Establishment of a Method for Analyzing the Zilpaterol Residue in Beef Using Tandem Mass Spectrometry

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Establishment of an analytical method on zilpaterol (ZPT), the beta-agonists, and a growth promoting agent for livestock was undertaken. Analytical method for ZPT in the beef by LC-ESI-MS/MS was adopted. Before sample extraction, a beef sample was hydrolyzed by beta-glucuronidase/arylsulfatase and 0.2 M ammonium acetate buffer solution. Afterwards, the mixture was extracted with ethylacetate and then defatted by n-hexane/methanol partitioning. Subsequently, the separated organic layer was evaporated and the dissolved solution was purified by using a Molecular Imprinted Polymer solid phase extraction. The quantitation was carried out using LC-ESI-MS/MS with C18, a non-polarity column under the gradient condition with 10 mM ammonium acetate buffer solution (pH 4.3) : acetonitrile (95 : 5 (v/v), a mobile phase A) and acetonitrile (a mobile phase B). The standard calibration curve presented linearity of correlation coefficient (r2) > 0.999, analyzed from 0.2 to 10 ng/mL. The limit of quantitation in a beef showed 0.2 μg/kg. The average recoveries of intra- and inter-day experiment ranged from 100.9 to 108.5% and 103.3 to 104.5%, respectively. The repeatability and reproducibility for intra- and inter-day measurement expressed as a relative standard deviation was less than 10.0%, respectively. Therefore, the established analytical method for ZPT residue in beef was applicable to the official analytical method with the acceptable level of sensitivity, repeatability and reproducibility.

Keywords: beta-agonists, beef, analytical method, LC-ESI-MS/MS, zilpaterol

Introduction
The veterinary drugs, including antibiotic, antiprotozoal, synthetically antimicrobial, anthelmintic, insecticide, sedative, synthetic hormone, anti-inflammatory, and beta-agonists agents have used much to the therapeutic and prophylactic purposes as well as for improving breeding efficiency (Bocca et al., 2003). However, the abuse of veterinary drug could not only adverse effects but also lead to health risks in humans. For this reason, the establishment regarding maximum residue limits (MRLs) of veterinary drug in livestock products was required, which is strictly regulated by the government.
regulated in the several countries. Particularly, in Korea, MRLs of veterinary drug residues were established to the livestock products or marine products of 124 (MFDS, 2013). Furthermore, in order to ensure the safety assurance of circulated livestock products, the continuous nation-wide veterinary drug residue monitoring programs were conducted (Yamamoto et al., 2009; MFDS, 2012). Also, the results of nation-wide veterinary drug residues monitoring were reflected in the policy of food safety/hygiene.

Growth promoting agent, beta-agonists are commonly used as bronchodilators for the treatment of pulmonary diseases and the economic advantage in mass gain of feedlot steers, has progressively forwarded the use of beta2 agonists at doses above therapeutic ones as feed additives for livestock (Bocca et al., 2003; Verhoeckx et al., 2006; Van Hoof et al., 2005). At higher doses, an anabolic growth-promoting effect is evidenced with increased muscle development and reduced fat deposition (Blanca et al., 2005; López-Carlos et al., 2010). These effects lead to the misuse of these drugs in livestock production. For that reason, in the European Union, the use of beta-agonists as growth promoters is banned by the council directive 96/23/EC (European Commission, 1996). On the other hand, in Korea, all beta-agonists excluding the ractopamine is banned and regulated (MFDS, 2013).

The species of the beta-agonists exist in clenbuterol, cimaterol, terbutaline, metaproterenol, salbuterol, ractopamine, isoxsuprine, zilpaterol, among others (Van Hoof et al., 2005; López-Carlos et al., 2010). Among these, a zilpaterol (ZPT) is a beta-adrenergic agonist which is used to a growth promoting agent for the livestock product (Verhoeckx et al., 2006). It was produced by Intervet and Merck KGaA (Germany). A beta-glucuronidase/arylsulfatase was obtained from Merck KGaA (Germany). Sample extraction and purification were carried out with Molecular Imprinted Polymer (MIP) solid phase extraction (SPE) cartridge from Supelco (USA). All other chemicals and reagents used throughout the study were of analytical-grade, unless stated otherwise.

**Materials and Methods**

**Samples** A beef of residue-free was purchased from the local markets in Korea. The samples were homogenized by using a blender, and then kept in a polyethylene container in a freezer at temperature below –50°C.

**Chemicals and reagents** A pure standard ZPT (certified analytical standard, 98.5%) was purchased from Toronto Research Chemical (Canada) and an internal standard (I.S.) clenbuterol-d9 (CBT-d9, 99.5%) was purchased from Dr. Ehrenstorfer (Germany). The chemical structures of ZPT and CBT-d9 are shown in Fig. 1. Analytical-grade acetic acid, ammonium acetate, hydrochloric acid, sodium chloride, and sodium hydroxide were acquired from Wako chemical (Japan). Acetone, acetonitrile, ethyl acetate, n-hexane, and methanol were of HPLC grade and were supplied by Merck KGaA (Germany). A beta-glucuronidase/arylsulfatase was obtained from Merck KGaA (Germany). Sample extraction and purification were carried out with Molecular Imprinted Polymer (MIP) solid phase extraction (SPE) cartridge from Supelco (USA).

**Standard solution preparation** For stock standard solution, ZPT and CBT-d9 were prepared in methanol at concentration of a 100 μg/mL and 10 μg/mL, respectively. The levels of the working standard solutions were prepared via the serial dilution using a mobile phase A, reaching the following concentration: 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 ng/mL. All standard solutions were maintained in an amber bottle and stored at –20°C pending analysis.

**Sample preparation** Precisely 5 grams (± 0.1 g) of the homogenized sample were placed into a 50 mL centrifuge tube, to which 200 μL of I.S. (0.04 μg/mL), 100 μL of beta-glucuronidase/arylsulfatase, 10 mL of 0.2 M ammonium acetate buffer solution (pH 5.2), and 10 mL of saturated sodium chloride were added, and

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**Fig. 1.** The chemical structure of zilpaterol and clenbuterol-d9.
the mixture was vigorously shaken. For enzymatic hydrolysis, mixture was carried out by incubating overnight at 37°C. After hydrolysis, the mixture was adjusted as pH 12.0 (± 0.5) using 5M NaOH solution and 10 mL of ethylacetate was added then followed by 5 min of centrifugation at 6,000 × g to separate the solvent (× 2). The collected supernatants were evaporated to near dryness by rotary evaporator at 55°C, and then reconstituted in 20 mL of n-hexane saturated with methanol. The dissolved solution was transferred separatory funnel and to which was added 20 mL of methanol saturated with n-hexane. After 3 min of vigorously shaking, the methanol phase was transferred to a round bottom flask, and then evaporated to near dryness by rotary evaporator at 55°C. Finally make up with 2 mL of 0.025 M ammonium acetate solution

**Purification procedure** For conditioning the MIP cartridge, 1 mL of methanol, 1 mL of water, and 1 mL of 0.025 M ammonium acetate solution were introduced and discarded. The dissolved solution from 2 mL of 0.025 M ammonium acetate solution was introduced into the MIP cartridge and the eluate was collected into a round bottom flask. The analytes were then eluted with 5 mL of methanol : acetic acid (9 : 1, v/v) in tube. The eluate was evaporated to near dryness by nitrogen-evaporator (N-EVAP III, Oranomation Associates, USA) under a nitrogen steam below 40°C. Finally, the residue was dissolved in 2 mL of mobile phase A (10 mM ammonium acetate : acetonitrile (95 : 5, v/v)).

**LC-ESI-MS/MS analysis** The LC-ESI-MS/MS system was conducted by Thermo accela high speed-TSQ Vantage (USA) and the analyte was separated on an Atlantis dC_{18} column (2.1 × 150 mm, 3 μm, Ireland). The binary solvent system was run in gradient mode with 10mM ammonium acetate (pH 4.3) : acetonitrile (95 : 5 v/v), a mobile phase A and acetonitrile (a mobile phase B). A linear mobile phase gradient started at 100% A (0 – 2 min), decreased to 0% A (2 – 8 min) and was maintained at 100% B (8 – 10 min), followed by a gradient to 100% A (10 – 14 min), after which the column was equilibrated at 100% A (14 – 17 min). The flow rate was 0.2 mL/min with a column temperature maintained at 40°C. The injection volume in each experiment was 10 μL. The main ions produced in tandem mass spectrometer were obtained in positive electrospray ionization (ESI+), then was optimized to increase sensitivity. The MS source conditions were as follows: The ion spray voltage was set at 5.0 kV and capillary temperature at 350°C.

**Results and Discussion**

**Establishment of instrument optimization** In this study, the ESI probe was selected as ionization technique due to its sensitivity, simplicity, easy handling and maintenance. Additionally, all compounds presented positive ionization and MS parameters were optimized in order to have at least two segments with acceptable sensitivity.

Therefore, according to the chemical structure property of ZPT, the mass spectra in ESI positive mode was recorded. Fig. 2 shows the full scan and the MS/MS spectra for ZPT. The full scan spectrum (optimized at a S-Lens RF amplitude voltage of 57 V) showed an abundant ion at a m/z 262.19 corresponding to the [M + H]^+ ion. The MS/MS spectrum presented a major peak at m/z 244.18 and 185.10 optimized at the collision energy of 24 and 12 eV, respectively. These fragmentations were judged that the fragment with m/z 244.18 was due to the loss of water, a subsequent loss of NH₃ and CH₃CCH₃ led to the fragment with m/z 185.10 (Van Hoof et al., 2005). Therefore, 262.19→244.18 and 262.19→185.10 were selected as quantitative and confirmative transitions, respectively.

A column was chosen for a C_{18} reverse phase considering polarity, and other instrument condition used the official method for clenbuterol and ractopamine residues in Korea food code (MFDS, 2013). However, the working standard solution made from methanol caused the peak tailing on operation conditions. In order to overcome the problem of the poor resolution, the final reconstitution solution was applied to a mobile phase A, resulting in a considerable increase in the peak symmetry and sharpness of ZPT standard solution.

**Establishment of sample preparation procedure** Beta-glucuronidase/arylsulfatase has been commonly used as hydrolysis enzyme to optimize and improve the detecting ability of residual beta-agonist compounds (Smith et al., 2002). Additionally, an ammonium acetate buffer solution was utilized usefully as part of a protein purification solution to remove contaminants via the diffusion (Wikipedia, 2013). Therefore, in order to improve the efficiently detecting ability of target compound, the sample was hydrolyzed in incubator at 37°C after adding beta-glucuronidase/arylsulfatase and ammonium acetate buffer. Subsequently, the hydrolyzed conjugates was controlled as pH 12 using a sodium hydroxide, and then added with an organic solvent. In this study, an ethylacetate was used as the extraction solvent because it has readily separated from an water by liquid-liquid separation with non-polar solvents.

Sample separation was used for liquid-liquid partition method. As beef includes substantial fat and non-polar coextractives, the n-hexane/methanol partition was added to remove non-polar coextractives. The n-hexane/methanol partition is a very efficient method for fat and non-polar coextractive elimination (Kwon et al., 2011). Sample purification was used for solid phase extraction method. ZPT is a beta-agonists which is efficiently purified via alumina A, HCX, HLB, MEX, MIP, and PCX SPE cartridge (Cho et al., 2013). Among these, MIP is applied to ZPT clean-up. It has been prepared and applied in selective recognition and enrichment of ZPT, and they exhibited high recognition selectivity. Meanwhile, because a zilpaterol-d₇ is hard to find, a CBT-d₉ of similar physicochemical property was selected as I.S.

**Method validation** The specificity of the analytical method was judged from the absence of interfering peaks at the retention
times of the analytes. The retention times of ZPT and CBT-d9 were constant at 6.62 ± 0.1 and 8.12 ± 0.1 min, and there were no interfering peaks at the retention times of ZPT and CBT-d9 (Fig. 3).

The linearity of ZPT working standard solution by the analytical method was conducted via an internal standard procedure (Using the response ratio of their strongest transition compared to a CBT-d9 internal standard). The equation of calibration curve was obtained by plotting peak areas in ‘y’ axis against concentrations of

Fig. 2. Precursor ion- and product ion-spectra of zilpaterol and clenbuterol-d9.
Fig. 3. Chromatogram results by LC-MS/MS analysis of zilpaterol in beef muscle. (A) chromatogram of zilpaterol (1 µg/kg) and clenbuterol-$d_9$ (4 µg/kg) standard solution, (B) chromatogram of a blank beef, (C) chromatogram of spiked beef at 0.4 µg/kg.
ZPT in ‘x’ axis (Hem et al., 2011), which was $y = 0.0257x + 0.0004$, with a correlation coefficient ($r^2$) of 0.999 (Table 1). These values demonstrate that the method’s quantification is sufficient for Codex Guideline ($r^2 > 0.95$) (Codex, 1993).

The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the standard deviation of blank sample responses (σ) and the slope of the calibration curve (S), which were calculated by multiplying σ/S by 3.3 and 10, respectively (ICH, 1996). Instrumental LOD and LOQ were determined to be 1.5 and 5 pg, respectively.

MLOQ (Method Limit of Quantitation) is not an instrumental LOQ, but instead is a practical LOQ for the total analytical method. It is usually calculated by using instrumental LOQ, injection volume, final extract volume, and sample weight in analytical method (Lee, 2012; Lee et al., 2012). MLOQ value for ZPT was 0.2 µg/kg. MRL for ZPT of animal products in U.S. FDA is set up in liver (12 µg/kg) (U.S.FDA, 2013). According to the guideline on the residue analytical method in SANCO/825/00 (European Commission, 2010), MLOQ of animal origin is recommended below 10 µg/kg or lowest MRL. Consequently, the proposed MLOQ value is adjudged reasonable for determination of MRL of ZPT residue in beef muscle.

Accuracy and precision were conducted via intra- and inter-day analyses (in a single laboratory), and precision was calculated in terms of intra-day repeatability and inter-day reproducibility (ICH, 1996; Thompson et al., 2002). Accuracy was expressed as a percentage of recovery and precision as a relative standard deviation (RSD). Intra- and inter-day analyses were conducted using fortified beef muscle (no Korea MRL criteria) at two different concentrations of MLOQ (0.2 µg/kg) and 2 × MLOQ (0.4 µg/kg). Intra-day analysis was conducted in six replicates at each concentration level, whereas inter-day analysis was performed for three consecutive days in triplicate at the same concentrations. The recovery averages of the intra-day experiment ranged from 100.9-108.5% and the recovery averages of inter-day experiment ranged from 103.3 to 104.5% (Table 2). The intra-day repeatability expressed as RSD was less than 9.2% in beef muscle, whereas inter-day reproducibility expressed as RSD was less than 9.5% in beef muscle (Table 2). These recovery and RSD values were consistent with the ranges listed in the Codex Guideline (Codex, 1993), and thus the method described herein can be considered excellent as a reliable, reproducible, and accurate routine analytical method. Additionally, it had the tendency to be similar to the results that the existing researcher reports (Van Hoof et al., 2005; Williams et al., 2004). Therefore, our newly established analytical method for ZPT residue in beef muscle can be confirmed as the suitable method.

### References


### Table 1. Standard curve range, linearity and $r^2$ of zilpaterol

<table>
<thead>
<tr>
<th>Compound</th>
<th>Matrix</th>
<th>Concentration range (µg/kg)</th>
<th>Linearity</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zilpaterol</td>
<td>Beef muscle</td>
<td>0.2 – 10</td>
<td>$y = 0.0257x – 0.0004$</td>
<td>0.999</td>
</tr>
</tbody>
</table>

### Table 2. Intra- and inter-day recoveries and RSD of zilpaterol in beef muscle

<table>
<thead>
<tr>
<th>Spiked concentration (µg/kg)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-day (n = 6)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>100.96 ± 8.19</td>
<td>8.11</td>
</tr>
<tr>
<td>0.4</td>
<td>108.45 ± 9.98</td>
<td>9.20</td>
</tr>
<tr>
<td><strong>Inter-day (n = 3 × 3 days)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>103.38 ± 8.46</td>
<td>8.18</td>
</tr>
<tr>
<td>0.4</td>
<td>104.51 ± 9.93</td>
<td>9.50</td>
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Analytical Method for Determination of Zilpaterol in Beef


**URL citations**


ii) http://en.wikipedia.org/wiki/Ammonium_acetate (June 30, 2014)