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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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Abstract

β-agonists are anabolic compounds that promote fat loss and muscle gain, and their administration to livestock may provide economic benefits by increasing growth rate and feed efficiency. For these reasons, β-agonists are also commonly added to livestock feed as growth promoters. This can introduce a significant risk of secondary human poisoning through intake of contaminated meat. 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 recoveries for clenbuterol, ractopamine, and zilpaterol ranged from 109.1% to 118.3%, 95.3% to 109.0%, and 94.1% to 120.0%, respectively. The recovery and coefficient of variation (CV) values fell within the acceptable range according to the Codex guidelines. This method reduced the analysis time without decreasing detection efficiency by modifying the pretreatment steps. This method could 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.

Key words: clenbuterol, ractopamine, zilpaterol

Introduction

Administration of high doses of β-agonists, which were originally developed to treat human bronchial diseases and premature birth, 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. Toxicity studies have demonstrated that long-term administration of clenbuterol, a member of the β-agonist group, might lead to serious adverse effects in the cardiovascular and nervous systems (Juan *et al.*, 2010; Martinez-Navarro, 1990). Several cases of adverse effects in humans have been linked to the consumption of clenbuterol-contaminated meat products (Brambilla *et al.*, 2000). This led to the prohibition of clenbuterol use in over 150 countries, including European countries (Commission of the European Communities, 1996).

Ractopamine and zilpaterol belong to the same drug class and have effects similar to those of clenbuterol. These compounds 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 (Health Canada, 2014; U.S. Food and Drug Administration, 2014). Likewise, zilpaterol is authorized for use in South Africa, Mexico, the United States, and Canada (Delmore *et al.*, 2010). Given Korea's high dependence on meat imported from areas that still use ractopamine and zilpaterol, analysis of the three commonly used β-

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agonists, clenbuterol, ractopamine, and zilpaterol, in meat products is an important tool for evaluating food safety.

Immunoassays (Shelver *et al.*, 2005; Shelver and Smith, 2011), high-performance liquid chromatography (HPLC) (Freire *et al.*, 2013; Zhao *et al.*, 2010), gas chromatography-mass spectrometry (GC-MS) (Wang *et al.*, 2010), and liquid chromatography-mass spectrometry (LC-MS) (Zhang *et al.*, 2009) have been used to analyze β-agonist residues. Recently, liquid chromatography-tandem mass spectrometry (LC/MS/MS) has been commonly used since it can quantitatively and qualitatively analyze a substance based on the unique characteristics of mass and fragment ions created by an electrical current (Juan *et al.*, 2010; Li *et al.*, 2010; Shao *et al.*, 2009). Previous studies that have simultaneously analyzed clenbuterol, ractopamine, and zilpaterol by LC/MS/MS 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).

A method that improved upon individual assays was developed in South Korea in 2013, and has been used for the simultaneous determination of clenbuterol and ractopamine (Cho *et al.*, 2013; National Institute of Food and Drug Safety Evaluation, 2013). However, this technique does not include measures for analyzing zilpaterol, which has seen increasing use due to its superior growth-enhancing effects.

Policies regarding permission to use zilpaterol vary among countries, and these differences are becoming increasingly problematic. International safety management efforts for zilpaterol are still lacking. In contrast, clenbuterol is prohibited in most countries and is strictly controlled in countries that permit its use. Similarly, the grounds for safety management of ractopamine were

Table 1. Comparison of tolerated ractopamine levels among the FDA, Codex, and Health Canada

Tissue Type	Animal	Tolerated Ractopamine Level (μg/kg)		
		Codex[3]	FDA[4]	Health Canada[5]
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; [3]Codex, Food and Agriculture Organization of the United Nations, 2014; [4]FDA, U.S. Food and Drug Administration, 2014; [5]Health Canada, Health Canada, 2014.

established through the adoption of maximum residue limits (MRLs) by the Codex Alimentarius Commission to ensure human health and safety. The limits shown in Table 1 were based on a risk assessment published by the Joint FAO/WHO Export Committee on Food Additives (JECFA) in July 2012 (Food and Agriculture Organization of United Nations, 2014). Therefore, a method that simultaneously measures all three β-agonists would be beneficial. To that end, a new method that met Codex guidelines and had a high detection efficiency was developed for the simultaneous detection of clenbuterol, ractopamine, and zilpaterol in muscle tissue, the primary source of meat. The method was subsequently validated using a collection of meats and meat byproducts.

Materials and Methods

Reagents and standards

Acetonitrile, *n*-hexane, and ethyl acetate (Burdick & Jackson, USA) were used as extraction solvents. Potassium carbonate (K_2CO_3; Merck, Germany), formic acid (Fluka, USA), and anhydrous sodium sulfate (Junsei Chemical, Japan) were used as pretreatment and mobile phase solvents. Triple distilled water was purified through a Milli-Q system (Millipore, USA). Clenbuterol (clenbuterol hydrochloride, 99.8%), ractopamine (ractopamine hydrochloride, 99.8%), and zilpaterol (zilpaterol hydrochloride, 99.8%) (Dr. Ehrenstorfer GmbH, Germany) were used as standards, whereas clenbuterol-d_9, zilpaterol-d_7 (Dr. Ehrenstorfer GmbH, Germany), and ractopamine-d_3 (CDN_ISOTOPES, Canada) were used as internal standards. All standards and internal standards were diluted with methanol (Burdick & Jackson, 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. Stock and working standard solutions were stored at -20°C and diluted to individual concentrations by using 0.1% formic acid(v/v), as required.

Sample treatment and extraction

Frozen chunks of beef, pork, and beef byproducts imported from several countries were used as samples. Prior

to analysis, the samples were thawed and then ground using a mixer (HMF-560/HK, Hanil, Korea). Five grams of each homogenized, refrigerated sample was placed into 50 mL centrifuge tubes. 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. Ten milliliters 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 solution was centrifuged for 10 min at 4,000 rpm and -4°C, and the supernatant was transferred to a new 50 mL centrifuge tube. Then, 10 mL of ethyl acetate was added to the solution, and the resulting supernatant was isolated using the same extraction procedure. The combined supernatants were gently enriched with nitrogen at 40°C to obtain a final volume of 2-3 mL. Ten milliliters of acetonitrile and 15 mL of n-hexane were added to the concentrate and vortexed for 10 min. The mixture was centrifuged for 10 min at 4,000 rpm and -4°C 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 dissolved 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 through a 0.2 μm polyvinylidenedifluoride syringe filter prior to injection.

Conditions and experimental set-up for chromatography and mass spectrometry

LC-MS (API 4000, AB SCIEX, USA) was used to develop a simultaneous determination method for clenbuterol, ractopamine, and zilpaterol. Separation was performed with X Bridge C_{18} (2.1 mm × 150 mm, 3.5 μm; Waters, Ireland), and the column temperature was maintained at 35°C. The analysis was carried out with 0.1% formic acid (solvent A) and acetonitrile (solvent B) as the mobile phase solvents. 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/min, and the injection volume was 10 μL. Positive electrospray ionization mode was selected, and the analysis was performed in multiple reaction monitoring mode. The source temperature was set at 600 °C. The precursor ion, product ions, collision energy, and dwell time for each substance are listed in Table 2.

MS/MS optimization

Conventional analysis methods for clenbuterol and ractopamine were used to determine the optimal assay conditions for mass spectrometry. Since ions at m/z 203 and 259 have been used in published literature regarding clenbuterol, the collision energy shown in Table 2 was applied to the product ion to obtain ions at these m/z ratios (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 daughter 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 2. 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 the present study, ions at m/z 244 and 185, which showed the highest sensitivity upon application of collision energy, were monitored as shown in Table 2. While the previous studies used ractopamine-d_5, cimaterol-d_7, and clenbuterol-d_9 as internal standards (Blanca et al., 2005; Mauro et al., 2014; Suo et al., 2013),

Table 2. Mass spectrometry transition conditions and retention times

Compounds	Transition	Dwell time (s)	Collision Energy (eV)	Retention time (min)
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.

zilpaterol-d$_7$ was used as an internal standard for zilpaterol to improve efficiency and reproducibility in the present study.

Validation

Linearity, recovery, limit of quantification (LOQ), and reproducibility tests were conducted to validate our method according to the Codex guidelines (Codex Alimentarius Commission, 1993). Three imported samples each of beef, pork, and beef byproducts were used for these validation experiments. Three different concentrations of each sample were tested. Calibration curves were prepared at concentrations of 0.2-16 μg/kg for clenbuterol and zil-

paterol, 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 greater than 10 was obtained for each substance. Coefficients of variation (CV) were calculated as the ratio of the standard deviation to the mean. Fig. 1 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 that were 0.5, 1, and 2 times the MRLs (0.005, 0.01, and 0.02 mg/kg) for ractopamine, and 1, 2, and 4 times the LOQ values (0.2, 0.4, and 0.8 μg/kg) for clenbuterol and zilpaterol.
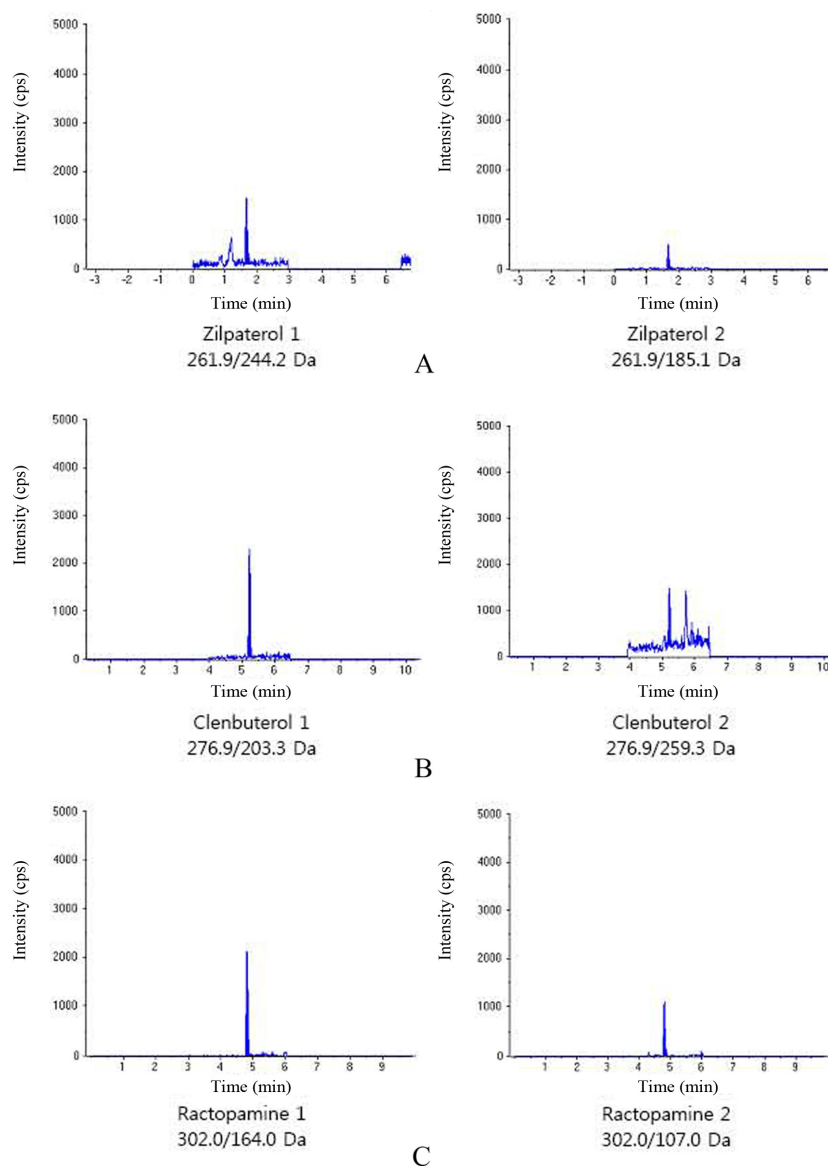

Fig. 1. Ion chromatograms of zilpaterol, clenbuterol, and ractopamine at the limit of quantification (LOQ). The daughter ion chromatograms for A, zilpaterol; B, clenbuterol;and C, ractopamineare shown for drugs injected into meat samples at the LOQ level (0.2 μg/kg for zilpaterol and clenbuterol and 0.4 μg/kg for ractopamine).
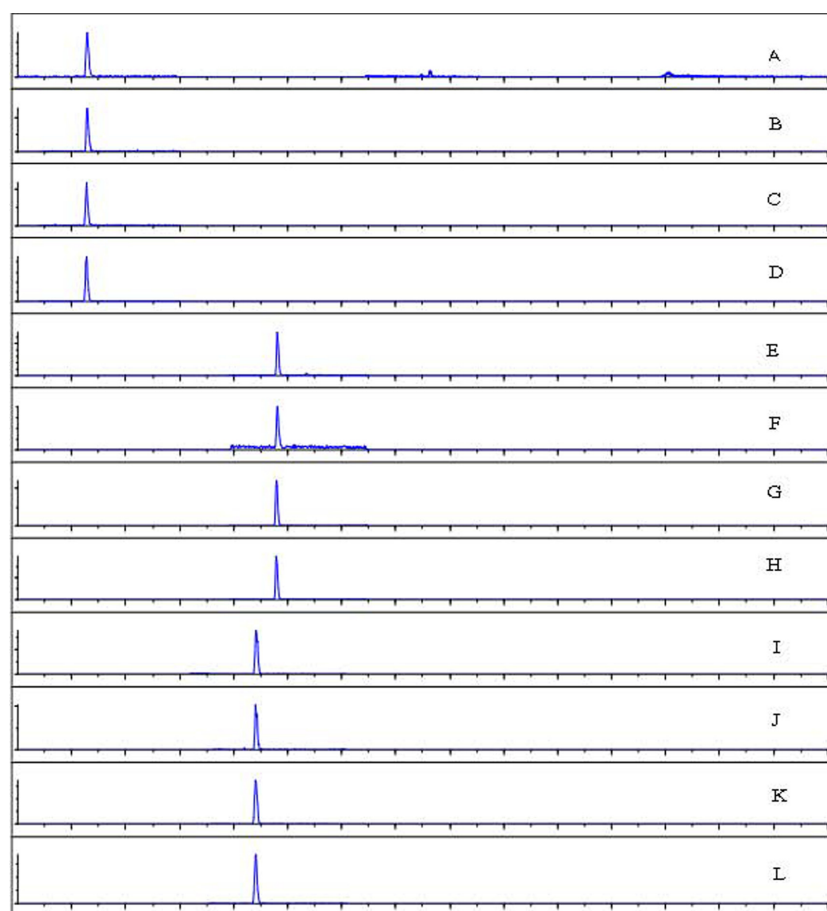
Fig. 2. Total chromatograms of standard solutions. Peaks: zilpaterol (A, B), zilpaterol-d$_7$ (C, D), clenbuterol (E, F), clenbuterol-d$_9$ (G, H), ractopamine (I, J), ractopamine-d$_3$ (K, L).

Peak retention times in the chromatogram were compared and adjusted according to the internal standards to determine recovery rates using the content values of clenbuterol, ractopamine, and zilpaterol. Each concentration was analyzed in triplicate to verify the experimental methods.

Results and Discussion

Comparison with current β-agonist detection methods

A method for the simultaneous determination of clenbuterol and ractopamine was developed in 2013 in South Korea and is currently used as a Food Code analysis method (KFDA, 2013). Fig. 3 compares the Food Code analysis and Food Safety and Inspection Service (FSIS) methods with the new method developed herein. The FSIS method comprises eight steps and does not include solid phase extraction and deproteinization steps, as is the case in our method (United States Department of Agriculture Food Safety and Inspection Service Office of Public Health Science, 2014). Notably, there are additional

examples of methods that do not use deproteinization steps for the analyses of feed and milk (Li *et al.*, 2010; Suo *et al.*, 2013). We used 4 M K$_2$CO$_3$ to adjust the pH of the solution to strongly basic, and 5 mL of triple distilled water to promote dispersion of the sample in the pretreatment solvent and to increase the recovery rate. We also used acetonitrile and hexane in place of saturated methanol and hexane to increase the liquid-liquid distribution effect (Juan *et al.*, 2010), and maintained the temperature during nitrogen decompression at 40°C, rather than 55°C, to minimize evaporation (Li *et al.*, 2010; Mauro *et al.*, 2014). Finally, we used 0.1% aqueous formic acid and acetonitrile as the mobile phase instead of buffered ammonium acetate to ensure no overlapping retention times, encourage optimal peak shape, and minimize run time (Fig. 2).

Applicability

In our validation tests, R^2 values for the clenbuterol, ractopamine, and zilpaterol calibration curves were 0.9992,

A	B	C

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 Na₂SO₄ evaporated at 55℃

↓

SPE with MIP cartridge

↓

Evaporation at 50℃

↓

LC-MS/MS analysis

B

5 g sample + 4 M potassium carbonate (K₂CO₃) + Internal standard + Distilled water

↓

Perform ethyl acetate extraction twice, centrifuge and collect the supernatant + Dehydration with Na₂SO₄ (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 Na₂SO₄ and 0.5 g MgSO₄ 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
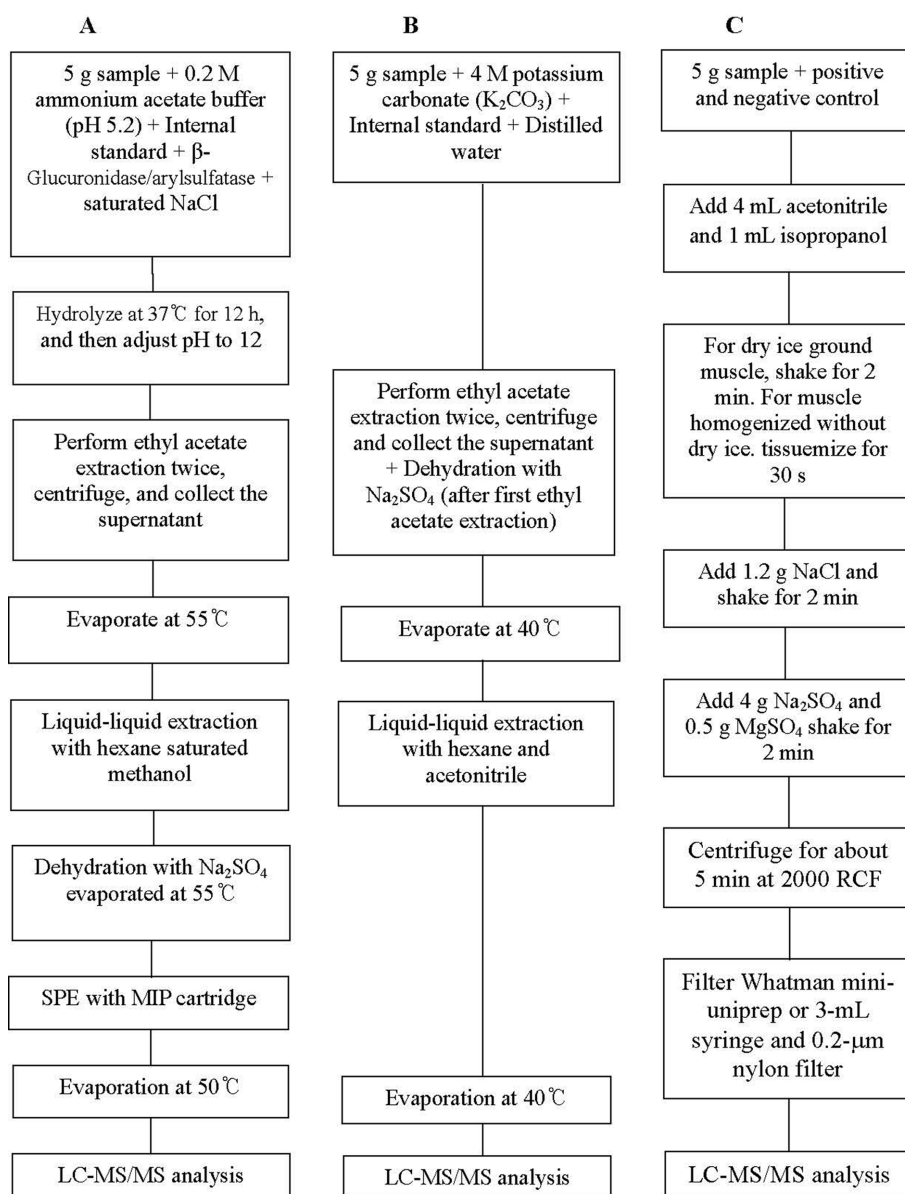
↓

LC-MS/MS analysis

Fig. 3. Comparison of the Food Code analysis, the new analysis method, and an analysis by the United States Food Safety and Inspection Service (FSIS). A, The Food Code method; B, the new analysis method; C, the U.S. FSIS method.

0.9998, and 0.9979, respectively. The LOQ was 0.2 μg/kg for clenbuterol and zilpaterol, and 0.4 μg/kg for racto-pamine. The average recovery rates were 109.1-118.3% for clenbuterol, 95.3-109.0% for ractopamine, and 94.1-120.0% for zilpaterol. The CV values were less than 10.58% for all three compounds. These results met the Codex criteria for linearity, recovery rate, LOQ, CV, and reproducibility (Table 3). Accordingly, these findings confirmed the applicability of our method.

International trends in β-agonist drug permissibility
Based on safety concerns, β-agonist drugs are now con-

sidered more controversial 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. The Codex Alimentarius Commission, the United Nations food standards body, established racto-pamine 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 these Codex MRLs. Although all β-agonists are prohibited for use as growth pro-moters in the EU (Commission of the European Commu-

Table 3. Validation data for clenbuterol, ractopamine, and zilpaterol in meat samples

Compounds	R^2 [1]	LOQ[2] (μg/kg)	Peak concentration (mg/kg)	Beef		Pork		Beef byproducts	
				Recovery (%)	CV[3]	Recovery (%)	CV	Recovery (%)	CV
Clenbuterol	0.9992	0.2	0.0002	113.33	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	0.0050	95.33	9.46	104.00	5.77	102.00	3.40
			0.0100	101.17	8.22	98.83	2.04	109.00	10.58
			0.0200	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]R^2, correlation coefficient; [2]LOQ, limit of quantification; [3]CV, coefficient of variation.
Data are expressed as the mean value of 3 experiments.

nities, 1996), the United States permits the use of a level that is 3-5 times higher than the Codex MRLs (U.S. Food and Drug Administration, 2014). The United States uses a separate standard for turkey, for which Codex has not yet established MRLs. Unlike the United States, Canada follows the limits suggested by Codex, although a separate national 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 Codex guidelines and Canadian laws, but no limits have been set in the United States (Table 1). In the United States, zilpaterol detection is permitted at concentrations below 12 μg/kg (U.S. Food and Drug Administration, 2014). Similarly, in Canada zilpaterol at concentrations up to 5, 5, and 2 μg/kg is permitted in cow kidney, liver, and muscle, respectively (Health Canada, 2014). During the 35th session (2012) of the Codex Alimentarius Commission, JECFA decided to conduct a risk assessment for zilpaterol, and new MRLs for zilpaterol are expected to be established in the near future. Based on trends, it is expected that the new limits maybe controversial and not universally adopted. Consequently, β-agonist use in livestock may remain important, and further studies on human health risk of β-agonists will be necessary. Therefore, our simultaneous determination method of β-agonists in products destined for human consumption may prove timely and beneficial.

Safety issues associated with zilpaterol

Despite international trends toward establishing MRLs for zilpaterol, continued controversy surrounding the safety issue of this drug is expected. Although clinical toxicology studies have been conducted in horses (Hepworth-Warren and Alcott, 2014; Wagner et al., 2008), case studies on the toxicity in other animals and humans are still lacking. 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). Herein is reported the validation of a method to simultaneously measure clenbuterol, ractopamine, and zilpaterol in muscle tissue, the primary source of meat.

Application of the new method

Using our method, we performed residue analysis for levels of the three β-agonists in the following 299 imported meats and byproducts samples: 154 beef, 57 pork, and 88 beef byproducts. Our results revealed that ractopamine was detected in two beef samples, and zilpaterol was detected in one beef and one beef byproduct sample (Table 4). Based on these results, we believe that there is non-negligible contamination of imported meat and meat byproducts. In particular, a high detection rate was observed for zilpaterol, for which MRLs have not yet been established, indicating a need for the continued surveillance of this drug. Other less frequently used β-agonists that still pose a health risk, such as cimaterol and salbutamol, could be assessed using similar simultaneous determination method in future studies. In addition, the scope of the method may be extended to include processed meat products.

Conclusion

A new method for the simultaneous determination of

Table 4. Monitoring data for three β-agonists from 299 samples

Type	N	Compounds	Samples detected (n)	Residue level (µg/kg)	MRLs[1] (µg/kg)
Beef	154	Clenbuterol	-	-	NE[2]
		Ractopamine	2	0.6, 2.0	10
		Zilpaterol	1	0.01	NE
Pork	57	Clenbuterol	-	-	NE
		Ractopamine	-	-	10
		Zilpaterol	-	-	NE
Beef byproduct	88	Clenbuterol	-	-	NE
		Ractopamine	-	-	10
		Zilpaterol	1	6.3	NE

[1]MRLs, maximum residue limits; [2]NE, not established.

three β-agonists, including zilpaterol, was developed. 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 and was applicable to muscle samples, making it appropriate for further studies of β-agonists in meat. Also, it had the advantage of determining zilpaterol, which is not included in the Food Code method, at lower levels than those detectable using the US FSIS method. 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 could 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.

References

1. Blanca, J., Muñoz, P., Morgado, M., Méndez, N., Aranda, A., Reuvers, T., and Hooghuis, H. (2005) Determination of clenbuterol, ractopamine and zilpaterol in liver and urine by liquid chromatography tandem mass spectrometry. *Anal. Chim. Acta.* **529**, 199-205.

2. Brambilla, G., Cenci, T., Franconi, F., Galarini, R., Macri, A., Rondoni, F., Strozzi, M., and Loizzo, A. (2000) Clinical and pharmacological profile in a clenbuterol epidemic poisoning of contaminated beef meat in Italy. *Toxicol. Lett.* **114**, 47-53.

3. Cho, Y. J., Chae, Y. S., Kim, J. Y., Kim, H. S., Kang, I., Do, J. A., Oh, J. H., Kwon, K., and Chang, M. I. (2013) Improvement of a simultaneous determination for clenbuterol and ractopamine in livestock products using LC-MS/MS. *Korean J. Food Sci. Technol.* **45**, 8.

4. Codex Alimentarius Committee (1993) Codex guidelines for the establishment of a regulatory programme for control of veterinary drug residues in foods. Codex, Rome, pp. 1-46.

5. Commission of the European Communities (1996) Council directive 96/22/EC. *Off. J. Eur. Commun.* **No. L125**, 10.

6. Delmore, R. J., Hodgen, J. M., and Johnson, B. J. (2010) Perspectives on the application of zilpaterol hydrochloride in the United States beef industry. *J. Anim. Sci.* **88**, 2825-2828.

7. Food and Agriculture Organization of United Nations (2014) UN food safety body sets limits on veterinary growth promoting drug. Available from: http://www.fao.org/media/news article. Accessed on April 7, 2014.

8. Freire, E. F., Borges, K. B., Tanimoto, H., Nogueira, R. T., Bertolini, L. C., and de Gaitani, C. M. (2013) Monitoring of ractopamine concentration in the mixture of this feed additive with vitamin mineral complex and with swine feed by HPLC. *Food Addit. Contam. Part A. Chem. Anal. Control. Expo. Risk. Assess.* **30**, 796-803.

9. Health Canada (2014) List of maximum residue limits (MRLs) for veterinary drugs in foods. Available from: http://www.hc-sc.gc.ca/Drugs & Health product/Veterinary Drugs/Maximum Residue Limits. Accessed on April 7, 2014.

10. Hepworth-Warren, K. L. and Alcott, C. J. (2014) Treatment and resolution of zilpaterol hydrochloride toxicity in a quarter horse gelding. *Equine Vet. Educ.* **26**, 81-85.

11. Juan, C., Igualada, C., Moragues, F., Leon, N., and Manes, J. (2010) Development and validation of a liquid chromatography tandem mass spectrometry method for the analysis of beta-agonists in animal feed and drinking water. *J. Chromatogr. A.* **1217**, 6061-6068.

12. KFDA (2013) Food Code. Korea Food and Drug Administration, Cheongwon, Korea, pp. 5.3.63.

13. Lawrence, T. E., Gasch, C. A., Hutcheson, J. P., and Hodgen,

J. M. (2011) Zilpaterol improves feeding performance and fabrication yield of concentrate-finished cull cows. *J. Anim. Sci.* **89**, 2170-2175.

14. Li, C., Wu, Y. L., Yang, T., Zhang, Y., and Huang-Fu, W. G. (2010) Simultaneous determination of clenbuterol, salbutamol and ractopamine in milk by reversed-phase liquid chromatography tandem mass spectrometry with isotope dilution. *J. Chromatogr. A.* **1217**, 7873-7877.

15. Martinez-Navarro, J. F. (1990) Food poisoning related to consumption of illicit β-agonist in liver. *Lancet* **336**, 1311.

16. Mauro, D., Ciardullo, S., Civitareale, C., Fiori, M., Pastorelli, A. A., Stacchini, P., and Palleschi, G. (2014) Development and validation of a multi-residue method for determination of 18 β-agonists in bovine urine by UPLC-MS/MS. *Microchem. J.* **115**, 70-77.

17. National Institute of Food and Drug Safety Evaluation (2013) Hand book of hazardous residue analysis for livestock foods and marine products. National Institute of Food and Drug Safety Evaluation, Cheongwon, Korea, pp. 130-133 (in Korean)

18. Shao, B., Jia, X., Zhang, J., Meng, J., Wu, Y., Duan, H., and Tu, X. (2009) Multi-residual analysis of 16 β-agonists in pig liver, kidney and muscle by ultra performance liquid chromatography tandem mass spectrometry. *Food Chem.* **114**, 1115-1121.

19. Shelver, W. L., Keum, Y. S., Li, Q. X., Fodey, T. L., and Elliott, C. T. (2005) Development of an immunobiosensor assay for the beta-adrenergic compound zilpaterol. *Food Agric. Immunol.* **16**, 199-211.

20. Shelver, W. L. and Smith, D. J. (2011) Immunochemical-based zilpaterol measurement and validation in urine and tissues. *Food Agric. Immunol.* **22**, 247-258.

21. Suo, D. C., Zhao, G. L., Wang, P. L., and Su, X. O. (2013) Simultaneous determination of beta-agonists and psychiatric drugs in feeds by LC-MS-MS. *J. Chromatogr. Sci.* **2013**, 1-5

22. United States Department of Agriculture Food Safety and Inspection Service Office of Public Health Science (2014) Screening and confirmation of beta-agonists by HPLC/MS/MS. Washington DC, USA, pp. 1-16.

23. U.S. Food and Drug Administration (2014) The code of federal regulations, title 21. Available from: http://www.fda.gov/medicaldevices/databases. Accessed Apr. 7, 2014.

24. Wagner, S. A., Mostrom, M. S., Hammer, C. J., Thorson, J. F., and Smith, D. J. (2008) Adverse effects of zilpaterol administration in horses: Three cases. *J. Equine Vet. Sci.* **28**, 238-243.

25. Wang, L., Li, Y. Q., Zhou, Y. K., and Yang, Y. (2010) Determination of four β2-agonists in meat, liver and kidney by GC-MS with dual internal standards. *Chromatographia* **71**, 737-739.

26. Williams, L. D., Churchwell, M. I., and Doerge, D. R. (2004) Multiresidue confirmation of β-agonists in bovine retina and liver using LC-ES/MS/MS. *J. Chromatogr. B.Analyt. Technol. Biomed. Life Sci.* **813**, 35-45.

27. Zhang, L. Y., Chang, B. Y., Dong, T., He, P. L., Yang, W. J., and Wang, Z. Y. (2009) Simultaneous determination of salbutamol, ractopamine, and clenbuterol in animal feeds by SPE and LC-MS. *J. Chromatogr. Sci.* **47**, 324-328.

28. Zhao, L., Zhao, J., Huangfu, W. G., and Wu, Y. L. (2010) Simultaneous determination of melamine and clenbuterol in animal feeds by GC-MS. *Chromatographia* **72**, 365-368.