ^{o}C water bath for 12 hours. After sufficient electrolysis, the homogenate was centrifuged with 5000r/min to separate the supernatant. Then 5mL 0.1 mol/L $HClO_4$ was added into the supernatant, followed by adjusted to pH 1±0.3 with $HClO_4$. After centrifuging with 5000 r/min for 10 minutes, transfer the supernatant (about 10 mL) to a new 50 mL centrifuge tube, and then adjust the solution to pH 11 with 10 mol/L NaOH. 10 mL saturated sodium chloride solution and 10 mL isopropanol/ethyl acetate (V/V=6:4) solution were added into the supernatant to extract the analyte, followed by centrifuging with 5000r/min for 10 minutes.

Transfer all organic phases and dried by N_2 under the condition of 40 ^{o}C water bath. 5 mL sodium acetate buffer was used to dissolve the residue with ultrasonic.

2.2.2 Purification

Weak cation exchange column (WCEC) was used to purify the analytes. The analytes solution prepared above was first added in the WCEC, followed by washed with 2 mL H_2O, 2 mL 2% formic acid aqueous solution and 2 mL CH_3OH successively. Then 2 mL 5% ammonia methanol solution was used to extract the analytes, followed by complete evaporation of the solvent.

200 μL 0.1% formic acid/water-methanol solution (95+5) was used to dissolve the residue with ultrasonic. Transfer the solution to 1.5 mL centrifuge tube, and centrifuge with 15000 r/min for 10 minutes. The supernatant was collected for HPLC-MS analysis.

2.3 Liquid chromatography with tandem-mass spectrometric analysis.

a) Chromatographic column: waters atlantics C18 column, 150 mm × 2.1 mm (internal diameter);

b) Mobile phase: 0.1% Formic acid solution (A), 0.1% Formic acid/acetonitrile (B);

Table S-2 Gradient elution procedures

Time/min	A/%	B/%
0	96	4
2	96	4
8	85	15
20	82	18
21	5	95
24	5	95
25	96	4

c) Flow rate: 0.2 mL/min;

d) Column temperature: 30 ^{o}C

e) Quantity of sample: 10 μL

f) Ionization source: electrospray ionization, positive ion mode

g) Scanning mode: multiple reaction monitoring

h) Desolvation gas, cone gas and collision gas are high-purification gas or other high-purification gas which is suitable; the gas flow rate should be adjusted to make the limits of mass spectrometry meet the analysis requirements.

i) Adjust the column voltage, cone voltage, collision voltage, etc. to optimal sensitivity.

j) Monitoring ion: see Table S-3 for monitoring ion

[2]. GB/T 22286-2008. *Determination of β-agonists residues in foodstuff of animal origin-Liquid chromatography with tandem-mass spectrometric method*, Standards Press of China: Bei Jing, 2008

3. The parameters of prepared standard pork samples

Table S-3 The parameters of prepared standard pork samples

Sample	Sal		Cle		Bro		Ter		Tur		Rac	
	Q (μg)	C (μg/Kg)	Q (μg)	C (μg/Kg)	Q (μg)	C (μg/Kg)	Q (μg)	C (μg/Kg)	Q (μg)	C (μg/Kg)	Q (μg)	C (μg/Kg)
1	1.44E-5	7.2E-3	1.42E-5	7.1E-3	1.36E-5	6.8E-3	1.70E-5	8.5E-3	2.00E-5	1.0E-2	1.70E-5	8.5E-3
2	1.48E-4	7.4E-2	1.58E-4	7.9E-2	1.48E-4	7.4E-2	1.58E-4	7.9E-2	2.00E-4	1.0E-1	1.86E-4	9.3E-2
3	1.58E-3	7.9E-1	1.46E-3	7.3E-1	1.58E-3	7.9E-1	1.46E-3	7.3E-1	1.96E-3	9.8E-1	2.04E-3	1.02E0
4	1.28E-2	6.4E0	9.40E-3	4.7E0	1.30E-2	6.5E0	1.34E-2	6.7E0	1.90E-2	9.5E0	2.20E-2	1.1E1
5	1.08E-1	5.4E1	1.08E-1	5.4E1	1.14E-1	5.7E1	1.10E-1	5.5E1	1.86E-1	9.3E1	1.84E-1	9.2E1
6	8.20E-1	4.1E2	8.20E-1	4.1E2	9.00E-1	4.5E2	8.00E-1	4.0E2	1.82E0	9.1E2	1.70E0	8.5E2
7	6.00E0	3.0E3	6.20E0	3.1E3	7.20E0	3.6E3	1.50E1	7.5E3	1.58E1	7.9E3	1.50E1	7.5E3
8	5.00E1	2.5E4	5.00E1	2.5E4	6.40E1	3.2E4	4.20E1	2.1E4	1.34E2	6.7E4	1.38E2	6.9E4

Q was the total amount of the *β*-agonists diffused into the pork tissue strips after the tissue strip was incubated for 10 h. C was the standard concentration of *β*-agonists in each pork tissue sample. The preparation process of the sample was illustrated in experimental section of manuscript.

4. EIC and TIC (CID fragments) analysis of four *β*-agonists in pork sample by iEESI-MS

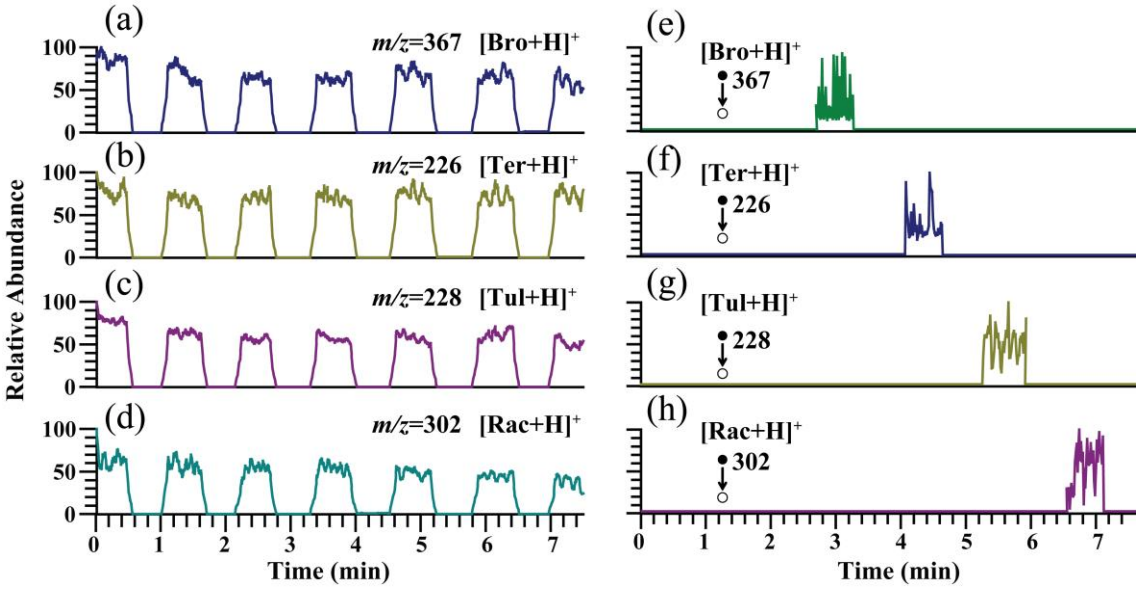

Figure S-1 EIC and TIC (CID fragments) of four *β*-agonists in pork sample analysed by iEESI-MS. (a) EIC of Bro, (b) EIC of Ter, (c) EIC of Tur, (d) EIC of Rac, (e) TIC of Bro in CID analysis, (e) TIC of Ter in CID analysis, (f) TIC of Tul in CID analysis, (g) TIC of Rac in CID analysis. The duration of 2.8-3.4 min, 4.0-4.6 min, 5.25-5.9 min, 6.5-7.1 min were used for CID analysis of Bro, Ter, Tul and Rac, respectively.

As shown in Figure 2a, the signals with approximate 100% relative abundance were attributed the total ion of pork sample, while the signals with approximate 0% relative abundance were the CID signal of selected *β*-agonists, where 0.5-1.1 min, 1.7-2.3 min, 2.8-3.4 min, 4.0-4.6 min, 5.25-5.9 min, 6.5-7.1 min were assigned for CID signal of Sal, Cle, Bro, Ter, Tul and Rac, respectively. Similarly, Figure 2b-c and Figure S-1 a-d were the extracted ion chromatography of 6 *β*-agonists extracted from total ion chromatography, respectively. The signals with approximate 100% relative abundance were attributed the salbutamol or clenbuterol, while the signals with approximate 0% relative abundance were the CID signal of selected *β*-agonists, where 0.5-1.1 min, 1.7-2.3 min, 2.8-3.4 min, 4.0-4.6 min, 5.25-5.9 min, 6.5-7.1 min were assigned for CID signal of Sal, Cle, Bro, Ter, Tul and Rac, respectively. Figure 2d-e and Figure S-1 e-h were the total ion chromatography of CID analysis of salbutamol (0.5-1.1 min) and clenbuterol (1.7-2.3 min), respectively.

5. iEESI-MS spectra of pork sample with *β*-agonists

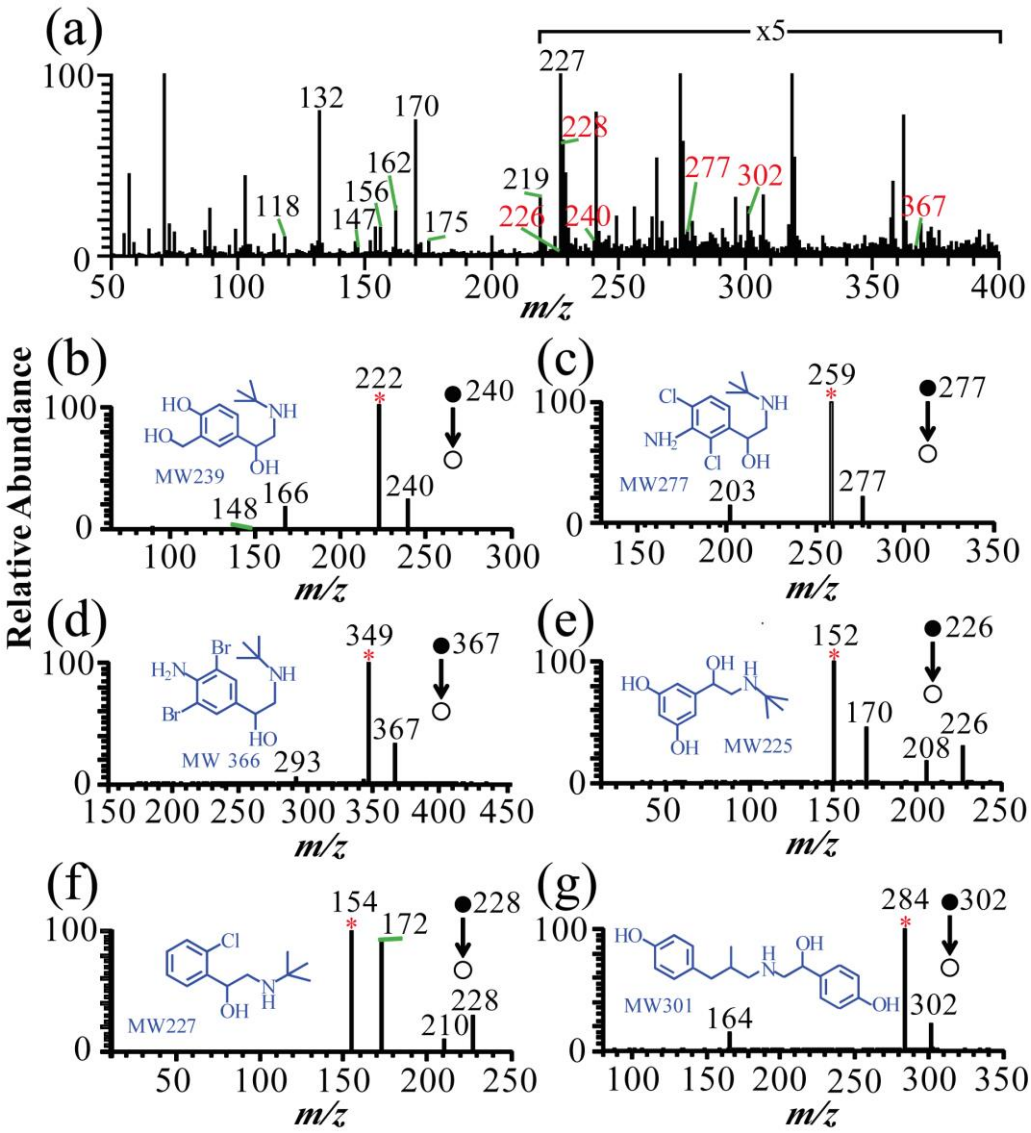

Figure S-2. Mass spectra of *β*-agonists in pork tissue samples recorded by iEESI-MS experiments using CH_3OH/H_2O (v/v=1:1) as the extraction solvent. (a) Mass spectral fingerprint of a pork sample; (b) CID mass spectrum of Sal, (c) CID mass spectrum of Cle, (d) CID mass spectrum of Bro, (e) CID mass spectrum of Ter, (f) CID mass spectrum of Tul and (g) CID mass spectrum of Rac. The concentration of the corresponding *β*-agonists were 7.6 μg/kg (Sal), 14.1 μg/kg (Cle), 49.3 μg/kg (Rac), 7.1 μg/kg (Ter), 9.5 μg/kg (Tul) and 15.3 μg/kg (Bro). The corresponding protonated ions were $[Sal+H]^+$ (*m/z* 240), $[Cle+H]^+$ (*m/z* 277) , $[Bro+H]^+$ (*m/z* 367), $[Ter+H]^+$ (*m/z* 226), $[Tul+H]^+$ (*m/z* 228) and $[Rac+H]^+$ (*m/z* 302). The fragments marked by red star were used for quantitation of the corresponding *β*-agonist.

All the *β*-agonists including Sal (MW 239), Cle (MW 276), Rac (MW 301), Ter (MW 225), Tul (MW 227) and Bro (MW 366) were detected as protonated molecules in the iEESI-MS

spectra (Figure S-2a), which were in agreement with the previous data obtained using LC-MS. Since the *β*-agonists concentrations were much lower than other compounds such as amino acids (*m/z* 118 [Valine + H]$^+$, *m/z* 132 [Creatine + H]$^+$, *m/z* 147 [Lysine + H]$^+$, *m/z* 156 [Histidine + H]$^+$, *m/z* 162 [L-carnitine + H]$^+$, *m/z* 170 [Vitamin B6 + H]$^+$, *m/z* 175 [Arginine + H]$^+$, *m/z* 203 [Spermine + H]$^+$), Carnosine (*m/z* 227 [M + H]$^+$), the corresponding signals of the *β*-agonists were relatively weak in the fingerprint spectra,[47] and could be predominant in 5-foldzoom view spectrum (Figure S-2a).

Tandem mass spectrometry experiments were carried out to confirm the molecular ions of six *β*-agonists. The corresponding MS^2 data (Figure S-2b-g, Table S-4) were matched using authentic *β*-agonist compounds, showing the CID fragmentation patterns which were very similar to the reported data in the literature and identical to those detected from the spiked pork tissue samples. For instance, the main fragment ions of protonated Sal (*m/z* 240) were detected at *m/z* 222, 166 and 148 (Figure S-2b), by the loss of H_2O, $CH_2=C(CH_3)_2$, and H_2O again, successively. The protonated Cle (*m/z* 277) yielded main fragment ions of *m/z* 259 and 203 (Figure S-2c), by the loss of H_2O and [$CH_2=C(CH_3)_2$], successively. Similarly, for protonated Bro (*m/z* 367), the main fragment ions of *m/z* 349 and 293 (Figure S-2d) were formed by loss of H_2O and [$CH_2=C(CH_3)_2$], successively. For protonated Ter (*m/z* 226), the main fragment ions were detected at *m/z* 208, 170 and 152 (Figure S-2e). The fragment ions *m/z* 208 and 170 could be formed by the loss of H_2O and [$CH_2=C(CH_3)_2$] from the protonated Ter, respectively, while the fragment ions of *m/z* 152 were formed by losing H_2O from the ions of *m/z* 170 or by losing [$CH_2=C(CH_3)_2$] from the precursor ions of *m/z* 208. For protonated Tul (*m/z* 228), the main fragment ions were found at *m/z* 210, 172, 154 (Figure S-2f), by following the similar pathway observed in the Ter case. For protonated Rac (*m/z*

302), the main fragment ions of *m/z* 284 and 164 were produced by the loss of H_2O and [HO-Ph-CH=CH_2] (*m/z* 120) (Figure S-2g), successively. All the fragmentation patterns were confirmed using the authentic compounds under the experimental conditions, showing the successful detection of the *β*-agonists. For each kind of *β*-agonists, the strongest fragment ions, marked with red star (Figure S-2b-g), was selected as the signal ions for quantitative measurements.

6. The characteristic mass spectra peak of six kinds of *β*-agonists

Table S-4 The characteristic mass spectra peak of six kinds of *β*-agonists

Substrate	Characteristic peak
Salbutamol	240, 222*, 166, 148
Clenbuterol	277, 259*, 203
Brombuterol	367, 349*, 293
Terbutaline	226, 208, 170, 152*
Tulobuterol	228, 210, 172, 154*
Ractopamine	302, 284*, 164

*Quantitative peak of corresponding substrate

7. Calibration curve data of 6 kinds of *β*-agonists by iEESI-MS

Table S-5 Calibration curve data for 6 *β*-agonists obtained by iEESI-MS

Substrate	Linear equation (lgI = algC + b)	Correlation coefficient (R^2)	Linear range (μg/kg)	LOD =C3σ/S (μg/kg)
Salbutamol	Y=0.317X+1.807	0.9910	0.0072 -415.6	0.002
Clenbuterol	Y=0.229X+1.408	0.9938	0.0085 - 415.6	0.005
Brombuterol	Y=0.185X+2.037	0.9964	0.0072 - 415.6	0.002
Terbutaline	Y=0.227X+1.686	0.9944	0.0085 - 415.6	0.003
Tulobuterol	Y=0.236X+1.080	0.9983	0.0103 - 897.6	0.006
Ractopamine	Y=0.197X+2.148	0.9953	0.0086 - 854.1	0.003

I: Signal intensity of *β*-agonists. C: Concentration of *β*-agonists in pork. σ: standard deviation. S: The average intensity of the quantitative peak of *β*-agonists

8. Recoveries obtained for iEESI-MS of 6 *β*-agonists

Table S-6 Recoveries obtained for iEESI-MS of 6 *β*-agonists

	Sal			Cle			Bro			Ter			Tur			Rac		
Sample	S	D	R	S	D	R	S	D	R	S	D	R	S	D	R	S	D	R
	μg/Kg	μg/Kg	%	μg/Kg	μg/Kg	%	μg/Kg	μg/Kg	%	μg/Kg	μg/Kg	%	μg/Kg	μg/Kg	%	μg/Kg	μg/Kg	%
1	7.4E-2	6.7E-2	91.2	7.9E-2	7.4E-2	93.3	9.3E-2	8.9E-2	95.0	7.9E-2	7.3E-2	92.3	1.0E-1	9.3E-2	93.1	7.4E-2	7.1E-2	98.3
2	7.9E-1	7.3E-1	93.7	7.3E-1	7.0E-1	95.0	1.02E0	9.8E-1	95.7	7.3E-1	6.9E-1	93.8	9.8E-1	9.3E-1	94.8	7.9E-1	8.1E-1	102.2
3	6.5E0	4.7E0	94.2	4.7E0	4.5E0	96.2	1.1E2	1.0E1	97.4	6.7E0	6.4E0	94.8	9.5E0	9.1E0	95.3	6.5E0	6.3E0	98.1
4	5.4E1	5.4E1	96.5	5.4E1	5.3E1	97.5	9.2E2	9.3E2	101.1	5.5E1	5.3E1	96.4	9.3E2	9.51	101.6	5.7E1	5.4E1	98.8
5	4.1E2	4.0E2	97.8	4.1E2	4.1E2	98.2	8.5E2	8.4E2	98.7	4.0E2	3.9E2	97.9	9.1E2	9.0E2	98.8	4.5E2	4.32	96.3
6	3.0E2	2.9E2	99.1	3.2E3	3.1E3	98.9	7.5E3	7.3E30	97.7	7.5E3	7.3E3	102.7	7.9E3	7.8E3	99.2	3.6E3	3.5E3	98.8

S: The concentration of *β*-agonists spiked in pork samples. D: The concentration of *β*-agonists detected by iEESI-MS R: The recovery of *β*-agonists obtained by iEESI-MS

9. Calibration curve data of 6 kinds of *β*-agonists by direct infusion ESI-MS

Table S-7 Calibration curve data for 6 *β*-agonists obtained by direct infusion ESI-MS

Substrate	Linear equation (lgI = algC + b)	Correlation coefficient (R^2)	Linear range (μg/kg)	LOD =C3σ/S (μg/kg)
Salbutamol	Y=0.156X+0.943	0.9891	0.87 - 50.3	0.52
Clenbuterol	Y=0.209X+2.264	0.9863	1.0 - 50.3	0.30
Brombuterol	Y=0.322X+1.366	0.9938	0.87 - 50.3	0.26
Erbutaline	Y=0.254X+0.904	0.9944	1.0 - 50.3	0.60
Tulobuterol	Y=0.589X+0.057	0.9883	1.2 - 108.6	0.75
Ractopamine	Y=0.118X+4.364	0.9932	1.0 - 103.4	0.16

I: Signal intensity of *β*-agonists. C: Concentration of *β*-agonists in pork. σ: standard deviation. S: The average intensity of the quantitative peak of *β*-agonists

10. Calibration curve data of 6 kinds of *β*-agonists by GC-MS

Table S-8 Calibration curve data for 6 *β*-agonists obtained by GC-MS

Substrate	Linear equation	Correlation coefficient (R^2)	Linear range (μg/kg)	LOD =C3σ/S (μg/kg)
Salbutamol	Y=129218.8X-69342.7	0.9996	1.4 - 100	0.8
Clenbuterol	Y=53770.9X-52241.5	0.9996	1.8 - 100	1.1
Brombuterol	Y=74407.8X-71061.4	0.9999	1.7 - 100	0.9
Terbutaline	Y=95581.6X-67548.1	0.9996	1.5 - 100	0.8
Tulobuterol	Y=80219.7X-53820.5	0.9991	1.3 - 100	1.0
Ractopamine	Y=1343.5X+108.1	0.9954	1.2 - 100	0.4

Y: The area of the TIC peak of corresponding *β*-agonist. C: Concentration of corresponding *β*-agonist. σ: standard deviation. S: The average intensity of the quantitative peak of *β*-agonists

11. Calibration curve data of 6 kinds of *β*-agonists by LC-MS

Table S-9 Calibration curve data for 6 *β*-agonists obtained by LC-MS

Substrate	Linear equation	Correlation coefficient (R^2)	Linear range (μg/kg)	LOD =C3σ/S (μg/kg)
Salbutamol	Y=45299.7X+385.2	0.9997	0.1-50	0.04
Clenbuterol	Y=271614.3X+45171.9	0.9959	0.05-50	0.02
Brombuterol	Y=1065980X+38993.8	0.9997	0.05-50	0.01
Terbutaline	Y=34566.3X+2607.9	0.9992	0.1-50	0.05
Tulobuterol	Y=63480.2X+5687.6	0.9975	0.05-50	0.02
Ractopamine	Y=100437.5X+7953.2	0.9991	0.1-50	0.03

Y: The area of the TIC peak of corresponding *β*-agonist. C: Concentration of corresponding *β*-agonist. σ: standard deviation. S: The average intensity of the quantitative peak of *β*-agonists