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Postprint: Development of an Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry Method for the Determination of Five -Agonist Residues in Porcine Liver, Beef, and Mutton

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Abstract

This study aimed to establish a rapid UPLC-MS/MS method for the detection of five -agonist residues in pig liver, beef, and mutton. Samples were extracted with 80% acetonitrile, subjected to single-step cleanup using a PRiME HLB solid-phase extraction column, concentrated under a stream of nitrogen, and finally reconstituted in 10% methanol. Gradient elution was performed using 10 mmol/L ammonium acetate aqueous solution and 10 mmol/L ammonium acetate methanol solution (pH=5) as the mobile phase, detection was carried out in multiple reaction monitoring mode, and quantification was performed using the internal standard method. The five -agonists exhibited good linearity in the concentration range of 0.5-50.0 ng/mL, with R^2 values of 0.99963-0.99996. At spiking levels of 0.5, 1.0, and 5.0 g/kg, the recoveries of the five -agonists were 93.7%-106.1% in pig liver, 95.2%-104.0% in beef, and 95.1%-106.0% in mutton. The experimental results demonstrated that the method possesses good accuracy and repeatability. Based on a signal-to-noise ratio (S/N) of 3 for the characteristic ion chromatographic peaks, the limits of detection (LODs) for the five -agonists were 0.01-0.04 g/kg, and the limits of quantification (LOQs) were 0.02-0.15 g/kg. This method is simple to operate, highly sensitive, and accurate, making it suitable for the detection and analysis of -agonist residues in large batches of livestock products.

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Full Text

UPLC-MS/MS Method for the Determination of Five -agonists Residue in Pork Liver, Beef and Mutton

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Abstract

This study aimed to establish a rapid UPLC-MS/MS method for detecting five -agonists in pork liver, beef, and mutton. Samples were extracted with 80% acetonitrile, purified through a single-step PRiME HLB solid-phase extraction column, concentrated by nitrogen blowing, and finally reconstituted with 10% methanol. Gradient elution was performed using 10 mmol/L ammonium acetate aqueous solution and 10 mmol/L ammonium acetate methanol solution (pH=5) as the mobile phase. Detection employed multiple reaction monitoring (MRM) mode with internal standard quantification. The five -agonists showed good linearity in the concentration range of 0.5–50.0 ng/mL, with correlation coefficients (R^2) between 0.99963 and 0.99996. At spiked concentrations of 0.5, 1.0, and 5.0 g/kg, the average recoveries were 93.7%-106.1% in pork liver, 95.2%-104.0% in beef, and 95.1%-106.0% in mutton. The method demonstrated good accuracy and repeatability. Based on a signal-to-noise ratio (S/N) of 3, the detection limits ranged from 0.01 to 0.04 g/kg, while the quantitation limits ranged from 0.02 to 0.15 g/kg. This simple, sensitive, and accurate method is suitable for high-throughput detection of -agonist residues in animal products.

Keywords: UPLC-MS/MS; -agonists; animal products

-agonists are animal stimulants that promote protein synthesis and reduce fat deposition. When added to livestock feed, they significantly improve carcass lean meat percentage and feed conversion efficiency. However, the high dosages and prolonged administration periods commonly used in livestock production often result in excessive -agonist residues in animal products, which can be transferred through the food chain and accumulate in the human body, causing health damage. Human poisoning from -agonists manifests as severe palpitations, nausea, vomiting, and increased heart rate, which can be life-threatening in severe cases. The Ministry of Agriculture of the People' s Republic of China has explicitly prohibited the use of -agonists in all animal-derived foods. Nevertheless, some farmers continue to use these drugs in livestock production driven by economic interests. Therefore, establishing detection methods for -agonist residues in animal products to trace illegal producers through the production chain plays a crucial role in food safety supervision.

Common detection methods for -agonists include immunoassays (such as

enzyme-linked immunosorbent assay, colloidal gold immunochromatography, and chemiluminescence immunoassay) and chromatographic methods (high-performance liquid chromatography, gas chromatography-mass spectrometry, and liquid chromatography-mass spectrometry). In recent years, with the widespread application of liquid chromatography-mass spectrometry, detection methods for -agonists have been continuously optimized. This study developed a rapid UPLC-MS/MS method for detecting five -agonists (shown in [Figure 1: see original paper]) in pork liver, beef, and mutton, aiming to provide a reference for high-throughput detection of these residues in livestock products.

Molecular Structures of Five -agonists

[Figure 1: see original paper] Molecular structures of five -agonists

1.1 Instruments and Reagents

Instruments: UPLC-TQS Micro ultra-high performance liquid chromatography-tandem mass spectrometer with electrospray ionization source (Waters, USA); electronic balance (Sartorius, Germany); high-speed refrigerated centrifuge (Sigma, Germany); solid-phase extraction apparatus (Supelco, USA); nitrogen evaporator (Organomation, USA); PRiME HLB solid-phase extraction columns (Waters, USA); precision pH meter (Shanghai Precision Scientific Instrument Co., Ltd.); basic orbital mixer and digital orbital shaker (IKA, Germany).

Reagents: Ractopamine (98% purity), clenbuterol (99.1% purity), salbutamol (99.2% purity), cimaterol (99.9% purity), and clorprenaline (99.9% purity). Internal standards: ractopamine-D3 (99% purity), clenbuterol-D9 (99.4% purity), salbutamol-D3 (98.4% purity), cimaterol-D7 (99.3% purity), and clorprenaline-D7 (99.8% purity). Methanol and acetonitrile were HPLC grade (Merck); water was ultrapure; other reagents were analytical grade.

Standard stock solutions: Appropriate amounts of the five -agonist standards and internal standards were dissolved in pure methanol to prepare individual stock solutions at 1 mg/mL, stored at -18 °C in the dark. Mixed standard solutions were prepared by accurately transferring appropriate volumes of individual standard solutions and diluting with pure methanol to obtain a 1 g/mL mixed solution. Mixed internal standard solutions were prepared similarly and stored at 4 °C.

1.2 Analytical Conditions

Chromatographic conditions: Column: Waters Acquity BEH-C18 (2.1 mm × 100 mm, 1.7 m); injection volume: 4 L; column temperature: 30 °C; mobile phase A: 10 mmol/L ammonium acetate (pH=5), mobile phase B: 10 mmol/L ammonium acetate in methanol (pH=5); flow rate: 0.3 mL/min; gradient elution program shown in .

Mass spectrometry conditions: Electrospray ionization source in positive

ion mode; capillary voltage: 0.6 kV; ion source temperature: 500 °C; desolvation gas flow rate: 1000 L/hr.

1.3 Sample Pretreatment

Accurately weigh 2.00 g of homogenized sample into a 50 mL centrifuge tube, add appropriate internal standards, then add 10 mL of 80% acetonitrile aqueous solution. Vortex for 1 min, shake for 10 min extraction, and centrifuge at 10,000 r/min for 10 min. Transfer 6 mL of supernatant directly onto a 6 mL PRiME HLB solid-phase extraction column at a flow rate of 1 s per drop, collect all eluate, and mix thoroughly by shaking. Evaporate 4 mL of eluate to near dryness under nitrogen at 50 °C. Reconstitute with 1 mL of 10% methanol aqueous solution, filter through a 0.2 m microporous membrane, and the solution is ready for analysis.

2.1 Optimization of Chromatographic Conditions

Three mobile phases were compared: 0.2% formic acid aqueous solution–40% methanol acetonitrile solution, 0.1% formic acid acetonitrile solution–0.1% formic acid aqueous solution, and 10 mmol/L ammonium acetate aqueous solution–10 mmol/L ammonium acetate methanol solution (pH=5). The presence of ammonium acetate in the mobile phase yielded better peak shapes and higher response values. Therefore, 10 mmol/L ammonium acetate aqueous solution–10 mmol/L ammonium acetate methanol solution (pH=5) was selected for gradient elution. The characteristic ion chromatograms of the five -agonists under positive ion scanning are shown in [Figure 2: see original paper].

[Figure 2: see original paper] Positive ion scanning characteristic ion chromatograms of five -agonists and isotopic internal standards

MRM: multiple reaction monitoring; RAC: ractopamine; CLE: clenbuterol; SAL: salbutamol; CIM: cimaterol; CLO: clorprenaline; RAC-D3: ractopamine-D3; CLE-D9: clenbuterol-D9; SAL-D3: salbutamol-D3; CIM-D7: cimaterol-D7; CLO-D7: clorprenaline-D7.

2.2 Optimization of Mass Spectrometry Conditions

Mass spectrometry conditions were optimized in positive electrospray ionization mode (ESI+). A 100 ng/mL mixed standard solution was infused at 5.0 L/min for primary mass spectrometry scanning to obtain suitable precursor ion conditions. Secondary scanning of precursor ions was performed while adjusting collision energy and cone voltage. The fragment ion with the strongest signal was selected as the quantification ion, and the second strongest as the qualification ion. The optimized MRM conditions including ion pairs, cone voltage, and collision energy are shown in .

MRM conditions of five -agonists and isotopic internal standards

2.3 Method Linearity and Detection Limits

Mixed standard solutions of the five -agonists were accurately measured and diluted with 10% methanol aqueous solution to prepare standard working solutions at concentrations of 0.5, 2.0, 5.0, 10.0, 20.0, and 50.0 ng/mL, each containing 5 ng/mL internal standards for LC-MS/MS analysis. Using the internal standard method, standard curves were plotted with the peak area ratio of each characteristic ion to its corresponding isotopic internal standard as the y-axis and the standard solution concentration as the x-axis. The regression equations and correlation coefficients are shown in . The five -agonists exhibited good linearity in the concentration range of 0.5-50 ng/mL, with linear correlation coefficients (R^2) between 0.99963 and 0.99996. Method detection limits were calculated based on a signal-to-noise ratio (S/N) of 3, yielding values of 0.01-0.04 g/kg. Quantitation limits based on S/N=10 were 0.02-0.15 g/kg.

Linear equation and correlation coefficient of five -agonists

2.4 Recovery

Blank samples of pork liver, beef, and mutton were spiked at three different concentrations (0.5, 1.0, and 5.0 g/kg) and subjected to recovery tests following the procedure in section 1.3. Each level was repeated three times to calculate average recovery and relative standard deviation (RSD). The results are shown in . Recoveries ranged from 93.7% to 106.1% in pork liver, 95.2% to 104.0% in beef, and 95.1% to 106.0% in mutton. These results indicate satisfactory recoveries that meet the requirements for routine detection of the five -agonists in pork liver, beef, and mutton.

Average recovery ratio and RSD of five -agonists in pork liver, beef and mutton (n=3)

2.5 Precision

Pork liver, beef, and mutton samples were prepared with positive spikes at three concentrations (0.5, 1.0, and 5.0 g/kg) following the pretreatment method in section 1.3. Each level was analyzed six times to calculate within-day precision RSD. The same procedure was repeated over three consecutive days to determine day-to-day precision RSD, with results shown in . Within-day precision RSDs were 0.6%-4.5% in pork liver, 0.9%-4.1% in beef, and 1.2%-3.6% in mutton. Day-to-day precision RSDs were 0.5%-4.4% in pork liver, 0.9%-5.3% in beef, and 1.2%-4.1% in mutton. These results demonstrate good repeatability and day-to-day precision, satisfying the requirements for routine detection.

Precision RSD of five -agonists in pork liver, beef and mutton (n=6)

3.1 Optimization of Pretreatment Steps

Livestock products contain substantial amounts of protein and fat. In this study, acetonitrile/water solvent was used to precipitate proteins, which were

removed by centrifugation. PRiME HLB cartridges were then employed for simple and rapid purification. Conventional solid-phase extraction requires four steps: activation, loading, washing, and elution. In this procedure, sample extracts were directly loaded onto PRiME HLB columns, eliminating the three steps of activation, washing, and elution while simultaneously removing complex matrices such as fats, thus achieving one-step purification. The purified sample solutions were concentrated using a 24-well nitrogen evaporator for batch rapid processing. This pretreatment approach is time-saving and efficient, improving detection throughput.

3.2 Optimization of LC-MS Conditions

This method compared different mobile phases and ultimately selected 10 mmol/L ammonium acetate aqueous solution–10 mmol/L ammonium acetate methanol solution (pH=5) for gradient elution. By optimizing the gradient elution program, the retention times of all five -agonists were achieved within 6 minutes with good peak shapes. Using positive electrospray ionization mode, precursor and product ion information for the five -agonists was obtained to determine optimal quantification and qualification ion pairs. Ion source parameters were optimized to achieve satisfactory response values.

4 Conclusion

At concentrations of 0.5–5.0 g/kg, the method achieved recoveries of 93.7%–106.1% in pork liver and 95.2%–104.0% in beef. The detection limits for the five -agonists were 0.01–0.04 g/kg, with quantitation limits of 0.02–0.15 g/kg. This method offers good accuracy and repeatability, is simple to operate, highly sensitive, and accurate, making it suitable for high-throughput detection of -agonist residues in animal products.

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