# Determination of Salbutamol, clenbutesol, Ractopamine in pork meat by LC-MS/MS

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**Abstract:** A method for determination of Salbutamol, clenbutesol, Ractopamine in pork meat by liquid chromatography-Tandem mass spectrometry (LC-MS/MS)was established. The sample was injected into LC-MS/MS after cleaned by SPE tube and dissolved by methanol.The quantitative analysis was achived by ESI source under positive ion mode,$C_{18}$ liquid column . The RSD of the method was less than 6%, and average recovery was more than 80% ,the liner range was in 1-10ng/mL and the low detection limit of Salbutamol, clenbutesol, Ractopamine were 0.051ng/mL, 0.012ng/mL, 0.018ng/mL respectively.
**Key words：** LC-MS/MS; pork meat; Salbutamol; clenbutesol; Ractopamine

## 1. Introduction

Clenbuterol is neither veterinary drugs, nor is additive, adrenergic nerves, belonging to the beta stimulant hormone, the chemical composition is alpha - (TERT Ding Anji)]-4- 3,5- two amino methyl chlorobenzene methanol hydrochloride. Clenbuterol only uesd in medicine for the treatment of asthma early. In animals, the role of it is the redistribution of nutrients, which can promote animal growth, improving lean meat rate, reducing fat deposition, improving feed remuneration, etc. Clenbuterol is not protein hormone. It will form the residues in pig tissues, especially high residues in pig liver and other organs after used. It will directly endanger human health when the high level residue meat eaten. The main hazard are muscle tremor, tremor, headache, palpitation, nausea, vomiting and other symptoms, especially more harm to patients with hypertension, heart disease, hyperthyroidism and hypertrophy of the prostate disease, which can lead to serious death [1-2]. The toxic effects of ractopamine is unknown. But as a feed additive for long-term use of their carcinogenic, teratogenic and mutagenic effects which need to be further studied. Ractopamine belongs to the adrenal hormone, can produce like effects, can cause allergic reactions, the decreased immune function. At the same time, veterinary drugs through animal feces, urine excretion of pollution of the ecological environment, but also indirectly endanger human health. salbutamol, clenbuterol, ractopamine and other stimulants may not be detected in animal food in china. detection of salbutamol, clenbuterol and ractopamine residues use such methods as liquid chromatography [3], GC-MS[4-5], LC-MS [6-7], this experiment using liquid chromatography tandem mass spectrometry for the detection of clenbuterol, ractopamine and salbutamol in pork. This method can ensure the accuracy of the experiment and the sample pretreatment, and save the detection time.

## 2.Materials and methods

### 2.1 reagents and instruments

Methanol: chromatography purity, TEDIA; formic acid: analysis of pure, 98%, Sinopharm Chemical Reagent Co. Ltd.; clenbuterol standard: 100 μg/mL, environmental protection scientific research and monitoring institute of the Ministry of agriculture; clenbuterol -D9 isotope internal standard, 100 g/mL, Dr. Ehrenstorfer GmbH; ractopamine: 100 μg/mL, environmental protection scientific research and monitoring institute of the Ministry of agriculture; ractopamine -D5 isotope internal standard, 10mg/ bottle, Tianjin Alta Technology Co. Ltd; salbutamol standard: 100μg/mL, environmental protection scientific research and monitoring institute of the Ministry of agriculture; salbutamol -D3 isotope internal standard, 100 μg/mL, Dr. Ehrenstorfer GmbH; acetonitrile: chromatography purity, Germany Merck; Purified water, 1.25L/ bottle. Beta glucosidase hydrochloride glucose aldehyde / arylsulfatase: 10mL/ bottle, Roche Diagnostics: GmbH centrifuge: Neofuge 23R, Shanghai Lishen Scientific Instrument Co Ltd; balance: JY10002, Shanghai Hengping Scientific Instrument Co. Ltd; constant incubator: DHP-9082, Shanghai scientific instrument co.;

0.1% formic acid aqueous solution preparation method: 0.5mL formic acid was added to 500mL water and filtered by 0.22 M aqueous membrane filter.

0.2mol/L ammonium acetate buffer solution preparation method: 15.4g ammonium acetate, dissolved in 1000mL of water, with the amount of acetic acid to adjust the pH value to 5.2.

Ultimate 3000 high performance liquid chromatography, three triple quadrupole mass spectrometry detector

(electric spray ion source ESI): Thermo Fisher TSQ QUANTUM ULTRA; Hypersil Gold HPLC column (2.1mm * 100mm, 3μm), Thermo Fisher; 0.22 μm organic phase needle filter head, Agela company; vortex oscillator: MS3 Digital, IKA; solid phase extraction column : MCX,60mg/3mL, Shanghai ANPEL company.

## 2.2 instrument conditions

### 2.2.1 liquid chromatography conditions

Column: C18 column: 100 x 2.1mm (I, d), 3 μm; column temperature: 35 ℃;

The mobile phase of acetonitrile (A), 0.1% formic acid water solution (B), gradient: 0min ~ 3min, A phase increased from 10% to 90%; 3min ~ 8min, A keep 90%;8.0min ~ 8.1min ,A change to 10%; 8.1min ~ 14min A keep10%.

Flow rate: 0.3mL/min.

### 2.2.2 mass spectrometry reference conditions

a) Ionization mode: electrospray ionization( positive ion mode);
b) spray voltage: 3.5kV;
c) evaporator temperature: 150;
d) sheath gas ($N_2$) pressure: 35arb;
e) auxiliary gas ($N_2$) flow: 10 arb;
f) collision chamber (Ar) pressure: 1.5 m Torr;
g) scanning mode: selective response monitoring (SRM);
h) mass divert valve state: 0min-2min is in the Inject \Waste state, 2min-7.5min is in the Load \ Detector state, 7.5-14min is in the Inject \ Waste state;
i) collision energy is shown in Tab.1, Tab.2, Tab.3.

Tab. 1 collision energy & product ions of Salbutamol and Salbutamol-$D_3$

| Analyte | Parent ion (m/z) | Daughter ion (m/z) | Collision energy (eV) |
|---|---|---|---|
| salbutamol | 240.1 | 121.1 | 28 |
| | 240.1 | 133 | 28 |
| | 240.1 | 148.1 | 18 |
| salbutamol-$D_3$ | 243.1 | 124.1 | 29 |
| | 243.1 | 133 | 28 |
| | 243.1 | 151.1 | 18 |

Tab. 2 collision energy & product ions of clenbutesol and clenbutesol-$D_9$

| Analyte | Parent ion (m/z) | Daughter ion (m/z) | Collision energy (eV) |
|---|---|---|---|
| clenbuterol hydrochloride | 277.1 | 132 | 27 |
| | 277.1 | 168 | 29 |
| | 277.1 | 203 | 15 |
| clenbuterol hydrochloride -$D_9$ | 286.1 | 133.1 | 30 |
| | 286.1 | 169 | 29 |
| | 286.1 | 204 | 16 |

Tab. 3 collision energy & product ions of Ractopamine and Ractopamine-$D_5$

| Analyte | Parent ion (m/z) | Daughter ion (m/z) | Collision energy (eV) |
|---|---|---|---|
| Ractopamine | 302.1 | 107 | 32 |
| | 302.1 | 121 | 22 |
| | 302.1 | 164 | 15 |
| Ractopamine-$D_5$ | 307.1 | 107 | 31 |
| | 307.1 | 121 | 21 |
| | 307.1 | 167.1 | 15 |

## 2.3 preparation of standard solution

Imbibe 0.5mL salbutamol -D3 standard solution to a 50mL volumetric flask, filled with methanol to capacity,

get 1μg/mL salbutamol -D3 standard solution.

Imbibe 0.5mL clenbuterol hydrochloride -D9 standard solution to a 50mL volumetric flask, filled with methanol to capacity, get 1 μg/mL clenbuterol -D9 standard solution.

Weigh accurately 10mg ractopamine -D5 standard to a 50mL volumetric flask, filled with methanol to capacity, get ractopamine -D5 standard solution 0.2mg/mL. From the ractopamine -D5 standard solution of 0.2mg/mLdraw 0.25mL into 50mL volumetric flask. Filled with methanol to Volume,get 1 μg/mL ractopamine -D5 standard solution.

Imbibe 0.5mL salbutamol standard solution to a 50mL volumetric flask, filled with methanol to capacity, get 1μg /mL standard salbutamol solution. From the 1μg /mL standard salbutamol solution draw 5mL into 50mL volumetric flask, filled with methanol to volume, get 100ng/mL salbutamol standard solution.

Accurate draw 0.5mL clenbuterol standard solution to a 50mL volumetric flask, filled with methanol to capacity, get 1μg/mL clenbuterol standard solution. Accurate draw 5mL1μg /mL clenbuterol standard solution into 50mL volumetric flask, filled with methanol to volume 50mL, get 100ng/mL clenbuterol hydrochloride standard solution.

Learn 0.5mL accurate standard ractopamine solution to a 50mL volumetric flask, with methanol to capacity, get 1μg /mL ractopamine standard solution. Draw 5mL 1μg /mL ractopamine standard solution into 50mL volumetric flask, filled with methanol to volume, get 100ng/mL ractopamine standard solution.

Draw 0.5, 1, 2, 4, 5mL 100ng/mL clenbuterol standard solution and 100ng/mL salbutamol standard solution 100ng/mL ractopamine standard solution to 5 different 50mL flasks, then draw 0.5mL 1μg /mL ractopamine -D5 standard solution ,0.5 mL 1μg /mL clenbuterol hydrochloride -D9 standard solution , 0.5mL 1μg /mL standard solution salbutamol -D3 to each flask, add methanol to volume 50mL. Get the1,2,4,8,10.0ng/mL clenbuterol ,salbutamol and ractopamine standard solution. Each mass concentration was injected 10 μL into liquid chromatography tandem mass spectrometry, and the standard curve was drawn by internal standard method.

### 2.4 Sample pretreatment

#### 2.4.1 Sample extraction

Weigh 5.0g (accurate to 0.01g) samples in 50 mL centrifuge tube, add 50 μL the 1000ng/mL clenbuterol–D9 standard solution, 1000ng/mL salbutamol-D3 standard solution and 1000ng/mL ractopamine –D5 standard solution respectively, add 0.2mol/L ammonium acetate buffer solution 5mL, adding beta Glucuronidase / Arylsulfatase solution 40 μL. One minute vortex oscillation. Then put into the incubator under 37℃ constant temperature for 17 hours, after the time out add methanol to 25mL, vortex extracted for 1 min. centrifuge for 5 minutes by high speed centrifuge with 8000rpm/min, take 5 mL supernatant to be purified.

#### 2.4.2sample purification

MCX solid phase extraction column should be active by 3mL methanol, 3mL water and 3mL 2% formic acid water Sequentially, take 5 mL supernatant through the column, then flush the MCX solid phase extraction column with 3mL 2% formic acid and 3mL methanol Sequentially, then eluted the MCX solid phase extraction column by 3% ammonia methanol solution, the eluent is dried by nitrogen at 37℃, volume to1mL by methanol, then vortex 1 minute.take the Sample solution filted by 0.22 μm organic membrane. then inject 10 μL Sample solution into liquid chromatography tandem mass spectrometry.

## 3 Results and discussion

### 3.1 Sample pretreatment

Because some components are not soluble in methanol, after adding methanol it can make some organic ingredients in sample solution precipitate, a better purified sample liquid can be getten after high-speed centrifugation.

### 3.2 Chromatography and mass spectrometry

The three components can be separated by the column C18 (100mm * 2.1mm, 3μm), and the retention time is suitable. By using mass spectrometry detector’s self optimizing function in the mass spectrometer, three ion pairs were auto selected for each component, the strongest abundance ion is choosen as quantitative ion, the other two are choosen as qualitative reference ion. The optimal collision energy of each ion pair is optimized by the workstation, and the collision energy is shown in Table 1 to table 3.

### 3.3 Chromatogram

Under the above 1.2 conditions of chromatography and mass spectrometry, get the total ion of three standard and three internal standard in SRM mode, the total ion flow diagram of blank sample, the total ion flow diagram

of spiked blank samples, as shown in Fig.1 to Fig.4.

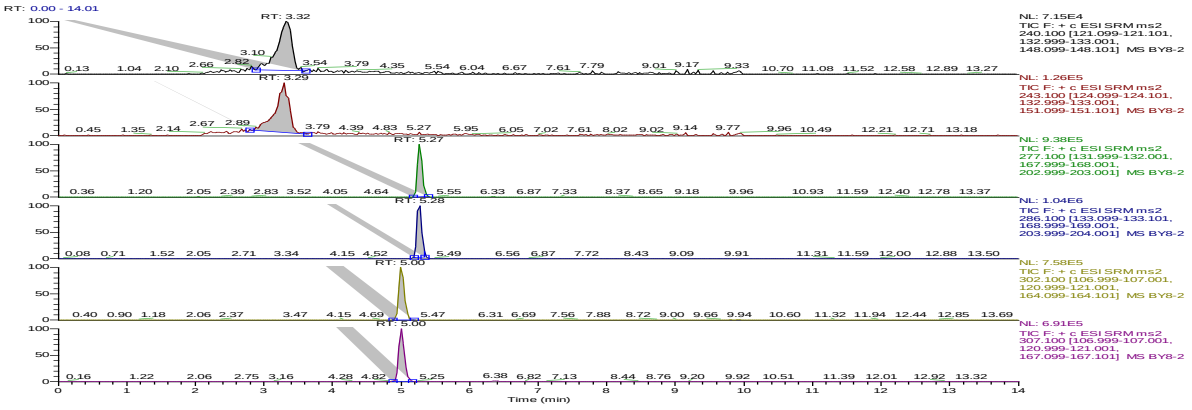

Fig.1 TIC chromatogram of standard sample in SRM mode

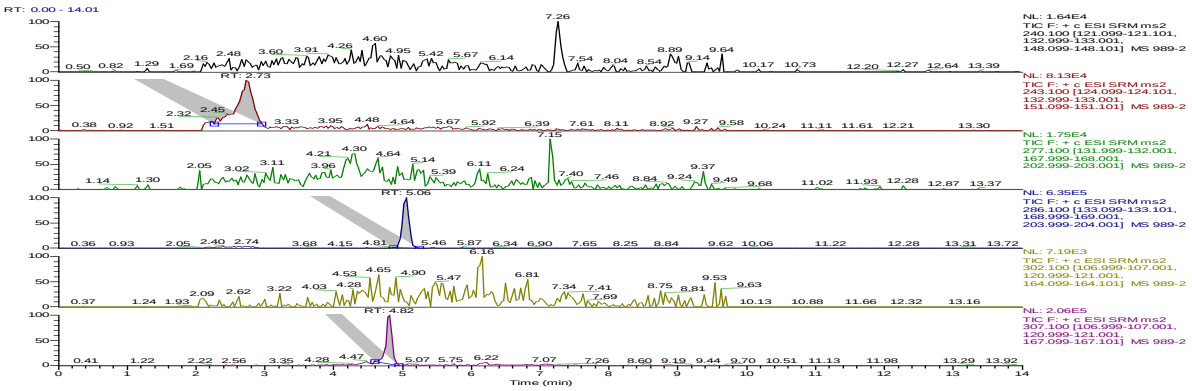

Fig.2 TIC chromatogram of unknown sample in SRM mode

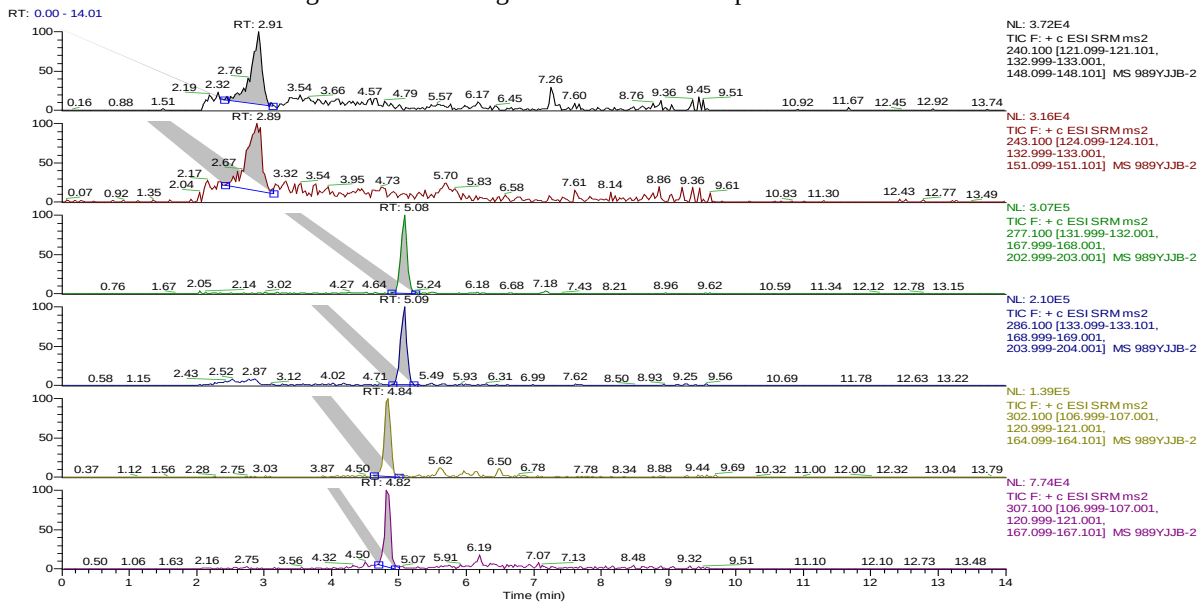

Fig.3 TIC chromatogram of unknown sample added with standard sample in SRM mode

## **3.4** Standard curve and minimum detection limit

Under the experimental conditions, select the area ratio of peak area of standard to isotope internal standard peak area as the Y, the mass concentration (ng/mL) as X to form standard curve and get the linear equation.Take the mass concentration of 3 times the baseline noise as the detection.[8] Regression equation, correlation coefficient and detection limit are shown in table 4.

## **3.5 Precision and recovery test**

Blank pork samples were spiked for recovery experiments. 100ng/mL salbutamol, clenbuterol and ractopamine standard solution were added to different 5g samples by 50,250,500μL respectively, and 1000ng/mL salbutamol-D3, 1000ng/mL clenbuterol -D9, 1000ng/mL ractopamine -D5 standard solution were added in by 50 μL, get the samples with the content of salbutamol, clenbuterol hydrochloride ractopamine were 1, 5,10 μg/kg respectively. According to the above 1.3.2 sample processing method for sample processing. According to the above 1.2 conditions of chromatography and mass spectrometry, the results of precision and recovery are shown in table 5. From table 5 we can see, the data obtained by this method can meet the test requirements.

Tab.4 Basic parameter of regression equation

| Analyte | Concentration range /（ng·mL$^{-1}$） | Linear regression equation | correlation coefficient | detection limit /（ng·mL$^{-1}$） |
|---|---|---|---|---|
| salbutamol | 1～10 | y=0.1442x+0.0164 | 0.9978 | 0.051 |
| clenbuterol hydrochloride | 1～10 | y=0.2205x+0.0284 | 0.9991 | 0.012 |
| Ractopamine | 1～10 | y=0.286x-0.0149 | 0.9997 | 0.018 |

Tab.5 Spiked recovery and precision

| Analyte | Spiked level /（μg·kg$^{-1}$） | average value /(μg.kg$^{-1}$) | Average recovery/% | RSD /% |
|---|---|---|---|---|
| salbutamol | 1 | 0.81 | 81 | 1.81 |
| | 5 | 4.55 | 91 | 2.65 |
| | 10 | 8.29 | 82.9 | 0.98 |
| clenbuterol hydrochloride | 1 | 0.88 | 88 | 3.36 |
| | 5 | 4.31 | 86.2 | 2.09 |
| | 10 | 9.45 | 94.5 | 2.61 |
| Ractopamine | 1 | 0.86 | 86 | 4.43 |
| | 5 | 4.65 | 93 | 5.10 |
| | 10 | 9.36 | 93.6 | 2.85 |

## 4 Conclusion

Through the experiment, a method of determination salbutamol, clenbuterol and ractopamine in pork by liquid chromatography tandem mass spectrometry was established. The method is simple, stable and accurate, and can meet the requirements of detection. This method can provide scientific reference and technical support for the inspection and monitoring salbutamol, clenbuterol and ractopamine in pork.

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