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Simultaneous determination of β-agonists in pork samples using liquid chromatography-tandem mass spectrometry

[1]Hanh, V.T., [2]Duong, N.V., [3]Ninh, L.T., [3]Vu, N.X. and [4,*]Chau, L.M.

[1]*Faculty of Food Science and Technology, Vietnam National University of Agriculture, Trau Quy town, Gia Lam district, Hanoi capital, Vietnam*
[2]*The Center for Technical Services and Professional Training in Agro-Forestry-Fishery Quality, Thai Nguyen Department of Agriculture, Forestry and Fishery Quality Assurance, Phan Dinh Phung ward, Thai Nguyen City, Vietnam*
[3]*Faculty of Biotechnology and Food Technology, Thai Nguyen University of Agriculture and Forestry, Quyet Thang commune, Thai Nguyen City, Vietnam*
[4]*Faculty of Animal Science and Veterinary Medicine, Thai Nguyen University of Agriculture and Forestry, Quyet Thang commune, Thai Nguyen City, Vietnam*

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Abstract

The misuse of β-agonists has been reported to be a potential risk to public health and is forbidden in many countries. The present study aimed to develop a simple, rapid, and reliable method for the multi-residue analysis of β-agonists including salbutamol, clenbuterol, and ractopamine in pork samples using liquid chromatography/tandem mass spectrometry (LC-MS/MS). In the proposed method, the pretreatment procedure was shortened to reduce time-consuming and chemical usage was minimized to decrease cost-consuming. Based on the LC-MS/MS experiments, linearities obtained ranged from 0.1–2 µg/kg for clenbuterol and 1–20 µg/kg for salbutamol and ractopamine, with a correlation coefficient of R^2 value of >0.998. The average recoveries of clenbuterol, salbutamol, and ractopamine were 105.3%, 101.2%, and 99.4%, respectively. The limits of quantification for clenbuterol, salbutamol, and ractopamine were 0.10, 0.54, and 0.63 µg/kg, respectively. These results suggested that this proposed method can be used for rapid and sensitive detection of β-agonists in pork with a simple treatment of samples and minimized solvent usage.

1. Introduction

Beta-agonists are a group of synthetic compounds which are commonly used in livestock as growth-promoting agents to improve carcass composition by decreasing fat to increase muscle mass due to increased economic benefits (Byrem *et al.,* 1998; Stolker and Brinkman, 2005). The misuse of β-agonists leads to potential risk factors for human health, particularly in patients with symptoms such as muscular tremors, cardiac palpitation, nervousness, headaches and muscular pain (Martínez-Navarro, 1990; Brambilla *et al.,* 2000). Although they are used in human and veterinary medicine for the treatment of lung diseases, the use of β-agonists as growth promoters in cattle is forbidden in many countries and the European Union (Stachel *et al.,* 2003). In Vietnam, many producers illicit the usage of β-agonist in animal feed due to economic benefits. A wide variety of β-agonists were added to feeds and their detection was often missed by government monitoring and inspection bodies. Therefore, it is highly required to develop a sensitive, rapid, reliable and simple method for analysis of these agents in meat products to monitor food quality and safety.

Several analytical approaches used to determine β-agonists include enzyme immunoassay (EIA) (Degand *et al.,* 1993), enzyme-linked immunosorbent assay (ELISA) kits (Angeletti *et al*., 1993), liquid chromatography (LC) methods coupled with different detection systems such as ultraviolet (UV) (Yan *et al.,* 2016) and diode array detector (DAD) (Blomgren *et al.,* 2002), gas chromatography-mass spectrometry (GC-MS) (He *et al.,* 2007; Liu *et al.,* 2009; Cheng *et al.,* 2013) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Pan *et al.,* 2015; Lin *et al.,* 2017; Jeong *et al.,* 2018). Among them, LC-MS/MS-multiple reaction monitoring (MRM) is currently considered the most

*Corresponding author.
Email: *leminhchau@tuaf.edu.vn*

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suitable technique to detect multiple classes of veterinary drugs in foodstuffs due to its highly selective and sensitive detection of analytes, indicating that it provides unambiguous identification and reliable confirmation (Dasenaki and Thomaidis, 2015).

In the past years, many analytical methods using LC-MS/MS-MRM were developed for the determination of β-agonists in pork (Pan *et al.,* 2015), muscle and viscera (Lin *et al*., 2017) and bovine tissue (Jeong *et al*., 2018). However, these methods still suffer disadvantages such as being time-consuming or cost-consuming because complex treatments of samples (e.g. enzymolysis prior to organic solvent, solid-phase extraction, combining QueEChERS procedure) are needed prior to analysis of analytes.

Taking these factors into account, this study attempted to develop a rapid, simple, and reliable LC-MS/MS quantification assay for the analysis of three β-agonists in pork with simple sample preparation. Clenbuterol, ractopamine, and salbutamol were selected as target analytes in this study because they are the most commonly used β-agonists (Liu *et al.,* 2009).

2. Materials and methods

2.1 Materials

Pork samples were purchased from the local market (Thai Nguyen, Vietnam). The tissues were homogenized in ice-cold and stored at -30℃ until analysis was begun.

2.2 Chemicals

Distill water, acetonitrile (ACN), methanol (MeOH), formic acid (FA) and ammonium acetate were of LC-MS grade and purchased from Merck (Darmstadt, Germany). Three β-agonists (salbutamol, clenbuterol, and ractopamine) and a corresponding internal standard (clenbuterol-d9, IS) were obtained from Dr. Ehrenstorfer GmbH (Germany). The minimum purity of all standards was 98.0%. All other reagents were of analytical reagent grade and were used without further purification.

2.3 Preparation of standards

Individual stock solutions (1000 µg/mL) and IS (1000 µg/mL) were in MeOH and then stored at -20℃ in brown glass to prevent photodegradation. Mixed standard solutions at a concentration of 10 µg/mL were prepared by a combination of three stock solutions. The mixed standard solutions were stored at 20℃ and prepared daily prior to experiments by further dilution with MeOH to give appropriate working solutions. Three separate replicates of the mixed working solution at six final different concentrations (between 0.1 and 2 µg/kg for clenbuterol; 1 and 20 µg for salbutamol and ractopamine) were conducted to obtain calibration standards.

2.4 Sample preparation

A 2.0 g aliquot of homogenized pork samples was transferred into a 50 mL falcon tube and spiked as appropriate with target analytes and 50 µL of IS at 1000 µg/mL. Thereafter, 10 mL of 0.1 M dipotassium hydrogen phosphate (K_2HPO_4) at pH 6 was added, and the tubes were vortexed for 1 min and shaken for 10 min. Subsequently, ultrasonic extraction was performed for 15 mins and then centrifuged at 4,000×*g* for 10 mins. The supernatant was transferred to a 15 mL falcon tube. An SCX cartridge was activated with 3 mL of MeOH, 3 mL of distilled water, and 3 mL of 0.1 M K_2HPO_4. The supernatants (6 mL) were applied to the SCX cartridge at an elution rate of 1–2 mL/min, which was then successively rinsed with 3 mL of distilled water and 1 mL of MeOH and dried in a vacuum. The elution of the β-agonist fraction from the SCX was performed with 3 mL of 5 ammonia in ACN. Finally, the effluent was evaporated by Syncrone at 40℃ and recontinued to 1 mL of the initial mobile phase. The resultant solution was filtered using a 0.22-µm membrane, and an aliquot (20 µL) of the filtrate was injected into the LC-MS/MS system.

2.6 LC-MS/MS conditions

A system for successive determination of three β-agonists in pork samples was performed on an Exion HPLC instrument using API Sciex Triple Quadrupole 3200 mass spectrometer equipped with electrospray ionization (USA), and data were collected in a positive ionization mode. Chromatographic separation was performed on a Phenomenex C_{18} column (3 µm pore size, 2×50 mm) maintained at 40℃. For the separation of the target analyte, and elution of 5–95% MeOH containing 0.1% FA at a flow rate of 0.35 mL/min was performed as follows (time in minutes per percent solvent B): 0.0/5, 1.5/5, 4/45, 6/70, 6.5/85, 7.2/95, 7.6/5, and 10/5 (total run time = 10 mins). The MS/MS parameters were as follows: capillary voltage, 5,500 V; curtain gar, 25 psi; collision gas, 5 psi; nebulizer gas (gas 1) and heater gas (gas 2), 50 each; temperature, 450℃. The optimal MRM conditions are summarized in Table 1.

2.7 Linearity

Muscle calibration curves were used to test the linearity of the developed method and quantify β-agonists in pork samples. The blank muscle samples were conducted at five different concentrations of 0.1–2 µg/kg for clenbuterol and 1–20 µg for salbutamol and ractopamine. Sample preparation was executed

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Table 1. Chromatographic conditions used in the detection and quantitation of three β-agonists residues in pork samples by LC-MS/MS-MRM.

Analyte	Retention time (min)	Polarity	Precursor ion (*m/z*)	Product ions (m/z)	Quantitative ions (*m/z*)	Collision energy (eV)
Clenbuterol	3.79	Positive	277	203.1	203.1	25
				259.2		19
Salbutamol	3.69	Positive	240	148.0	148.0	24
				222.2		13
Ractopamine	3.78	Positive	302	164.2	164.2	25
				284.0		19
Clenbuterol-d9	3.79	Positive	268.2	203.9	203.9	25

according to the aforementioned method. The internal standard (IS) clenbuterol-d9 was added with a final concentration of 0.5 µg/kg. The ratio of the peak area of analytes to that of IS was used for the assay. The linearity of this method was evaluated using the regression coefficient measured for the analyte. A correlation coefficient (R^2) > 0.99 was accepted to evaluate the linearity of the developed method.

2.8 Limits of detection and limits of quantification

Limits of detection (LODs) and limits of quantification (LOQs) were determined by analyzing blank samples spiked at 0.10 µg/kg for clenbuterol and 1.00 µg/kg for salbutamol and ractopamine for which a signal-to-noise ratio was >3 (S/N = 3) and 10 (S/N = 10), respectively.

2.9 Recovery and precision

The accuracy and precision were expressed as the percentage of recovery and the coefficient of variation (CV). Both the recovery and precision were assessed by repeatability and intra-laboratory reproducibility experiments. Recovery and precision were evaluated by spiking blank pork samples with mixed standard solutions at three concentration levels (0.1, 0.2 and 0.5 for clenbuterol; 1.0, 2.0 and 5.0 µg/kg for salbutamol and ractopamine) in ten replicates at each level.

3. Results and discussion

3.1 Development of the LC-MS/MS

The LC-MS/MS method was developed to provide confirmatory data for the determination of three β-agonists in pork. In the LC-MS/MS chromatogram (Figure 1), peaks of pure clenbuterol, salbutamol, and ractopamine spiked in pork samples were observed at the retention time of 3.79, 3.69, and 3.78, respectively. As shown in Figure 1 and Table 1, the production ions with the stronger signal (clenbuterol, *m*/z 203.1; salbutamol, *m*/z 148.0; and ractopamine, *m*/z 164.2) were selected as the ion for quantification. These results were in agreement with the retention time and production of ions reported by other studies (Zhang *et al.,* 2013; Pan *et al*., 2015; Jeong *et al*., 2018). Generally, to optimize the LC separation of the target analytes, it required several trials to select a suitable mobile phase composition and linear elution gradient for developing a method due to the complexity of the chemical compounds in biological samples, and affinities of the components toward various solvents. It was also recommended that the proportions of the organic solvents and aqueous phases need adjusting to obtain a reasonable and suitable retention time and sharp peaks. Some researchers reported that a mobile phase consisting of ammonium acetate as ion-pair reagents in water and methanol or acetonitrile greatly improved peak shape and facilitated resolving closely eluted compounds (Blomgren *et al.,* 2002; Pan *et al*., 2015; Yan *et al*., 2016; Lin *et al*., 2017). It was considered that the proportion of water in the mobile phase is low, inorganic acid or salt easily precipitates from the mobile phase, resulting in very tiny precipitate particles that can damage the pump head of the HPLC instrument (Yan *et al*., 2010).

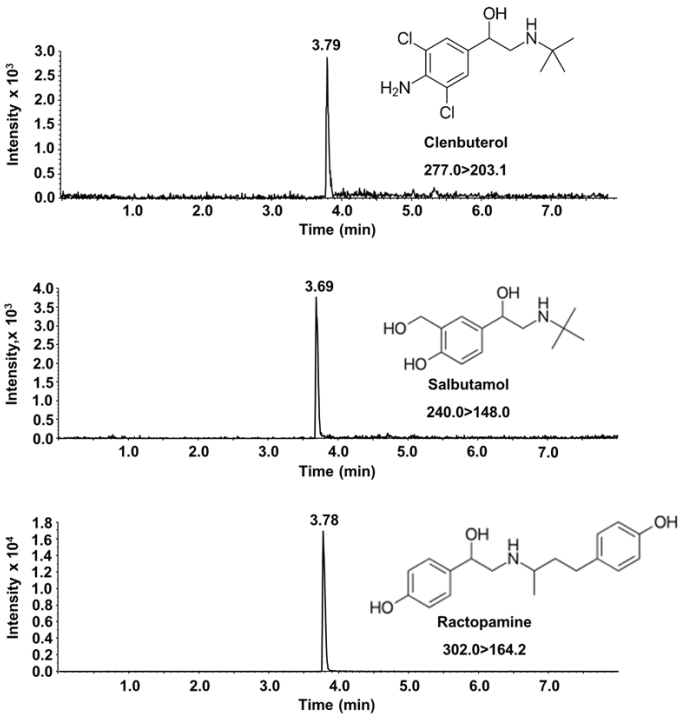

Figure 1. Typical chromatograms of three β-agonists residues spiked at 0.1 µg/kg for clenbuterol and 1 µg/kg for salbutamol and ractopamine in pork samples.

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Thus, formic acid (FA) was used and methanol (MeOH) as the mobile phase, which was readily soluble in organic solvents. The results showed that the retention time and sharp peaks of clenbuterol, salbutamol, and ractopamine were obtained, indicating that optimized mobile phase composition and linear elution gradient were reasonable (Figure 1).

3.2 Sample extraction and clean-up

The extraction procedure of β-agonists from meat and other tissues plays an essential part in analytical methods. Lin *et al.* (2017) showed that salt and endogenous compounds cannot be fully removed due to the complexity of the biological matrices and the trace levels in real samples, leading to possible effects. Because the complexity of the pork samples may be a major contributing source of matrix effects in LC-MS/MS-based bioanalytical methods, the solid-phase extraction (SPE) method using different reversed-phase absorbents was usually applied to extract β-agonists (Liu *et al.,* 2011). However, these techniques were time-consuming and required large amounts of organic solvents such as MeOH and ACN. To improve efficiency and reduce time-consuming sample preparation, Lin *et al*. (2017) used the QuEChERS method for the cleaning of target analytes in tissue extracts. The procedure began with sodium acetate buffer solution extraction and enzymatic hydrolysis and then used primary secondary amine (PSA) and C18EC for extraction of β-agonists from pork samples. In another study, Pan *et al*. (2015) compared the SPE clean-up technique followed by ultrasonic-assisted extraction with the enzymolysis method in the extraction of β-agonists from pork samples. It was reported that 0% recovery was obtained regardless of how many enzymes were used, and recovery rates were not significant differences between the two methods for extracting bound β-agonists. By contrast, Jeong et al. (2018) developed the LC-MS/MS method to determine β-agonists in bovine tissues without using the SPE technique, resulting in the low recovery of ractopamine (74%) and sensitivities of clenbuterol (LOD, 0.5 μg/kg) and ractopamine (1.7 μg/kg) were observed. It was also considered that very tiny matrics from biological samples can damage the pump head and the column of the HPLC instrument.

In this study, the extraction and purification of the analytes from fork samples were mainly performed by SCX cartridges. The SCX cartridges with a strong cation exchange stationary solid phase were used because they promote the isolation based on the ion exchange of the functional groups and consequently can separate the analytes of interest from other close polarity interference compounds which is not possible when applying regular C_{18} cartridges.

3.3 Linearity

The linearity of the analytical method was validated using the pork calibration curves for each analyte at different concentration levels spiked with IS at the final concentration of 0.5 μg/kg to prevent matrix effects. As shown in Table 2, linear ranges were 0.1–2 μg/kg for clenbuterol and 1–20 μg/kg for salbutamol and ractopamine with coefficients of correlation (R^2) >0.998 under the optimal LC-MS/MS conditions. These results indicated that the linearity was good for all analytes in the range of tested concentration as proved by the above coefficient correlation for all calibration curves.

3.4 Limit of detection and limit of quantification

The limit of detection (LOD: 3.3 SD/slope) and the limit of quantitation (LOQ: 10 SD/slope) for the three β-agonists analyzed in this work were >0.03 μg/kg and >0.1 μg/kg (Table 2). The sensitivity of clenbuterol (LOD: 0.03 μg/kg) and ractopamine (LOD: 0.19 μg/kg) in this work were compatible with that of the previous report (LODs of clenbuterol and ractopamine, 0.5 and 1.7 μg/kg, respectively) (Jeong *et al.,* 2018), and much higher than that of these two β-agonists by using modified QuEChERS method (clenbuterol, 1.0 μg/kg; ractopamine, 1.46) (Zhang *et al.,* 2015). Although LODs of salbutamol (0.16 μg/kg) and ractopamine (0.19 μg/kg) were slightly higher than those for both by the previous report (salbutamol, 0.049 μg/kg; ractopamine, 0.06 μg/kg), an extraction procedure of these β-agonists in the present study was more simple and faster, such as no need to adjust pH or using many chemicals during liquid -liquid extraction, evaporation and clean-up procedure (Pan *et al*., 2015).

In general, the developed method has higher

Table 2. The linearity, LOD, and LOQ of three β-agonists residues by LC-MS/MS-MRM.

Analyte	Linearity	Linear range (μg/kg)	R^2	LOD	LOQ
Clenbuterol	y = 0.195 x + 0.00269	0.1 – 2	0.9988	0.03	0.10
Salbutamol	y = 0.1 x	1– 20	0.9998	0.16	0.54
Ractopamine	y = 0.0249 x	1 – 20	0.9996	0.19	0.63

Three β-agonists were subjected to an LC-MS/MS-MRM analysis. Detailed assay conditions were described in the method section. Linearity (x), concentration (μg/kg), and y, peak area ratio (observed peak area of analytes against 0.5 μg/kg as the internal standard).

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sensitivity compared with the previous methods using LC-MS/MS analysis to determine β-agonists residues in meat products. In addition, the Codex Alimentarius Commission (CAC) recommends maximum residue limits of clenbuterol for cattle of 0.2 µg/kg in muscle and fat, 0.6 µg/kg in liver and kidney and 0.05 µg/L in cattle milk, expressed as a parent drug (CAC, 2015). The acceptable maximum residue limits of ractopamine hydrochloride for cattle and pigs are 10.0 µg/kg in muscle and fat, 40 µg/kg in the liver and 90 µg/kg in the kidney (CAC, 2015). Thus, the achieved signal intensities of the developed method were sufficient to determine β-agonists residues in pork samples.

3.5 Recovery and precision

The validation procedure was performed according to the requirements outlined in the Codex guidelines (CAC/GL 16 and 71) to evaluate the performance of the analytical method (CAC, 2014). As shown in Table 3, the mean recoveries of clenbuterol, salbutamol, and ractopamine were 105.3%, 101.2% and 99.4%, respectively, when the pork was spiked with pure analytes at three concentration levels (0.1, 0.2 and 0.5 µg/kg for clenbuterol; 1.0, 2.0 and 5.0 µg/kg for salbutamol and ractopamine) in ten replicates at each level.

The mean recoveries of all three analytes in the present study were highly satisfactory compared to those of the previous reports by using LC-MS/MS method (Zhang *et al.,* 2013; Pan *et al*., 2015; Jeong *et al*., 2018). These results also showed that more excellent recoveries of all three analytes were obtained compared to those of the HPLC-UV method (Blomgren *et al*., 2002; Yan *et al*., 2016). Jeong *et al.* (2018) reported the low recovery of analytes such as ractopamine due to the interference of the sample matrix or slowness of extraction of those compounds from the extracted matrix. Recovery experiment results for these analytes were acceptable according to the CODEX guidelines as they were higher than the 90% recovery criteria, indicating that the accuracy of the developed method was satisfactory for analyzing β-agonists residue levels in pork samples.

The precision of the assay was assessed at 0.1, 0.2 and 0.50 µg/kg for clenbuterol; 1.0, 2.0 and 5.0 µg/kg for salbutamol and ractopamine in spiked samples and expressed as the CV. The results were summarized in Table 3. The mean CV values of clenbuterol, salbutamol, and ractopamine spiked in samples were 8.65%, 4.19% and 4.12%, respectively. The CV values were less than 10% at three different concentration levels of analytes except for clenbuterol spiked at 0.1 µg/kg in samples (13.87%). The recoveries and CVs indicated that the present LC-MS/MS method would be sufficient to quantify three β-agonists in pork samples without any consideration of the matrix effect. The simple extraction procedure required a short time and minimized solvent usage but excellent sample recoveries were obtained, suggesting that the developed LC-MS/MS method was suitable for real-time and field-ready analysis.

Table 3. Recoveries and CVs of three β-agonists residues by LC-MS/MS-MRM using clenbuterol-d9 as an internal standard.

Analyte	Spiked (µg/kg)	Found (µg/kg)	Recovery (%)	CV (%)
Clenbuterol	0.10	0.11±0.02	114.00	13.87
	0.20	0.20±0.01	102.00	4.75
	0.50	0.50±0.04	100.00	7.32
Salbutamol	1.00	1.00±0.06	100.00	5.58
	2.00	2.00±0.05	100.05	2.71
	5.00	5.18±0.22	103.56	4.28
Ractopamine	1.00	1.03±0.06	102.68	5.87
	2.00	1.90±0.06	94.85	3.30
	5.00	5.03±0.16	100.66	3.18

4. Conclusion

In the present study, a simple, fast, and sensitive analytical method involving LC-MS/MS was developed and validated for the simultaneous determination of clenbuterol, salbutamol, and ractopamine in pork. The target analytes of interest were extracted SCX extraction method and analyzed using LC-MS/MS. Based on the results obtained for linearity, the limit of detection, the limit of quantification, accuracy, and precision of the developed LC-MS/MS method, it served as an accurate and reliable method for the identification and quantification of these β-agonists in pork samples.

Conflict of interest

The authors declare no conflict of interest

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