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Original Article

Determination of multiresidue analysis of β-agonists in muscle and viscera using liquid chromatograph/tandem mass spectrometry with Quick, Easy, Cheap, Effective, Rugged, and Safe methodologies

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ABSTRACT

The official analytical method of the Taiwan Food and Drug Administration, Ministry of Health and Welfare for testing for veterinary drug residues in foods is the multiresidue analysis of β-agonists. Samples are pretreated through liquid–liquid extraction and solid-phase extraction. This method is time consuming and requires the intensive use of solvents. To improve analytical efficiency and reduce costs, our study incorporated QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) techniques to establish a new method of multiresidue analysis of β-agonists in animal muscle and viscera. The pretreatment time was shortened and solvent usage was minimized. The modified analysis was conducted using liquid chromatography/tandem mass spectrometry (LC–MS/MS) and quantification was performed using multiple reaction monitoring. The results demonstrated that the correlation coefficients of the tissue calibration curve were higher than 0.99 and the limit of quantification (LOQ) was 1 ppb. The average recoveries in spiked samples varied from 70% to 120%, and the relative difference between duplicated analysis results was lower than 10%. On the basis of the results, the proposed method was concluded to be an appropriate procedure for determining the presence of β-agonists, and demonstrated the advantages of high recovery rates in spiked samples, high precision, reduced analysis time and solvent usage, and lower costs.

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1. Introduction

In recent years, the safety of meat products has been a source of great public concern [1,2] and methods of detecting drug residues have become a focus of research worldwide. Effective determination methods are required to guarantee meat safety [3,4]. Veterinary drugs are widely used at therapeutic levels in livestock breeding systems for treating various diseases; one type of these drugs, β-agonists, was originally intended to treat asthma and preterm labor in humans [5]. However, these compounds also promote lipolysis in muscle tissue and exhibit a considerable nutrition redistribution function, resulting in higher feed efficiency and a greater muscle-to-fat ratio in livestock [6–8]. β-Agonists have been utilized in livestock such as pigs and ruminants to reduce carcass fat and increase muscle mass, while improving the growth rate and feed conversion [9–12]. Previous research indicated that the residues of six β-agonists are prone to accumulate in the retinal tissue of food-producing animals [13]. Moreover, the ingestion of β-agonists deposited in animal tissues can cause acute poisoning in humans, particularly in patients with symptoms such as muscular tremors, cardiac palpitation, nervousness, headaches, and muscular pain [14]. Tissues contaminated with β-agonists appear to be harmful and pose a potential risk to human health [15]. Although their use in the therapeutic treatment of cattle with respiratory diseases is permitted, the use of β-agonists as growth promoters in cattle is forbidden in certain countries and the European Union [16]. However, illicit usage in animal feed persists in numerous countries [17]. Sensitive and specific analytical methods are therefore required to determine the level of β-agonists in meat, in order to monitor food safety. These analytical methods are essential for assessing human exposure to β-agonists and supporting the enforcement of laws and regulations.

A review of the literature shows that the methods used to monitor β2-agonist residues include high-performance liquid chromatography [18,19], gas chromatography–mass spectrometry [20,21], and liquid chromatography–mass spectrometry or liquid chromatography–tandem mass spectrometry (LC–MS/MS) [22,23]. LC–MS/MS is currently considered the most suitable technique used to detect multiple classes of veterinary drugs in foodstuffs because it provides unambiguous identification and reliable confirmation [24]. In order for a method to be deemed confirmatory, identification must be carried out according to relative retention times, the identification points of each analyte, and the relative ion ratios of selected multiple reaction monitoring (MRM) transitions. Despite the use of selective detection techniques such as MS, appropriate sample preparation is a challenging but necessary step in analytical procedures that reduces interference and averts possible matrix effects. Techniques such as immunoaffinity chromatography [25], liquid–liquid extraction [26], solid-phase extraction (SPE) [27], and matrix SPE [28] are used for extraction and purification. Most of the current extraction methods are time consuming and tedious. Additionally, the general SPE cleanup techniques lack specificity and selectivity and can mix analytes and interferents. This leads to interference and matrix effects and reduces the reliability of determination. In addition, the large quantities of organic solvents employed in these methods may cause environmental pollution [29].

Consequently, analytical laboratories are increasingly interested in developing new analytical methods that are quicker and enable higher sample throughput. In 2003, the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method for the multiclass and multiresidue analysis of pesticides in fruits and vegetables was first published by Anastassiades et al [30]. It has received widespread attention since its development and expanded beyond its application in traditional samples to include meat products, fish, blood, and even soil. The QuEChERS method is now a widely known methodology for the extraction of several classes of drugs, including pesticides and veterinary drugs, from different matrices. This method minimizes the time required for the extraction and cleaning processes and reduces the sample size, costs, required quantities of laboratory glassware, and levels of solvent consumption [31]. Additionally, the established QuEChERS pretreatment procedure without covering is simple and economical and requires only small amounts of organic solvents. This method can reduce the sample volume, solvent consumption, and required analytical time. The adaptation of the QuEChERS method for β-agonist monitoring is therefore strongly recommended. The aim of this study was to develop a rapid and easy multiresidue analytical method involving the QuEChERS procedure to determine the levels of seven β-agonists in muscle and viscera samples.

2. Materials and methods

2.1. Samples

Samples (muscle and viscera) were purchased from local supermarkets and were confirmed to be free of the target drugs. The tissues were homogenized and stored at −20°C until analysis was begun.

2.2. Chemicals, reagents, and solutions

All chemicals were of analytical grade. Acetonitrile, methanol (high-performance liquid chromatography grade), and ammonium acetate were obtained from Merck (Darmstadt, Germany). Ultrapure water (Milli-Q, Millipore Corporation, Bedford, MA, USA) was used to prepare all aqueous solutions.

All β-agonists (cimaterol, clenbuterol, ractopamine, salbutamol, terbutaline, tulobuterol, and zilpaterol) and corresponding internal standards (cimaterol-d7, clenbuterol-d9, ractopamine-d6, clenbuterol-d9, salbutamol-d6, terbutaline-d9, and zilpaterol-d7) were purchased from Sigma (St. Louis, MO, USA). The minimum purity of all standards was 98.0%. The chemical structures of all seven β-agonists are shown in Figure 1.

2.3. Preparation of standards

Individual stock solutions (100 μg/mL) were prepared by dissolving 1 mg of each compound in 10 mL of methanol. These were stored at −20°C in brown glass to prevent

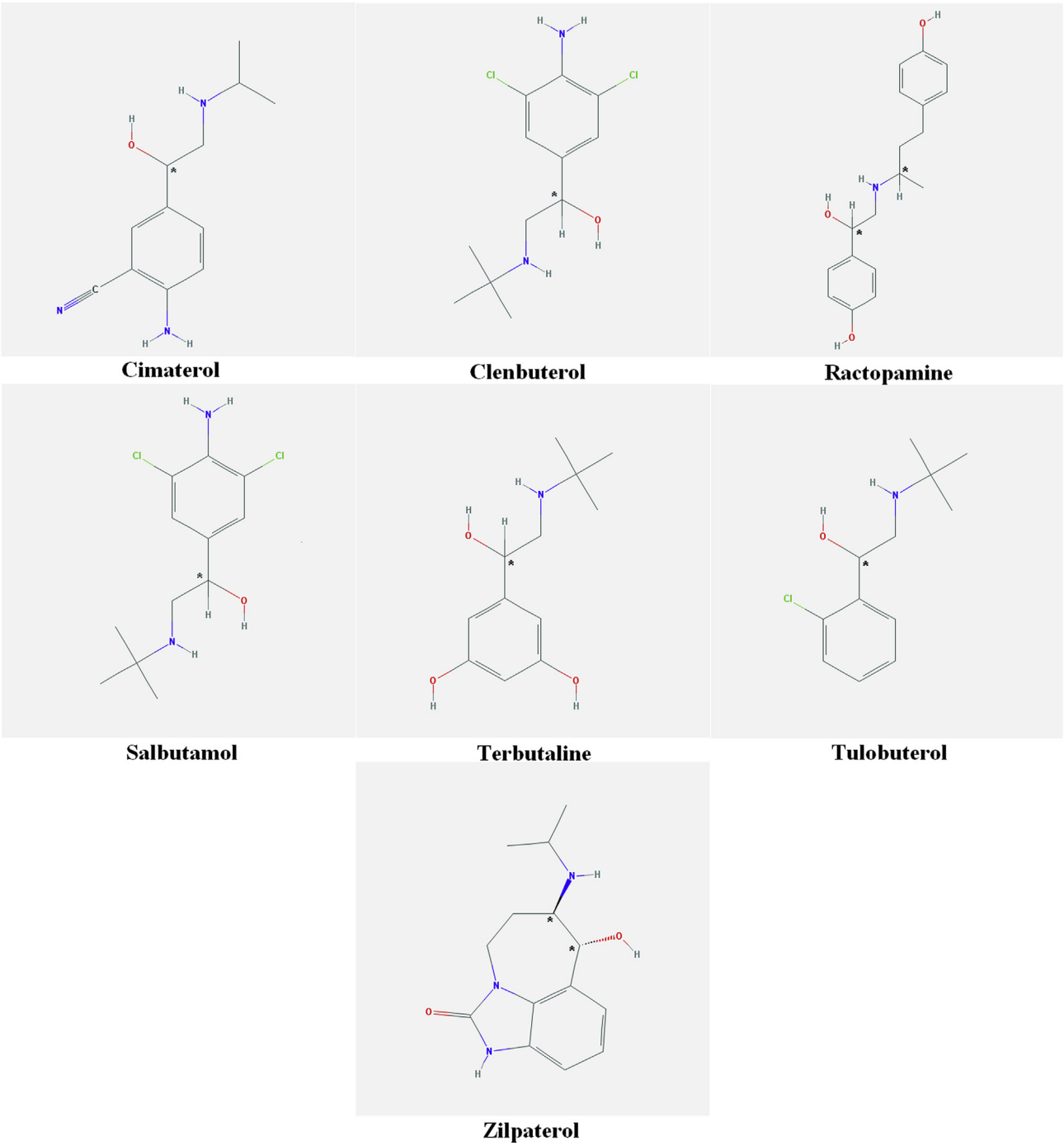

Figure 1 – Chemical structures of the β-agonists in this study.

photodegradation. Mixed standard solutions at concentrations of 1 μg/mL of each standard were prepared by mixing 100 μL of additives with each stock solution and diluting the solution to 10 mL with methanol. The mixed standard solutions were stored in amber bottles at −20°C. Six mixed IS solutions were prepared and stored in the same manner. All working solutions and calibration standards were obtained through gradient dilution of the intermediate solutions in concentrations varying from 1 μg/mL to 1 ng/mL. A working standard solution of ISs at a concentration of 1 μg/mL was determined with subsequent dilutions of their stock solutions in methanol. When not in use, the working solutions were kept at −20°C and renewed weekly.

2.4. *Sample preparation*

Five grams of finely chopped muscle and viscera homogenates was weighed into a 50 mL Falcon tube and spiked as appropriate with target compounds and the IS. Subsequently, 10 mL of 0.2 M sodium acetate solution was added, and the tubes were

shaken for 10 minutes. Following the addition of the IS and 100 μL of β-glucuronidase-arylsulfatase, the sample was incubated in a 37°C water bath for 1 hour before extraction. One milliliter of 1% acetate–acetonitrile was added and the mixture was stirred in a shaker for 30 seconds. A ceramic homogenizer, 6 g of anhydrous magnesium sulfate, and 1.5 g of anhydrous sodium acetate were then added and the tubes were shaken vigorously for 1 minute. Following centrifugation at 4032 × g and 15°C for 5 minutes, the supernatant was transferred to a new tube containing 900 mg of anhydrous magnesium sulfate, 150 mg of primary secondary amine (PSA), and C18EC and then subjected to high-speed homogenization for 1 minute and a second round of centrifugation under the same conditions. The supernatant was evaporated to dryness by a nitrogen blowing concentrator in a water bath at 65°C. The muscle and viscera residues were redissolved with 1 mL of an acetonitrile–H_2O solution (9:1, v/v) and acetonitrile, respectively. The sample extract was filtered through a 0.22-μm polytetrafluoroethylene filter into an autosampler vial for the LC–MS/MS analysis. A

Table 1 – Gradient program of the mobile phase for HPLC separation of the seven β-agonists in the present study (flow rate 1 mL/min).

Time (min)	5 mM ammonium acetate in methanol (%)
0	15
1	15
6	25
14	70
16	80
17	80
17.5	95
19.5	95
20	15
25	15

5 mM ammonium acetate in deionized water (%) + 5 mM ammonium acetate in methanol (%) = 100

HPLC = high performance liquid chromatography.

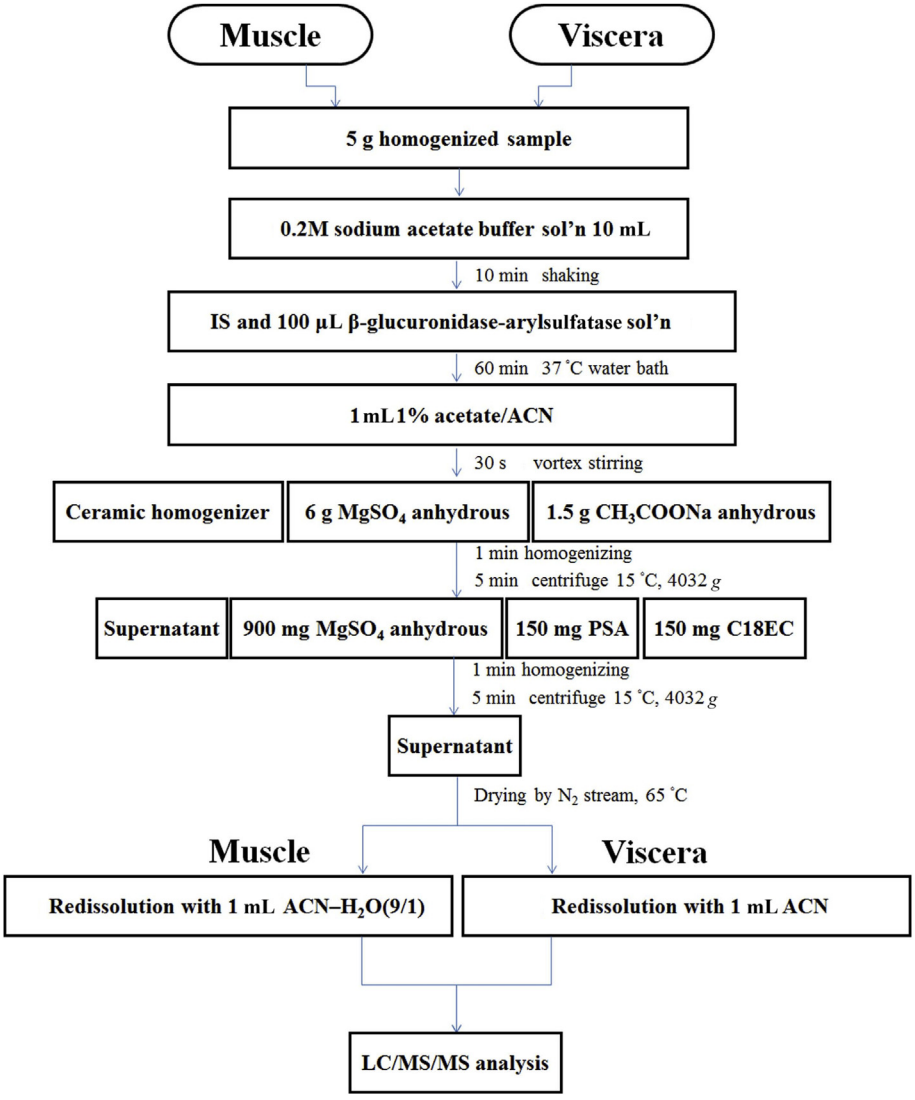

Figure 2 – Diagram of sample preparation.

scheme diagram of this sample preparation is shown in Figure 2.

2.5. LC–MS/MS analysis

LC was performed on a Dionex Ultimate 3000 Rs system (Sunnyvale, CA, USA) coupled to an AB SCIEX Q TRAP 5500 mass spectrometer (Framingham, MA, USA). Chromatographic separation was performed using an Agilent Zorbox SB-C18 column (150 mm × 4.6 mm, 5 μm, Palo Alto, CA, USA). Linear gradient elution was performed as shown in Table 1. The injection volume was 10 μL. Mass analysis was carried out using an electrospray ionization source in positive mode. The operation conditions were as follows: ionspray voltage, 5.5 kV; source temperature, 650°C; curtain gas, 20 psi; ion source gases 1 and 2, 65 psi. The optimal MRM parameters are summarized in Table 2.

2.6. Method validation

For the method validation, various parameters such as linearity, accuracy, precision, and limits of quantification (LOQs) were evaluated. The peak area of the most intense transition versus the concentration was used to establish the linear regression equation. The linearity of the method was evaluated on the basis of tissue calibration.

2.6.1. Linearity

Tissue calibration curves were used to quantify and test the linearity of the developed method. The blank muscle and viscera samples were fortified at five concentrations of 1–50 μg/kg of the target analytes. Three replications of each concentration were performed. Sample preparation was executed according to the aforementioned procedures. The tissue calibration curves for β-agonists were constructed by calculating the ratio of each peak area relative to the corresponding IS. The linearity of the LC–MS/MS method was evaluated using the regression coefficient measured for each analyte. The acceptance criterion was a correlation coefficient (R^2) >0.99.

2.6.2. Accuracy and precision

The results for accuracy and precision were expressed as the percentage of recovery and the coefficient of variation (CV). Recovery and repeatability were assessed by spiking blank muscle and viscera samples at two concentration levels (1.5 μg/kg and 3.0 μg/kg) for target analytes in five replicates at each level.

2.6.3. LOQs

LOQs were calculated by analyzing blank samples fortified at 1.0 μg/kg and defined as the lowest concentration of an analyte for which the signal-to-noise ratio was >10.

3. Results and discussion

3.1. Optimization of LC–MS/MS parameters

The LC–MS/MS method was developed to provide confirmatory data for the analysis of seven β-agonists in animal muscle and viscera tissue. Separation was performed in an Agilent Zorbox SB-C18 column (150 mm × 4.6 mm, 5 μm). Chromatographic parameters such as the choice of column, mobile phase composition, gradient conditions, and flow rate were tested to obtain the optimal separation of β-agonists. With reference to previous reports, a mobile phase consisting of 5 mM ammonium acetate in water and methanol was chosen [32–34]. The addition of ammonium acetate greatly improved peak shape and facilitated resolving closely eluted compounds. After optimization, the ammonium acetate concentration was set at 5 mM, and its addition to the aqueous phase further improved separation and overall peak shapes [8]. β-Agonists belong to Group A of Annex I, Council Directive 96/23/EC [35], therefore a minimum of four identification points are required, and were obtained by monitoring one parent ion (1 point) and two transitions (1.5 points each). The product ion with the stronger signal was selected as the ion for quantification, and the product ion with the weaker signal was selected as the ion for identification. The selected transitions for β-agonists and the optimal MS–MS conditions are described in Section 2.6. The gradient program in Table 1 was used for chromatographic separation. The flow rate was set at 1.0 mL/min and the analysis was completed in 25 minutes for the seven β-agonists. The MRM chromatograms of the seven β-agonists in the present study are shown in Figure 3.

3.2. Optimization of sample preparation

Sample preparation plays an important role in analytical methods. Various pretreatment methods have been proposed for monitoring the illegal use of β-agonists [25,26,36]. Salts and endogenous compounds cannot be fully removed due to the

Table 2 – Parameters of MRM condition and retention times of the β-agonists.

Compound	ESI	Retention time (min)	Precursor ion (m/z)	Product ions (m/z)	Decluster potential (V)	Entrance potential (V)	Collision energy (eV)	Collision cell exit potential (V)
Cimaterol	+	4.40	220	202, 160	45	10	12, 10	13
Clenbuterol	+	7.62	277	203, 259	58	10	20,15	13
Ractopamine	+	6.34	320.01	284, 107	59	10	18, 33	13
Salbutamol	+	4.53	240.01	148, 222	45	10	24, 13	13
Terbutaline	+	4.45	226	152, 125	60	10	20, 32	13
Tulobuterol	+	8.48	228	154, 118	40	10	22, 33	13
Zilpaterol	+	4.70	262.2	244, 185	54	10	19, 33	13

ESI = electrospray ionization; MRM = multiple reaction monitoring.

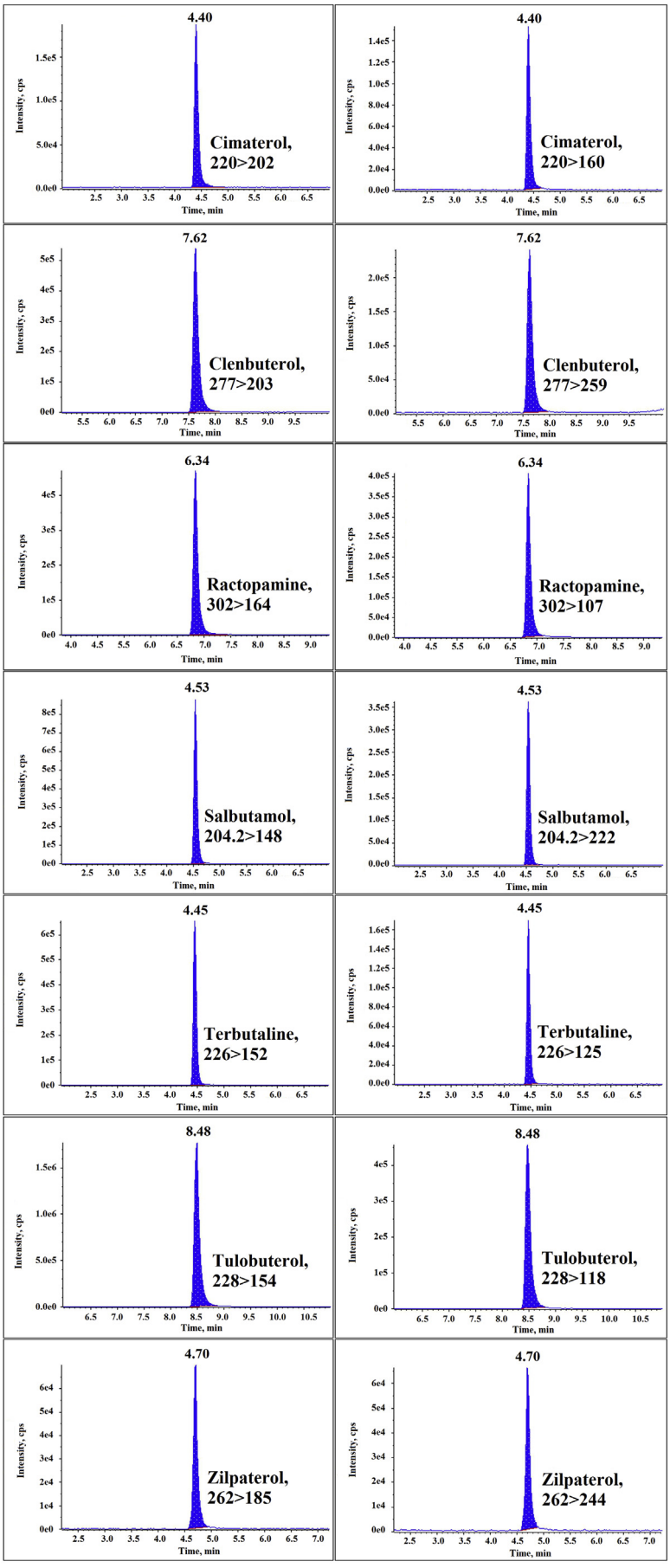

Figure 3 – Extract chromatograms of the seven β-agonists spiked at 3.0 μg/kg in muscle samples.

complexity of the biological matrices and the trace levels in real samples, leading to possible matrix effects. In addition, these techniques are time consuming, and the large quantities of organic solvents required, including acetonitrile and methanol [2,29], may cause environmental pollution. By contrast, the established QuEChERS pretreatment procedure without covering is simple and economical and requires only small amounts of organic solvents. The principle of the QuEChERS method relies on sample cleaning using various dispersive SPE sorbents, including PSA, C18, and silica, as well as magnesium sulfate for the elimination of residual water prior to analysis [37]. To improve efficiency and reduce time-consuming sample preparation, the QuEChERS method was developed for the cleaning of target analytes in tissue extracts. The procedure begins with sodium acetate buffer solution extraction and enzymatic hydrolysis. Previous studies have demonstrated that favorable recoveries were obtained for most compounds when acetonitrile acidified with 1% acetic acid was used as the extraction solvent [38]. In this work, 10 mL of acetonitrile acidified with 1% acetic acid was used as the extraction solvent. While the analytes are transferred to an organic phase, some polar matrix impurities are left in the aqueous layer. A combination of 1.5 g sodium acetate and 6 g magnesium sulfate was used as a salting-out agent to partition analyte residues into the acetonitrile layer. Several sorbents such as PSA and C18 enabled satisfactory analyte recovery. The muscle and viscera extracts were evaporated under a stream of nitrogen at 65°C and the final residue was dissolved in 1 mL of an acetonitrile–H_2O solution (9:1, v/v) and acetonitrile, respectively. The optimized sample preparation protocol enabled a high percentage of recovery for the seven β-agonists.

3.3. *Method validation*

3.3.1. *Linearity*

The linearity of the analytical method was validated using the tissue calibration curves for each compound at different concentration levels to prevent matrix effects. Table 3 shows the tissue calibration parameters of the correlation coefficients. Table 3 shows $R^2 > 0.99$ for all seven β-agonists in muscle and viscera samples, which revealed good linearity in the concentration range for each β-agonist.

Table 3 – Linearity and LOQs of the β-agonists.

β-Agonists	Muscle		Viscera	
	R^2	LOQ (ng/g)	R^2	LOQ (ng/g)
Cimaterol	0.998	1	0.997	1
Clenbuterol	1.000	1	0.999	1
Ractopamine	0.998	1	0.997	1
Salbutamol	0.998	1	0.995	1
Terbutaline	0.996	1	0.995	1
Tulobuterol	0.999	1	0.998	1
Zilpaterol	0.993	1	0.993	1

LOQ = limit of quantification.

Table 4 – Recovery rates and CVs of the β-agonists from muscle samples.

β-Agonists	Spiked level (ng/g)	Recovery (%)	CV (%)
Cimaterol	1.5	90.5	5.4
	3.0	95.1	3.1
Clenbuterol	1.5	99.2	1.7
	3.0	87.6	5.8
Ractopamine	1.5	98.5	4.3
	3.0	100.3	4.6
Salbutamol	1.5	101.2	4.2
	3.0	90.8	5.8
Terbutaline	1.5	96.3	6.3
	3.0	100.2	3.5
Tulobuterol	1.5	97.9	7.7
	3.0	102.5	3.2
Zilpaterol	1.5	94.4	3.2
	3.0	97.4	6.4

CV = coefficient of variation.

Table 5 – Recovery rates and CVs of the β-agonists from viscera samples.

β-Agonists	Spiked level (ng/g)	Recovery (%)	CV (%)
Cimaterol	1.5	102.0	3.2
	3.0	95.0	4.2
Clenbuterol	1.5	101.1	2.5
	3.0	102.1	2.1
Ractopamine	1.5	99.6	5.7
	3.0	103.6	3.6
Salbutamol	1.5	110.3	4.2
	3.0	106.9	3.4
Terbutaline	1.5	100.8	4.2
	3.0	111.5	2.9
Tulobuterol	1.5	102.4	1.8
	3.0	105.5	6.2
Zilpaterol	1.5	90.7	6.6
	3.0	91.7	6.9

CV = coefficient of variation.

3.3.2. *Recovery*

The rates of recovery of each compound from muscle and viscera samples were evaluated at two concentration levels (1.5 μg/kg and 3.0 μg/kg) by determining the ratios of the measured and added amounts of the target analyte. The results of the recovery test for the seven β-agonists in muscle and viscera samples are listed in Tables 4 and 5. In muscle samples, the recovery rate of the 1.5 μg/kg and 3.0 μg/kg spiking levels ranged from 90.5% to 101.2% and 87.6% to 102.5%, respectively. In viscera samples, the recovery rate of the two spiking levels varied from 90.7% to 110.3% and 91.7% to 111.5%, respectively. These results showed that the accuracy of this method was satisfactory.

3.3.3. *Precision*

The precision of the assay was assessed at 1.5 μg/kg and 3.0 μg/kg for each analyte in the spiked samples and expressed as the CV. The results are presented in Tables 4 and 5. The CV of the muscle samples spiked at 1.5 μg/kg and 3.0 μg/kg ranged from 1.7% to 7.7% and 3.1% to 6.4%, respectively, and the corresponding values for the viscera samples varied

Table 6 – Comparison of LODs and LOQs in selected methods.

Analytes	Matrix	Sample preparation	Detection	LOD (ng/g)	LOQ (ng/g)	Reference
β-Agonist (7)	Muscle, viscera	QuEChERS	LC–ESI(+)-MS	—	1.00	Our work
Cimateol	Milk	Modified QuEChERS approach	UPLC-QTOF-MS	1.00	3.34	[38]
Clenbuterol				0.53	1.78	
Ractopamine				1.46	4.87	
Clenbuterol	Butter, egg, fish, milk	SLE with ultrasonic-assisted extraction	LC-ESI(+)-MS	—	0.02–0.17	[25]
Clenbuterol	Pork	Acidic extraction, SPE/C18 clean-up	HPLC-UV	0.02	—	[39]
Ractopamine				0.05	—	
Salbutamol				0.05	—	
Clenbuterol	Bovine liver	extracted with acetonitrile and isopropanol	HPLC-UV	0.20	0.42	[18]
β-Agonist (5)	Feed	QuEChERS	LC-ESI(+)-MS	15	50	[40]
β-Agonist (7)	Feed, urine, serum, muscle, liver	SPE technology	UHPLC-Q-Orbitrap HRMS	0.02–2.18	0.07–7.26	[8]
β-Agonist (6)	Beef muscle, beef liver, goat muscle, goat liver	QuEChERS	LC-ESI(+)-MS	0.30–1.00	0.90–3.20	[41]
Clenbuterol	Liver	MSPD/MIP-SPE	LC–ESI(+)IT-MS	<0.10	—	[42]
Zilpaterol	Feed	LPE/C18-SPE/derivative	GC–EI-MS	8	—	[43]
Zilpaterol	Urine, plasma, tissues, retina	hydrol/LLE/mixurea-SPE tissues: hydrolyzed/hexane defatted/Extrelut-SPE	LC–ESI(+)QqQ-MS	<0.10	—	[16]
β-Agonists	Feed	LPE/Mixa-SPE or IA-SPE	LC–bioassay/Q-ToF-MS	5–50	—	[44]
β-Agonists	Retina	LPE/SPE/derivative	GC–EI-MS	4–10	—	[45]
β-Agonists	Liver	Deconjugation/HLB-SPE	SPR (screening)	0.02–0.2	—	[46]

LOD = limit of detection; LOQ = limit of quantification.

from 1.8% to 6.6% and 2.1% to 6.9%, respectively. Thus, all CV values were less than 10% at the two concentrations (1.5 μg/kg and 3.0 μg/kg).

3.3.4. *LOQ*

The LOQ was defined as the concentration at 10 times the signal intensity of noise. The LOQs of all β-agonists were 1.0 μg/kg for the spiked muscle and viscera sample. The LOQs of this method are generally more favorable than those of some traditional methods, but the LOQs obtained in the current study are equal to or less favorable than those of other methods. Nevertheless, very low LOQs were achieved for spiked muscle and viscera samples. These results clearly demonstrate the feasibility of the proposed method. The method ensured reliable preparation and precise quantification of seven β-agonists. This comparison is shown in Table 6 [8,16,18,25,38–46].

4. Conclusion

In the present study, a simple, fast, and sensitive multiresidue analytical method involving LC–MS/MS was developed and validated for the simultaneous determination of seven β-agonists from two types of animal tissue (muscle and viscera). Analytes were extracted using the QuEChERS extraction procedure and analyzed using LC electrospray ionization MS/MS. This method was validated with fortified blank samples and the extraction procedure was fully optimized. Favorable values of validation parameters such as linearity, recovery, precision, and LOQs were obtained, indicating the suitability of the proposed solvent extraction method for the analysis of β-agonists. The proposed method possesses the following advantages: simplicity, speed, reliability, low cost, and low solvent consumption. QuEChERS is therefore a green technique for sample preparation.

Conflicts of interest

The authors certify that they have no conflicts of interest with any financial organization regarding the material discussed in this manuscript.

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