A dissertation for the Degree of Master of Science

Development and application of a method for rapid and simultaneous determination of three β-agonists (clenbuterol, ractopamine, and zilpaterol) using liquid chromatography - tandem mass spectrometry

수입식육 및 부산물 중 β-agonists (clenbuterol, ractopamine, zilpaterol) 약물의 동시분석법 개발 및 적용

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ABSTRACT

Development and application of a method for rapid and simultaneous determination of three β-agonists (clenbuterol, ractopamine, and zilpaterol) using liquid chromatography - tandem mass spectrometry

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β-agonists are anabolic compounds that promote fat loss and muscle gain, and their administration into livestock may provide economic benefits by increasing growth rate and feed efficiency. For these reasons, β-agonists are commonly added to livestock feed

as growth promoters, which can introduce a significant risk of secondary human poisoning through intake of contaminated meat. Therefore, a new method for the simultaneous determination of three β-agonists (clenbuterol, ractopamine, and zilpaterol) was developed in this study and applied to various meat samples.

The limits of quantification, derived through a validation test following Codex guidelines, were 0.2 μg/kg for clenbuterol and zilpaterol and 0.4 μg/kg for ractopamine. The average recovery rates for clenbuterol, ractopamine, and zilpaterol were 109.1–118.3%, 95.3–109.0%, and 94.1–120.0%, respectively, all of which are acceptable according to the Codex guidelines. Notably, this pretreatment method reduced the analysis time without decreasing detection efficiency.

It is hoped that this method may be utilized to manage the safety of imported meat products from countries where zilpaterol use is still permitted, thereby improving public health and preventing β-agonist poisoning due to secondary contamination.

Keywords : Clenbuterol, Ractopamine, Zilpaterol

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LIST OF ABBREVIATION

HPLC	High−performance liquid chromatography
GC−MS	Gas chromatography−mass spectrometry
LC−MS	Liquid chromatography−mass spectrometry
LC/MS/MS	Liquid chromatography−tandem mass spectrometry
JECFA	Joint FAO/WHO Export Committee on Food Additives
PVDF	Polyvinylidene difluoride
ESI+	Positive electrospray ionization
MRM	Multiple Reaction Monitoring
LOQ	Limit of quantification
MRLs	Maximum residue limits
CAC	Codex Alimentarius Commission
FSIS	Food Safety and Inspection Service
SPE	Solid phase extraction

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Introduction

Characteristics of β-agonists

β-agonists are drugs that have mimic effects of transmitter substances of nervous system such as catecholamine, epinephrine and norepnephrine (Juan *et al.*, 2010). These drugs act upon the β-adrenergic receptors that have three subtypes, namely, beta 1, 2 and 3. Functions differ depending on activated subtype of β-adrenergic receptors. Activation of β1-adrenergic receptors evokes stimulation of cardiac inotropy and chronotropy (Yoo *et al.*, 2009). Activation of β2-adrenergic receptors stimulates smooth muscle relaxation in the respiratory tract, digestive organs, uterus and several type of blood vessels (McGraw & Liggett, 2005). Activation of β3-adrenergic receptors involves in regulation of fat metabolism (Lowell & Flier, 1997). For these effects, β-agonists have been used therapeutic purposes for bradycardia, hypotension, heart failure, respiratory disease including asthma and preterm labour in humans. However, these drugs are also used as growth promoters in livestock for their function in growth rates enhancement and feed efficiency (Elliott *et al.*, 1993). Among them clenbuterol is the most effective β-agonist as a growth promoter. Ractopamine and zilpaterol also have been commonly used for a growth promoter. These three drugs have chemical structures as shown in Fig.1. Administration of high doses of β-agonists, which were originally developed to treat human diseases could lead to improved weight gain and carcass yield in livestock (Blanca *et al.*, 2005; Lawrence *et al.*, 2011). Consequently, this approach has long been

used to increase livestock productivity. However, toxicity studies demonstrated that long-term administration of clenbuterol, a member of the β-agonist group, may lead to serious adverse effects on the cardiovascular and nervous system (Martinez-Navarro, 1990; Juan *et al.*, 2010). Several cases of adverse effects in humans have also been associated with the consumption of clenbuterol-contaminated meat products (Brambilla *et al.*, 2000).

The differences in policies on β-agonists among countries

Clenbuterol is prohibited in over 150 countries, including European countries (Commission of the European Communities, 1996). Ractopamine and zilpaterol, which belong to the β-agonist group and have effects similar to those of clenbuterol, are still used in some countries to promote livestock growth. Although ractopamine is prohibited in most European Union(EU) countries (Blanca *et al.*, 2005), it is still officially permitted in 27 countries worldwide, including the United States and Canada (Government Gazette, 1999; U.S. Food and Drug Administration, 1999). Likewise, zilpaterol is authorized for use in South Africa, Mexico, the United States, and Canada (Delmore *et al.*, 2010). Given south korea's high dependence on meat imported from areas that still use ractopamine and zilpaterol, analysis of the three commonly used β-agonists (clenbuterol, ractopamine, and zilpaterol) in meat products is an important tool for safety consideration.

Methods for detection of β-agonists

Immunoassays (Shelver *et al.*, 2005; Shelver & Smith, 2011), high-performance liquid chromatography (HPLC) (Freire *et al.*, 2013), gas chromatography-mass spectrometry (GC-MS) (Wang *et al.*, 2010; Zhao *et al.*, 2010), and liquid chromatography-mass spectrometry (LC-MS) (Zhang *et al.*, 2009) have all been used to analyze β-agonist residues. Immunoassays have been performed only for the screening method due to their high sensitivity, and HPLC methods are also unsuitable as a confirmation method that can meet EU criteria for their low selectivity. Though GC-MS methods have been used for β-agonist analysis, they all required a time-consuming, laborious sample derivation process. More recently, the commonly used method has been liquid chromatography-tandem mass spectrometry (LC/MS/MS), in which two mass spectrometers are connected to quantitatively and qualitatively analyze a substance based on the unique characteristics of mass and fragment ions created by an electrical current (Burnett *et al.*, 2012; Hong *et al.*, 2008; Juan *et al.*, 2010; Li *et al.*, 2010; Lu *et al.*, 2013; Shao *et al.*, 2009). Thus far, few adequate methods have been reported that can simultaneously analyze clenbuterol, ractopamine, and zilpaterol in meats and byproducts. The studies that have simultaneously analyzed all three substances were limited in scope to samples from the urine, kidney, liver, and feed (Blanca *et al.*, 2005; Suo *et al.*, 2013; Williams *et al.*, 2004). Herein is reported the validation of a method to simultaneously measure clenbuterol, ractopamine, and zilpaterol in muscle tissue, the primary source of meat by using LC/MS/MS.

Background of this study

One analysis method for simultaneous determination of clenbuterol and ractopamine was developed in South Korea in 2013 and was an improvement over individual assays of the two substances (Cho *et al.*, 2013; National Institute of Food and Drug Safety Evaluation, 2013). Unfortunately, this method was not validated for measuring zilpaterol, the usage of which is increasing due to its superior growth-enhancing effects. The differences among countries' policies regarding the permission to use of zilpaterol are becoming increasingly problematic, yet international zilpaterol safety management efforts are still lacking. The use of clenbuterol, on the other hand, is prohibited in most countries and is strictly controlled. Similarly, the grounds for safety management of ractopamine were established through adapting Maximum Residue Limits (MRLs) by Codex Alimentarius Commission (CAC) to ensure human health and safety. The established limits were based on a risk assessment by the Joint FAO/WHO Export Committee on Food Additives (JECFA) in July 2012 (Food and Agriculture Organization of United Nations, 2014). South Korea still highly depends on meat imported from countries where β-agonists are allowed and few previous studies on a simultaneous measure of clenbuterol, ractopamine, and zilpaterol in muscle tissue, the primary source of meat consumption, have been reported. For these reasons, a method that can simultaneously measure all three β-agonists would be quite beneficial. To that end, a rapid method for simultaneous detection of clenbuterol, ractopamine, and zilpaterol that meets the Codex guidelines and maintains high detection efficiency was

developed. Then the potential of the method by testing a collection of meats and meat byproducts was evaluated.

Materials and Methods

Regents and standards

Acetonitrile, *n*-hexane, and ethyl acetate (Burdick & Jackson, Muskegon, MI, USA) were used as the extraction solvents. Potassium carbonate (K_2CO_3) (Merck, Darmstadt, Germany), formic acid (Fluka, St Louis, MO, USA), and anhydrous sodium sulfate (Junsei Chemical, Tokyo, Japan) were used as the pretreatment and mobile phase solvents. Triple distilled water added to the samples was purified through the Milli-Q system (Millipore, Milford, USA). Clenbuterol (clenbuterol hydrochloride, 99.8%), ractopamine (ractopamine hydrochloride, 99.8%), and zilpaterol (zilpaterol hydrochloride, 99.8%) (Dr. Ehrenstorfer GmbH, Augsburg, Germany) were used as the standards, while clenbuterol-d_9, zilpaterol-d_7 (Dr. Ehrenstorfer GmbH, Augsburg, Germany), and ractopamine-d_3 (CDN_ISOTOPES, Quebec, Canada) were used as the internal standards. All standards and internal standards were diluted with methanol (Burdick & Jackson, Muskegon, MI, USA).

For each of the three standards and three internal standards, a 100 mg/kg stock solution was prepared in methanol based on the known purity and molecular weight of each

substance. From these stock solutions, working standard solutions of clenbuterol, ractopamine, and zilpaterol at concentrations of 100, 200, and 200 µg/kg, respectively, were prepared by dilution in methanol. The same procedure was followed for clenbuterol-d_9, ractopamine-d_3, and zilpaterol-d_7 to yield solutions of 100, 200, and 200 µg/kg, respectively. The stock and working standard solutions were stored in the freezer (-20°C) and diluted to individual concentrations using 0.1% formic acid (v/v) as required.

Sample treatment and extraction

Five grams of each homogenized, refrigerated sample was accurately weighed and placed into a 50 mL centrifuge tube. The working standard solution of each internal standard (50 µL), 1 mL of 4 M K_2CO_3, and 5 mL of distilled water were added to each sample, and the mixture was stirred for 10 min; 10 mL of ethyl acetate was then added, and the mixture was shaken for 10 min. Next, 10 g of anhydrous sodium sulfate was added and mixed for 10 min to absorb residual water. The mixed solution was centrifuged for 10 min at 4,000 rpm, and the supernatant was moved to a new 50 mL centrifuge tube. Then, 10 mL of ethyl acetate was added to the solution again to re-extract, and the resulting supernatant was isolated by the same extraction procedure. The combined supernatants were gently enriched with nitrogen at 40°C to obtain a final volume of 2–3 mL. To this concentrate, 10 mL of acetonitrile and 15 mL of *n*-hexane were added, followed by vortexing for 10 min. The mixture was then centrifuged at

4,000 rpm for 10 min to separate the organic layer (acetonitrile). The isolated organic layer was gently enriched with nitrogen at 40°C and evaporated to dryness. The residue was reconstituted in 1 mL of 0.1% formic acid, which served as solvent A for the chromatographic analysis, and 0.5 mL of the solution was filtered with a 0.2-μm polyvinylidene difluoride (PVDF) syringe filter prior to injection.

Conditions and experimental set-up for chromatography and mass spectrometry

LC-MS (API 4000, AB SCIEX, USA) was selected to develop a simultaneous determination method for the three substances: clenbuterol, ractopamine, and zilpaterol. Separation was performed with an XBridge C_{18} (2.1 mm × 150 mm, 3.5 μm; Waters, Dublin, Ireland), and the column was maintained at the temperature of 35°C. The analysis was carried out with solvent A (0.1% formic acid) and solvent B (acetonitrile). Initial mobile conditions were set to 90% A and held for 1 min. The fraction of A was linearly decreased to 10% over 7 min and held at 10% for 3 min. At 10.10 min, the compositions were returned to 90% A and held there until 15 min. The flow rate was maintained at 0.4 mL per min, and the injection volume was 10 μL. Positive electrospray ionization mode (ESI+) was selected, and the analysis was performed in multiple reaction monitoring (MRM) mode. The source temperature was set at 600°C. The precursor ions, product ions, collision energy, and dwell time for each substance are listed in Table 1.

Validation

The linearity, recovery, limit of quantification (LOQ), and reproducibility tests were conducted to validate our method according to the Codex guidelines (Codex Alimentarius Committee, 1993). Beef, pork, and beef byproducts were used as samples. Calibration curves were prepared at concentrations of 0.2–16 μg/kg for clenbuterol and zilpaterol, and 0.5–32 μg/kg for ractopamine, for which MRLs have been established. The correlation coefficient (R^2) for each calibration curve was calculated, and the LOQ with a signal-to-noise ratio (s/n) greater than 10 was obtained for each substance. Fig. 2 shows the chromatogram for each substance after injection at the LOQ level. To evaluate the accuracy and precision of our method, the recovery rate was also determined using concentrations of 0.5, 1, and 2 times the MRLs (0.005, 0.01, 0.02 mg/kg) for ractopamine, and 1, 2, and 4 times the LOQ (0.2, 0.4, 0.8 μg/kg) for clenbuterol and zilpaterol. Peak retention times in the chromatogram were compared and adjusted according to the internal standards to determine the recovery rates using the content values of clenbuterol, ractopamine, and zilpaterol. Each concentration was analyzed in triplicate to confirm the accuracy and precision of the method.

Results

Pretreatment and instrument conditions

In the present study, A 4 M concentration of K_2CO_3 was added to adjust the pH of the solution to strongly basic, and 5 mL of triple distilled water was used to promote dispersion of the sample in the pretreating solvent and to increase the recovery rate. Acetonitrile and *n*-hexane were selected for liquid-liquid extraction and the temperature of nitrogen decompression was set at 40°C. Mobile phase solvents of this study were 0.1% aqueous formic acid and acetonitrile. The optimal assay conditions for mass spectrometry were found to be the m/z 203 and 259 ions for two daughter ions of clenbuterol, the m/z 164 and 107 ions for those of ractopamine, and the m/z 244 and 185 ions for those of zilpaterol, which showed the highest sensitivity (Table 1). Clenbuterol-d_9 was used as Interrnal standard for clenbuterol, ractopamine-d_3 was used as Internal standard for ractopamine and zilpaterol-d_7 was used as Internal standard for zilpatrol.

Applicability

In our validation tests, the R^2 values for calibration curves of clenbuterol, ractopamine, and zilpaterol were 0.9992, 0.9998, and 0.9979, respectively. The LOQ was 0.2 μg/kg for clenbuterol and zilpaterol, and 0.4 μg/kg for ractopamine. The average recovery rate was 109.1–118.3% for clenbuterol, 95.3–109.0% for ractopamine, and 94.1–120.0% for zilpaterol. The results meet the Codex criteria for linearity, recovery rate, LOQ, and

reproducibility (Table 2). Accordingly, the applicability of the new method was confirmed.

Application of the new method

Using the above-confirmed method, residue analysis for the levels of the three β-agonists in the following 299 samples was performed: 154 beef, 57 pork, and 88 beef byproducts. Our results revealed that ractopamine was detected in 2 beef samples, and zilpaterol was detected in 1 beef and 1 beef byproducts sample (Table 3). In particular, a high detection rate was observed for zilpaterol, for which MRLs have not yet been established.

Discussion

MS/MS optimization

To determine the optimal assay conditions for mass spectrometry, conventional analysis methods for clenbuterol and ractopamine were referred. Because the m/z 203 and 259 ions were used in all reference literature regarding clenbuterol, the collision energy shown in Table 1 was applied on the precursor ion to obtain the m/z 203 and 259 ions (Blanca *et al.*, 2005; Cho *et al.*, 2013; Suo *et al.*, 2013). For ractopamine, Blanca *et al.* (2005) and Cho *et al.* (2013) monitored m/z 284 and 164 or m/z 284 and 107 ions for

product ions, respectively. In this study, m/z 164 and 107 ions were used to track ractopamine because they showed high sensitivity upon application of collision energy, as shown in Table 1. To detect zilpaterol, Blanca *et al.* (2005) used m/z 244 and 202 ions, and Suo *et al.* (2013) used m/z 244 and 185 ions. In this study, the m/z 244 and 185 ions, which showed the highest sensitivity upon application of the collision energy, were monitored as shown in Table 1. In addition, while the previous studies (Blanca *et al.*, 2005; Mauro *et al.*, 2014; Suo *et al.*, 2013) used ractopamine-d_5, cimaterol-d_7, and clenbuterol-d_9 as an internal standard for zilpaterol, in the present study, zilpaterol-d_7 was used to improve efficiency and reproducibility.

Comparison with current β-agonist detection methods

A simultaneous determination method for the clenbuterol and ractopamine was developed in 2013 in South Korea and is currently used as a Food Code analysis method (KFDA, 2013). Fig. 4 compares the Food Code analysis method to the new method developed herein. For the development of our method, a protocol for simultaneous detection of β-agonists that is used by the Food Safety and Inspection Service (FSIS) of the Department of Agriculture in the United States (United States Department of Agriculture Food Safety and Inspection Service Office of Public Health Science, 2014) was referred. Their method comprises eight steps and does not include solid phase extraction (SPE) and deproteinization steps, as is the case in our method (Fig. 4). Notably, there are additional examples in the literature of methods that do not use deproteinization steps for the analyses of feed and milk (Li *et al.*, 2010; Suo *et al.*, 2013).

Acetonitrile and hexane were used in place of saturated methanol and hexane to increase the liquid-liquid distribution effect (Juan *et al.*, 2010), and the temperature during the nitrogen decompression process was maintained at 40°C, rather than 55°C to minimize evaporation (Li *et al.*, 2010; Mauro *et al.*, 2014). Finally, 0.1% aqueous formic acid and acetonitrile were used as the mobile phase instead of buffered ammonium acetate to ensure no overlapping retention times, to encourage optimal peak shape, and to minimize run time (Fig. 3).

International trends of β-agonist drug permissibility

β-agonist drugs are now considered more controversial for safety concerns than any other veterinary drug internationally. Unlike clenbuterol, which is prohibited from use in livestock in most countries, ractopamine is still authorized for use in over 27 countries, including South Korea. Codex, the United Nations food standards body, set ractopamine MRLs in 2012, proposing residue levels that have no known impact on human health (Food and Agriculture Organization of the United Nations, 2014). Unfortunately, not all countries comply with the Codex MRLs. Although all β-agonists are prohibited for use as growth promoters in the EU(Commission of the European Communities, 1996), a level that is 3–5 times higher than the MRLs of Codex is permitted in the United States (U.S. Food and Drug Administration, 2014). Furthermore, in the United States, a separate standard is used for turkey, for which Codex has not yet established MRLs. Unlike the United States, the limits suggested by Codex are followed in Canada, although a separate standard for turkey does exist. Differences also exist between

countries with respect to renal residue tolerance; the permissible tolerance is 90 μg/kg according to the Codex guidelines and Canadian laws, but no limits have been set in the United States (Table 4). Zilpaterol is permitted in cow liver in the United States at concentrations not exceeding 12 μg/kg (U.S. Food and Drug Administration, 2014). Similarly, zilpaterol is permitted in Canada at concentrations up to 5, 5, and 2 μg/kg in cow kidney, liver, and muscle, respectively (Health Canada, 2014). Furthermore, during the 35th session (2012) of the Codex Alimentarius Commission, the Joint FAO/WHO Expert Committee on Food Additives decided to conduct a risk assessment for zilpaterol, and new MRLs for zilpaterol are expected to be established in the near future. Based on previous trends it is expected that the new limits may be controversial and not universally adopted. Consequently, β-agonists will continue to remain important, and further studies on β-agonists are required.

Safety issues associated with zilpaterol

Apart from the international trends toward establishing MRLs for zilpaterol, controversy surrounding the safety issue of the drug is expected to continue (Taiwan Food and Drug Adimistration, 2014). Although clinical toxicology studies have been conducted with horses (Hepworth-Warren and Alcott, 2014; Wagner *et al.*, 2008), case studies on the toxicity in other animals and humans are still lacking. Moreover, since simultaneous analysis of the three β-agonists in livestock food products, the primary source of potential human exposure, has been performed in relatively few studies, it is evident that these issues have not been sufficiently studied at both the domestic and international

levels (Blanca *et al.*, 2005; Mauro *et al.*, 2014; Williams *et al.*, 2004). Based on Table 3 results, it is believed that there is non-negligible contamination of imported meat and meat byproducts, indicating a need for the continued surveillance of this drug. Moreover, through further studies, other β-agonists such as cimaterol and salbutamol, which are not frequently used but still pose a threat, are expected to be included in the simultaneous determination method in future. In addition, the scope of the method may be extended to include the processed meat products.

Conclusion

A new method for the simultaneous determination of three β-agonists, including zilpaterol, was developed in this study. The pretreatment method was modified to reduce the analysis time while enhancing detection efficiency. Moreover, the validation test results met the international Codex guideline standards, verifying that the method is applicable for actual practice. This method should enable rapid and efficient analysis of β-agonists. Additional analyses of zilpaterol may facilitate continued management of the drug and prove to be useful for assessing the risk and establishing MRLs for zilpaterol in the future. Furthermore, it is hoped that this method may be utilized to manage the safety of imported meat products from countries where zilpaterol use is still permitted, thereby improving public health and preventing β-agonist poisoning due to secondary contamination.

Table 1. Mass spectrometry transition conditions and retention time

Compounds	Transition	Dwell time (s)	Collision Energy (eV)	Retention time (m)
Clenbuterol	276.9 to 203.3[1]	0.05	22.68	5.2
	276.9 to 259.3[2]	0.05	15.33	
Clenbuterol-d_9	286.2 to 204.2	0.05	23.08	5.2
	286.2 to 268.2	0.05	15.41	
Ractopamine	302.0 to 164.0[1]	0.05	22.43	4.8
	302.0 to 107.0[2]	0.05	40.04	
Ractopamine-d_3	305.0 to 124.0	0.05	31.00	4.8
	305.0 to 107.0	0.05	35.00	
Zilpaterol	261.9 to 244.2[1]	0.05	17.00	1.7
	261.9 to 185.1[2]	0.05	35.00	
Zilpaterol-d_7	269.1 to 251.3	0.05	19.00	1.7
	269.1 to 185.2	0.05	35.00	

[1)] Ion for quantification ; [2)] Ion for identification

Table 2. Validation data for clenbuterol, ractopamine, and zilpaterol in meat samples

Compounds	[2)] R^2	LOQ[3)] (μg/kg)	Peak Concentration (mg/kg)	Beef		Pork		Beef by-products	
				Recovery (%)	CV[4)]	Recovery (%)	CV	Recovery (%)	CV
Clenbuterol	0.9992	0.2	0.0002	113.33[1)]	5.09	116.67	4.95	113.33	5.09
			0.0004	118.33	2.44	118.33	2.44	116.67	4.95
			0.0008	112.50	2.22	109.17	3.50	110.00	2.27
Ractopamine	0.9998	0.4	0.005	95.33	9.46	104.00	5.77	102.00	3.40
			0.010	101.17	8.22	98.83	2.04	109.00	10.58
			0.020	103.33	2.79	104.17	6.04	103.33	2.79
Zilpaterol	0.9979	0.2	0.0002	110.00	0.00	113.33	5.09	120.00	0.00
			0.0004	106.67	2.71	110.00	9.09	105.00	0.00
			0.0008	95.83	3.98	98.83	3.98	94.17	3.07

[1)] Mean value (n=3); [2)] R^2 : correlation coefficient ; [3)] LOQ : limit of quantification ; [4)] CV : coefficient of variation

Table 3. Monitoring data for β-agonists from 299 samples

Type	Number of samples	Compound	Number of samples detected	Residue level (μg/kg)	MRLs[1)] (μg/kg)
Beef	154	Clenbuterol	-	-	NE[2)]
		Ractopamine	2	0.6, 2.0	10
		Zilpaterol	1	0.1	NE
Pork	57	Clenbuterol	-	-	NE
		Ractopamine	-	-	10
		Zilpaterol	-	-	NE
Beef By-product	88	Clenbuterol	-	-	NE
		Ractopamine	-	-	10
		Zilpaterol	1	6.3	NE

[1)]MRLs : maximum residue limits ; [2)]NE: not established

Table 4. Ractopamine tolerance comparison between the FDA, Codex, and Health Canada

Area	Animal	Tolerated ractopamine Level (μg/kg)		
		Codex	FDA	Health Canada
Muscle	Cattle	10	30	10
	Pig	10	50	10
	Turkey	NE[1)]	100	30
Liver	Cattle	40	90	40
	Pig	40	150	40
	Turkey	NE	450	200
Kidney	Cattle	90	NT[2)]	90
	Pig	90	NT	90
	Turkey	NE	NT	NT

[1)] NE : not established; [2)] NT : no tolerance

Fig. 1. Chemical structures of clenbuterol, ractopamine, and zilpaterol

A, clenbuterol; **B**, ractopamine; **C**, zilpaterol

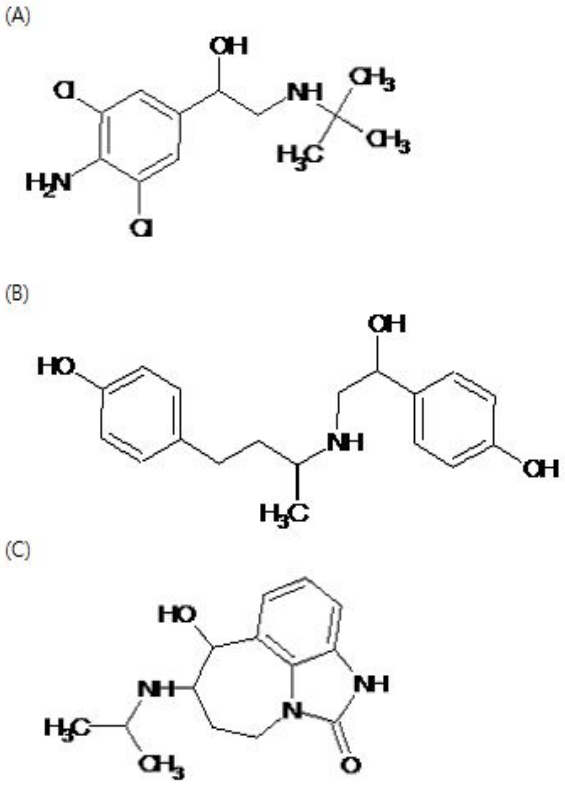


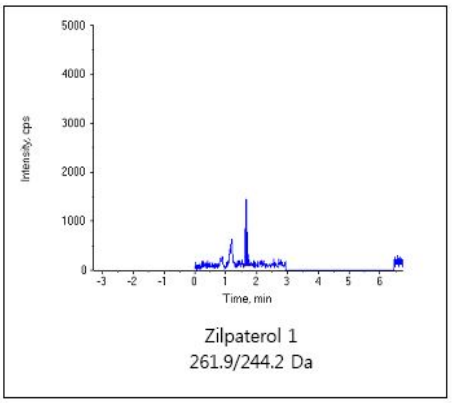
Fig. 2. Ion chromatograms of zilpaterol, clenbuterol and ractopamine at the limit of quantification (LOQ) level

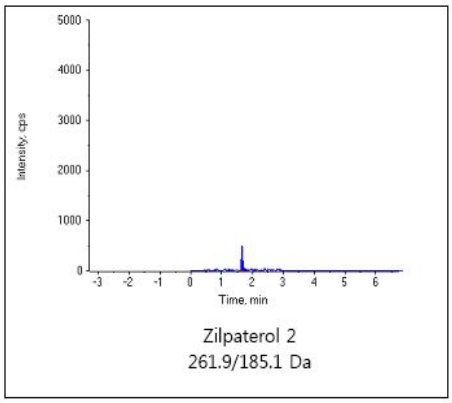
Two product ion chromatograms of each substance as displayed on the device are shown when drugs were injected at the LOQ level (0.2 μg/kg for zilpaterol and clenbuterol and 0.4 μg/kg for ractopamine) in meat samples. X and Y-axis represent time and intensity each.

A, Two product ions (m/z 244 and 185) of zilpaterol are shown as Peaks of 1.7 minute each (both have same retention time) and left chromatogram has a small matrix peak before zilpaterol ion peak ; **B**, Two product ions (m/z 203 and 259) of clenbuterol are shown as Peaks of 5.2 minute each (both have same retention time) and right chromatogram has a matrix peak at around 6 minute, which does not interfere with the product ion peak; **C**, Two product ions (m/z 164 and 107) of ractopamine are shown as Peaks of 4.8 minute each (both have same retention time).

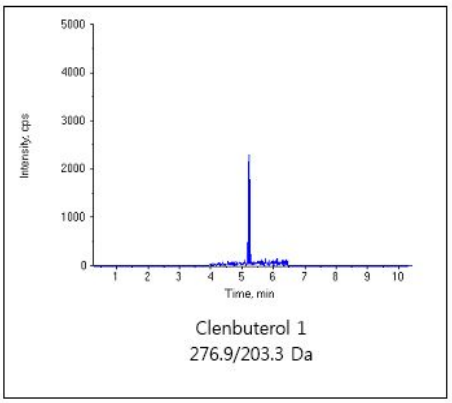
Fig. 2

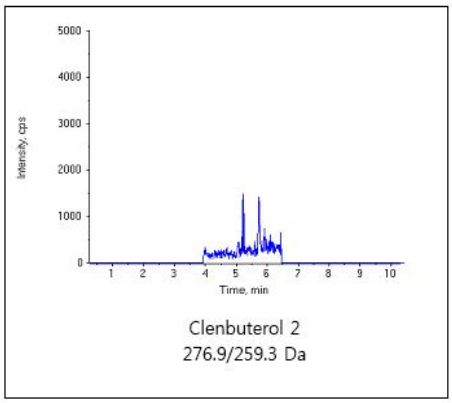
A


Intensity, cps
Time, min
Zilpaterol 1
261.9/244.2 Da

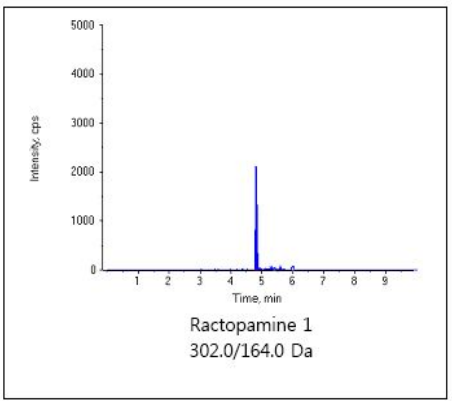

Intensity, cps
Time, min
Zilpaterol 2
261.9/185.1 Da

B


Intensity, cps
Time, min
Clenbuterol 1
276.9/203.3 Da


Intensity, cps
Time, min
Clenbuterol 2
276.9/259.3 Da

C


Intensity, cps
Time, min
Ractopamine 1
302.0/164.0 Da

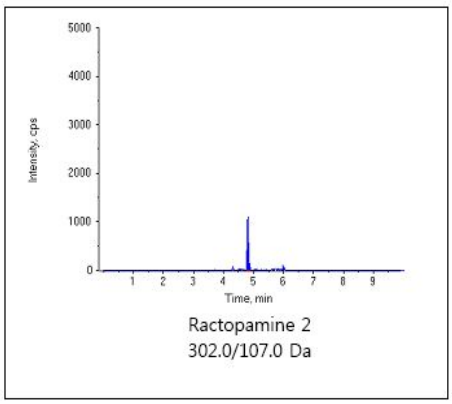

Intensity, cps
Time, min
Ractopamine 2
302.0/107.0 Da

Fig. 3. Total chromatogram of standard solutions of β-agonists

Peaks: zilpaterol (**A,B**), zilpaterol-d_7 (**C,D**), clenbuterol (**E,F**), clenbuterol-d_9 (**G,H**), ractopamine (**I,J**), ractopamine-d_3 (**K,L**)

Each substance has two product ion chromatograms. Standards and their internal standards have same retention time. Therefore, from A to D have same retention time of 1.7 minute, from E to H have same retention time of 5.2 minute, and from I to L have same retention time of 4.8 minute.

Fig. 3

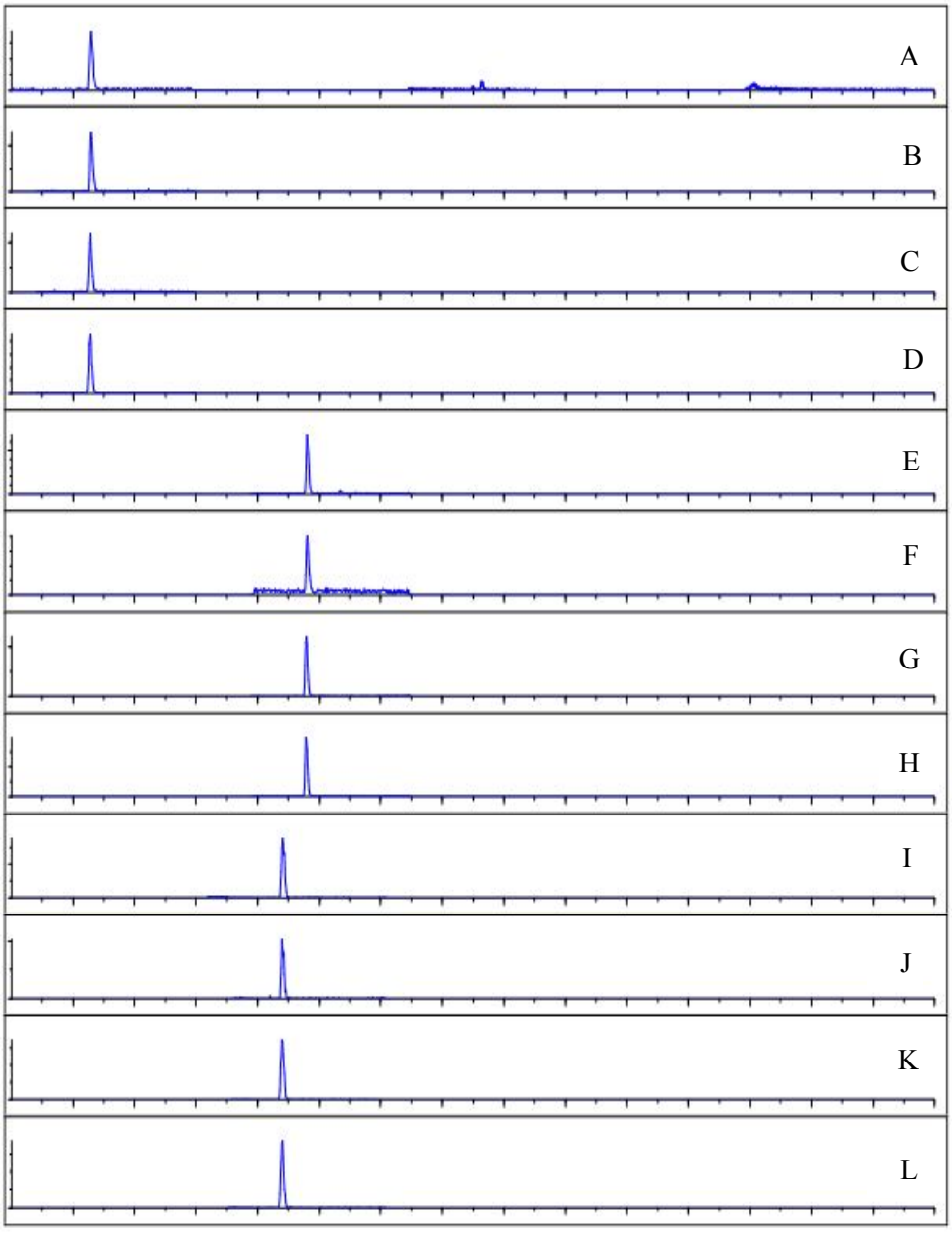
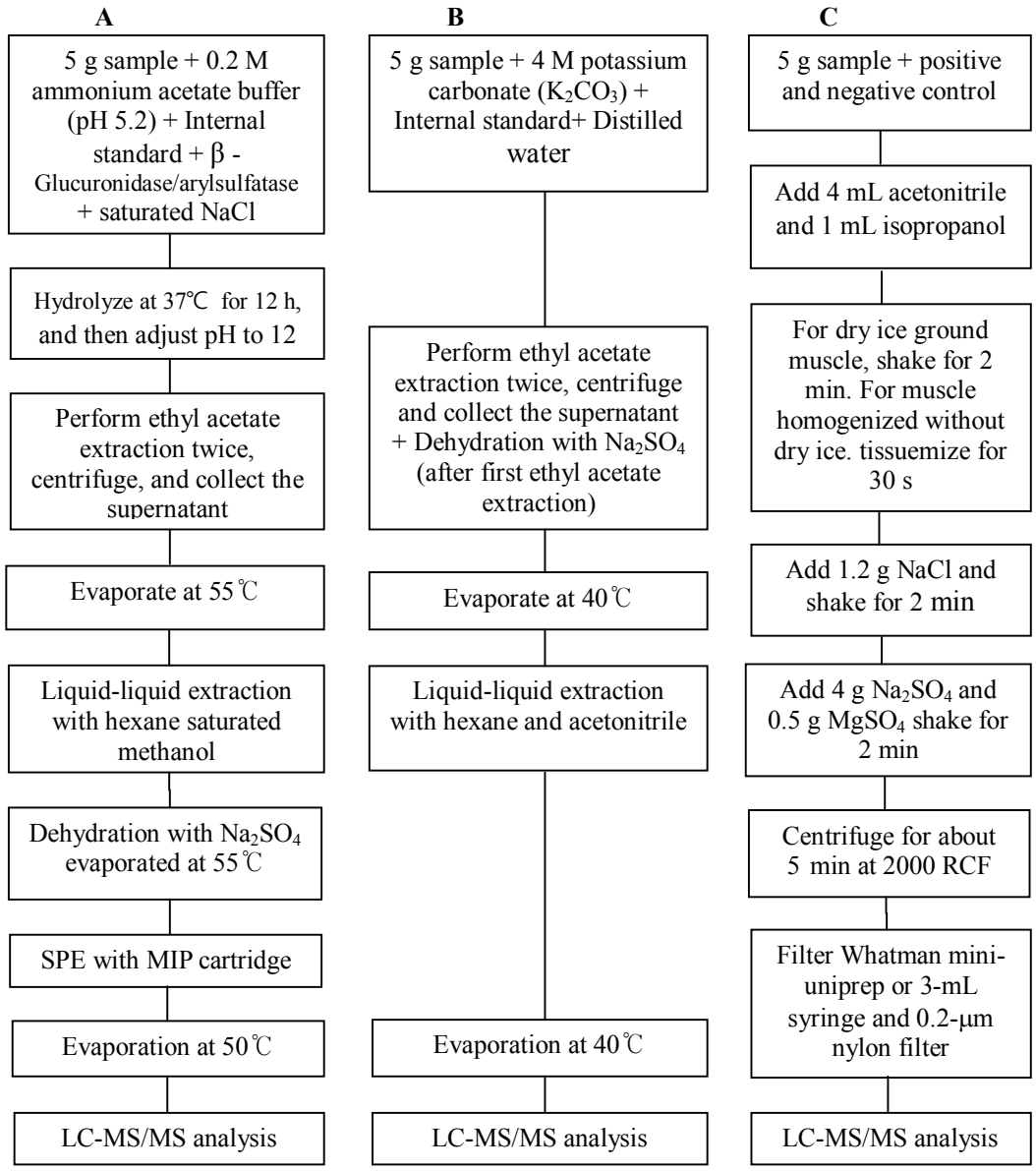


Fig. 4. Comparison of the Food Code analysis, the new analysis method, and an analysis by the United States Food Safety and Inspection Service (FSIS)

A, Food Code method; **B**, The new analysis method; **C**, U.S. FSIS method

Fig. 4


A
5 g sample + 0.2 M ammonium acetate buffer (pH 5.2) + Internal standard + β - Glucuronidase/arylsulfatase + saturated NaCl
Hydrolyze at 37℃ for 12 h, and then adjust pH to 12
Perform ethyl acetate extraction twice, centrifuge, and collect the supernatant
Evaporate at 55℃
Liquid-liquid extraction with hexane saturated methanol
Dehydration with Na_2SO_4 evaporated at 55℃
SPE with MIP cartridge
Evaporation at 50℃
LC-MS/MS analysis
B
5 g sample + 4 M potassium carbonate (K_2CO_3) + Internal standard+ Distilled water
Perform ethyl acetate extraction twice, centrifuge and collect the supernatant + Dehydration with Na_2SO_4 (after first ethyl acetate extraction)
Evaporate at 40℃
Liquid-liquid extraction with hexane and acetonitrile
Evaporation at 40℃
LC-MS/MS analysis
C
5 g sample + positive and negative control
Add 4 mL acetonitrile and 1 mL isopropanol
For dry ice ground muscle, shake for 2 min. For muscle homogenized without dry ice. tissuemize for 30 s
Add 1.2 g NaCl and shake for 2 min
Add 4 g Na_2SO_4 and 0.5 g $MgSO_4$ shake for 2 min
Centrifuge for about 5 min at 2000 RCF
Filter Whatman mini-uniprep or 3-mL syringe and 0.2-μm nylon filter
LC-MS/MS analysis

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국 문 초 록

수입식육 및 부산물 중 β-agonists (clenbuterol, ractopamine, zilpaterol) 약물의 동시분석법 개발 및 적용

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β-agonist계 약물은 특수한 동화작용(anabolic effect)에 의해 지방을 분해하고 근육을 증가시키는 효능이 있어, 이 약물을 투여한 가축은 비육률과 사료효율이 높아져 경제적인 효과를 얻을 수 있다. 이런 이유로 오랫동안 가축의 성장촉진제로 사료에 첨가되고 있으며, 그로 인해 오염된 식육을 섭취함으로써 발생하는 2차적인 인체중독의 위험성이 가중되고 있다. 따라서 본 연구에서는 3가지 β-agonist계 약물 (clenbuternol,

ractopamine, zilpaterol)에 대한 신속한 동시분석법을 개발하여 여러 종류의 샘플(쇠고기, 돼지고기 그리고 쇠고기 부산물)에 이 분석법을 적용하였다.

Codex guidelines에 따라 실시한 validation 검사를 통해 도출된 clenbuterol과 zilpaterol의 정량한계는 0.2 ug/kg이었으며, ractopamine은 0.4 ug/kg이었다. 또한 평균 회수률은 clenbuterol 109.1-118.3%, ractopamine 95.3-109.0% 이었으며, zilpaterol은 94.1-120.0%를 보여 모든 약물에서 Codex guidelines에 적합한 수준을 보였다. 또한 변경된 전처리법을 통해 분석시간은 줄었지만 감도는 감소하지 않았다.

이와 같이 본 연구를 통해 zilpaterol 을 추가하여 β-agonist 계 3 가지 약물에 대한 새로운 동시분석법을 개발하였다. 이 방법을 적용하면 β-agonist 약물에 대해 신속하지만 검출효율이 감소되지 않는 분석이 가능할 것으로 기대된다. 또한 zilpaterol 을 추가분석 함으로써 이 약물에 대한 지속적인 관리가 가능하여 앞으로 MRLs 설정 및 이 약물의 위해성 평가에도 도움이 될 것으로 생각된다. 나아가 이 약물이 허가된 나라에서 수입되는 축산물의 안전관리에 활용하여, 2 차 오염에 의한 중독사고 예방 등 국민 보건 수준 향상에 일조 할 수 있으리라 기대한다.

주요어 : 클렌부테롤, 락토파민, 질파테롤

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