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Improvement of simultaneous multi-residue analysis of 5 β-agonists in livestock and fishery products using LC-MS/MS

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Abstract: β-agonists, veterinary drugs used as bronchodilators and labor suppressants in humans and livestock, are also employed as growth promoters in livestock by inhibiting fat biosynthesis and stimulating muscle growth. Residues of these drugs in animal–derived food products, however, pose potential health risks to consumers. Numerous studies have been conducted on analytical methods for β-agonists, typically involving extraction with enzymes or acid treatment. However, these existing methods present challenges, including safety risks associated with reagents, lengthy extraction times, and high costs. This study developed a safe and rapid analytical method using hydrochloric acid instead of perchloric acid for extraction solvent and 0.1 % formic acid for redissolution solvent. The method demonstrated a correlation coefficient greater than 0.99, with an average recovery and a coefficient of variation that met the requirements of the CODEX guidelines. This method is expected to increase the efficiency of β–agonist residue monitoring and management.

Key words: β-agonist, veterinary drug, method validation, food safety, LC–MS/MS

Introduction

β-agonists, also known as phenylethanolamine derivatives, possess a conjugated aromatic ring with an amino group.[1] These compounds, which include zilpaterol, salbutamol, cimaterol, ractopamine, and clenbuterol, are well known for activating various physiological activities such as cardiac stimulation, inhibition of intestinal motility, vasodilation, and smooth muscle relaxation.[1-2] Consequently, these β-agonists are used as bronchodilators and labor suppressants in both humans and livestock. In particular, in livestock, β-agonists are known to inhibit fat biosynthesis and promote muscle growth. However, their use as growth supplements is generally prohibited, with exceptions for a few specific β-agonists,[3-4] owing to the potential health risks posed by their residues in animal tissues and, consequently, in foods

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derived from these animals.[5-7]

Extraction methods for β-agonists typically include enzyme treatment or acid treatment.[8-10] Enzyme–based methods often require time–consuming and complex sample pretreatment procedures to mitigate matrix effects. Currently, the method 8.3.24 of Food Code employs an acid extraction method, which provides a short analysis time but poses safety risks. To enhance safety and improve the analytical process, a modification of the method was considered, involving changes to the reagents used, thereby creating a safer experimental environment.

The method 8.3.24 is a certified procedure outlined in the Food Code by the Ministry of Food and Drug Safety (MFDS) of the Republic of Korea for the analysis of β-agonists, specifically salbutamol and cimaterol, which are veterinary drug residues.[11] Methods for the analysis of other β-agonists, such as zilpaterol, ractopamine, and clenbuterol, are included in the multi-residue analytical method 8.3.1.[12] Currently, the limit of quantification (LOQ) of zilpaterol in meat is set at 0.001 mg/kg, but the maximum residue limit (MRL) for beef is set to be revised to 0.0005 mg/kg. MRL refers to the highest level of a residue legally allowed in food, established to protect human health. This revision necessitates the modification of the LOQ to align with the updated regulations. Therefore, there is a need to improve the 8.3.24 method, not only to ensure regulatory compliance but also to combine the analysis of all five β-agonists into a single, more efficient method.

Previous research has developed various analytical methods for β-agonists, based on HPLC, GC–MS, LC–MS, and LC–MS/MS.[13-15] HPLC methods often lack specificity, while GC–MS methods require derivatization because of the high polarity of β-agonists, making them time–consuming, laborious, and expensive. In contrast, LC–MS/MS offers superior sensitivity and selectivity for quantitative determination and has become the preferred method.[16-18] Consequently, this study employs LC–MS/MS analysis for β-agonists.

This study focused to develop a safe and efficient LC–MS/MS analytical method by improving Food Code method 8.3.24. In addition, an effort was made to enhance the management of each group of veterinary drugs by developing a simultaneous analytical method for five β-agonists, incorporating ractopamine, zilpaterol, and clenbuterol into the existing target compounds of method 8.3.24. This developed method is designed to improve the efficiency of veterinary drug residue monitoring in domestic livestock and fishery products.

2. Experimental

2.1. Chemicals and reagents

Salbutamol (99.6 %), cimaterol (98.7 %), ractopamine (94.0 %), and clenbuterol (99.8 %) standards were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Zilpaterol (96.0 %) was purchased from Toronto Research Chemicals (Canada). All standard solutions were individually prepared in methanol at a concentration of 1,000 μg/mL and stored at -20°C. HPLC–grade methanol, acetonitrile, and distilled water (DW) were purchased from Merck (Darmstadt, Germany). Formic acid, perchloric acid, hydrochloric acid (HCl), and 5 M sodium hydroxide were purchased from Sigma–Aldrich (MO, USA). Polytetrafluoroethylene (PTFE) filters (15 mm diameter, 0.2 μm pore size) were purchased from Teknokroma (Barcelona, Spain).

The following equipment was used for extraction and clean–up: a mechanical shaker (MMV–1000W, Eyela, Tokyo, Japan), a centrifuge (Heraeus Megafuge 16R, Thermo Fisher Scientific, Dreieich, Germany), and a nitrogen evaporator (EC–1648N pro, Goojung EnT, Seoul, Republic of Korea).

2.2. Sample collection and preparation

In this study, method validation for five β-agonists was conducted on nine samples, including livestock products (beef, pork, chicken, eggs, milk, and fat) and fishery products (flatfish, eel, and shrimp). All samples were purchased from a domestic market in Republic of Korea. Gloves were worn to minimize variations in sample quality and composition, and each sample was individually packaged to prevent cross–contamination. Tissue samples (except milk) were homogenized using a blender and stored in a

resealable plastic bag, while milk samples were poured into a 50 mL centrifuge tube. All samples were stored at -20 °C. Preliminary analysis confirmed that none of the target analytes were present in these samples.

Homogenized samples (2 g) were prepared in 50 mL centrifuge tubes, to which 10 mL of 0.1 N HCl was added. The mixtures were shaken for 10 min and then centrifuged at 4,800 × g for 15 min at 4 °C. Each supernatant, excluding the fat layer, was transferred to a new 50 mL centrifuge tube, and the pH was adjusted to 12.0 using 5 M sodium hydroxide. Next, 10 mL of ethyl acetate was added, after which the mixture was shaken for 3 min and then centrifuged at 4,600 × g for 3 min at 4 °C. A 5 mL aliquot of the supernatant was transferred into a 15 mL centrifuge tube and evaporated to dryness under a nitrogen stream at 40 °C. The concentrate was dissolved in 1 mL of 0.1 % formic acid in DW and centrifuged at 4,600 × g for 3 min at 4°C. The resultant supernatant was filtered through a 0.2 μm PTFE syringe filter before analysis by LC–MS/MS.

2.3. LC–MS/MS analysis

Quantitative analysis was performed using a Shimadzu LC–MS–8060 high–performance liquid chromatograph coupled with a triple–quadrupole mass spectrometer. Chromatographic separation was achieved with an Imtakt Unison UK–025 C_{18} column (150 mm × 2 mm, 3.0 μm). Solvents A and B comprised 0.1 % formic acid in DW and 0.1 % formic acid in acetonitrile, respectively. The flow rate was set at 0.3 mL/min. The linear elution gradient profile consisted of 5 % B (0 min), increased up to 40 % B (6.5 min), increased up to 100 % B (7.0 min), held at 100 % B (11.0 min), rapidly decreased B to 5 % (11.2 min), and held for stabilization (15.0 min). The injection volume was set to 5 μL. Mass analyses were performed using an electrospray ion source in positive ionization mode, with multiple reaction–monitoring (MRM) experiments conducted. The operation conditions comprised a capillary voltage of 5.0 kV and a capillary temperature of 300 °C. *Tables* 1 and 2 list the optimized parameter values for MRM transitions and LC–MS/MS conditions used in the analysis of the five β-agonists.

2.4. Method validation

The validation of the method developed in this study was conducted according to the accepted criteria for analytical method validation, as indicated in decision CAC/GL 71–2009 of the CODEX guidelines.[19] The evaluated parameters were specificity, linearity, limit of detection (LOD), LOQ, and intra– and inter–day accuracy and precision.

The peaks of the five β-agonists were identified for specificity by distinguishing them from other substances on the chromatogram. The presence of the five β-agonists in samples was assessed by comparing the relative retention times and ion ratios of the MRM transitions with those of matrix–matched calibration solutions. The LOD and LOQ values of the analytical method were statistically calculated based on averaging the results for seven calibration curves. The LOD was determined using the standard deviation of the y–intercept divided by the average slope, multiplied by 3.3. The LOQ was similarly calculated using a multiplier of 10 instead of 3.3. The linearity of the calibration curves was expressed as the coefficient of determination (R^2). The calibration curve was constructed with five points: 1/2 × LOQ, 1 × LOQ, 2 × LOQ, 4 × LOQ, and 20 × LOQ or 1/4 × MRL, 1/2 × MRL, 1 × MRL, 4 × MRL, and 8 × MRL.

A sample that did not contain any of the target compounds was used as the control. The accuracy and precision were evaluated by repeating the procedure five times at three different concentration levels and were expressed as recovery and coefficient of variation (CV), respectively. Validation was conducted for three concentration levels: 0.5 × MRL, MRL, and 2 × MRL for analytes with established MRLs and LOQ, 2 × LOQ, and 10 × LOQ for analytes without an MRL or those prohibited for use. Method validation was performed in three different laboratories to confirm inter–laboratory reproducibility.

3. Results and Discussion

3.1. Optimization of MS conditions

The MS/MS conditions were optimized to establish sufficient sensitivity for detecting low concentrations

Table 1. LC-MS/MS parameters for five β-agonists

Compound	Retention time (min)	Ionization	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)
Zilpaterol	3.3	$[M+H]^+$	262.2	244.1[a)]	12
				185.1	23
				202.1	18
Salbutamol	3.5	$[M+H]^+$	240.2	148.1	18
				222.2	10
				166.2	13
Cimaterol	3.7	$[M+H]^+$	220.0	160.1	16
				202.1	10
				143.1	21
Ractopamine	5.4	$[M+H]^+$	302.2	164.1	16
				284.1	12
				107.0	33
Clenbuterol	6.1	$[M+H]^+$	277.1	203.0	16
				259.0	11
				132.0	27

[a)]Quantitative ion

of the target analytes in the samples. Both positive and negative ESI modes were tested for quantifying the five β-agonists in the nine samples. In a full spectrum scan in the range of m/z 200 – 400, zilpaterol, salbutamol, cimaterol, ractopamine, and clenbuterol could only be detected in positive ion mode. Therefore, the positive ion mode was selected for the simultaneous detection of the analytes. This showed that the mass of each precursor ion was equal to the exact molecular weight of the compound plus one. Given that the molecular weight of hydrogen (H) is approximately one, these results indicated that one hydrogen atom attached to each compound was ionized. Consequently, it concluded that all the analytes formed protonated $[M+H]^+$ molecular ions (*Table* 1).

Compounds such as clenbuterol, which contain halogen elements such as chlorine (Cl), can be difficult to analyze because they are easily dissociated in DW and may be unstable during LC–MS/MS. However, lowering the pH stabilizes halogen elements, making it easier to analyze such compounds.[20-21] In this study, an acid was added to the redissolution solvent to stabilize clenbuterol. If adding acid is not feasible, the molecular weight of the compound excluding Cl is often used as the exact mass, and the ionized value is typically considered as the precursor ion.[22-24]

After selecting the precursor ions, product scans were recorded to test various collision energy values. The collision energy that provided the maximum intensity for the obtained product ions was selected.

3.2. Optimization of sample preparation methods

3.2.1. Extraction

Method 8.3.24 of the Food Code involves using perchloric acid ($HClO_4$) to hydrolyze and remove proteins. However, this method has issues. Perchloric acid is a strong oxidant that poses explosive hazards and decomposes upon heating, releasing toxic and corrosive fumes.[25] It is also highly hygroscopic and readily disassociates to form perchlorate anions.[26] Consequently, perchloric acid is considered a highly dangerous reagent for use in the analytical methods of the Republic of Korea Food Code.

In this study, the aim was to replace perchloric acid with HCl. To optimize extraction conditions and enhance extraction solvent efficiency, the recovery rates of different HCl concentrations were compared (*Fig*. 1A). After extracting samples using 0.1 N, 0.4 N, and 1 N HCl, the best recovery was achieved with

0.1 N HCl. Therefore, 0.1 N HCl was determined to be the optimal extraction solvent for the method. By replacing perchloric acid with HCl, this study has developed a safer analytical method, which is expected to be adopted by various institutions to enhance food safety.

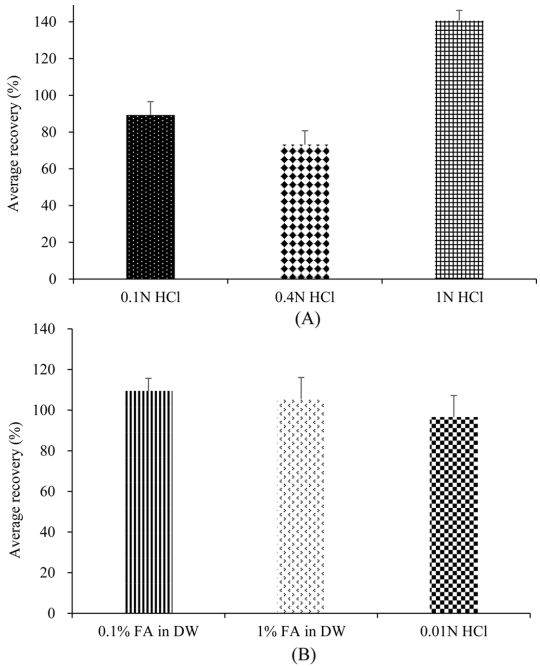

Fig. 1. Comparison of extraction (A) and redissolution (B) methods.

3.2.2. Redissolution solvent

HCl, which is used for redissolution in the current Food Code β-agonists analytical method, is a very strong acid that can place a significant burden on the equipment when directly injected into the LC–MS/MS. After nitrogen evaporation during the sample preparation process, the redissolution solvent is generally the same as or similar to the mobile phase solvent to stabilize the baseline, as using a solvent with significantly different polarity may lead to peak distortion.[27-28] Therefore, a redissolution solvent with polarity closely matching that of the mobile phase is preferred to minimize both instrumental burden and analytical errors.

An acid is added to the redissolution solvent to stabilize the analytes. Specifically, the polarity that is reduced during extraction for improved efficiency must be increased again before analysis. Therefore,

Table 2. LC-MS/MS conditions for analysis of five β-agonists

Parameter	Conditions		
LC	Shimadzu LC		
Column	Imtakt Unison UK-025 C_{18} column (150 mm × 2 mm, 3.0 μm)		
Flow rate	0.3 mL/min		
Injection volume	5 μL		
Column temperature	40 ℃		
Mobile phase	A: 0.1 % formic acid in DW B : 0.1 % formic acid in acetonitrile		
	Time (min)	A (%)	B (%)
	0.0	95	5
	0.5	95	5
	6.5	60	40
	7.0	0	100
	11.0	0	100
	11.2	95	5
	15.0	95	5
Mass spectrometer	Shimadzu LC–MS–8060		
Ionization mode	Positive		
Capillary temperature	300 ℃		
Capillary voltage	4.0 kV		
Collision gas	Ar		

Table 3. Matrix effect of five β-agonists in livestock and fishery products

Sample	Matrix effect (%)				
	Zilpaterol	Salbutamol	Cimaterol	Ractopamine	Clenbuterol
Beef	-57.1	336.7	67.2	139.5	94.4
Pork	37.2	478.2	55.7	157.5	56.7
Chicken	23.3	285.9	-18.3	32.0	-24.8
Egg	-22.7	275.3	-21.1	-10.0	-10.0
Milk	-6.6	278.4	12.5	19.6	9.0
Fat	-11.5	349.3	-19.4	-11.5	5.1
Flatfish	-0.2	429.2	-9.9	37.7	-3.5
Eel	183.7	666.1	211.1	278.7	137.1
Shrimp	9.4	266.9	-18.8	107.4	24.7

to redissolve the sample in highly polar water, it is necessary to increase the reduced polarity again using sodium hydroxide solution. Consequently, by redissolving it in acidic water and lowering the pH, the polarity of the sample is increased, allowing the target analytes to exist in their ionized form.

To replace HCl with formic acid, 0.1 % formic acid in DW and 1 % formic acid in DW, which have properties similar to those of the previously used HCl, were compared as redissolution solvents (*Fig*. 1B). All three acidic solvents exhibited similar recoveries and met the CODEX guidelines.[19] Therefore, to reduce the burden on the analytical instruments, 0.1 % formic acid was selected, as it is the same as the mobile phase and has the lowest acidity among the solvents tested.

In this study, the mobile phase for LC–MS/MS analysis of β-agonists was 0.1 % formic acid in DW. Similarly, previous studies have used 0.1 % formic acid in DW to redissolve β-agonists.[29,30]

3.3. Matrix effect

In LC–MS/MS analysis, sample matrices such as lipids and proteins can significantly affect the target analyte response and performance. These matrices may lead to either signal suppression or enhancement, affecting the accuracy and reliability of the analytical results.[31] The matrix effect (ME) was calculated using the equation.

$$ME(\%) = (Slope_{Spiked}/Slope_{Standard} - 1) \times 100$$

where $Slope_{spiked}$ is the mean value of the slopes obtained from samples spiked with the tissue standard and $Slope_{standard}$ is the mean value of the slopes obtained from the standard solutions. For ME values less than -50 % and greater than 50 %, the matrix effects on analyte ion suppression were considered to be strong.

In this study, the matrix effects were significant, as indicated by the calculated values (*Table* 3). Among the five analytes tested, salbutamol exhibited the greatest matrix effect. Conversely, clenbuterol and zilpaterol displayed the lowest matrix effects, with no significant matrix effects observed in any samples except for beef and eel. The best way to eliminate matrix effects is through the use of tissue standard curves. Therefore, tissue standards were used to construct calibration curves throughout this study to diminish the matrix effects.

3.4. Method validation

3.4.1. Linearity, specificity, and LOQ

Method validation for five β-agonists was conducted on nine types of samples (beef, pork, chicken, egg, milk, fat, eel, flatfish, and shrimp) and evaluated for linearity, accuracy, precision, and detection limits.

Linearity was represented as the coefficient of determination (R^2), which exceeded 0.99 in all sample types. The coefficients of determination (R^2) were 0.9974 – 0.9996 for beef, 0.9936 – 0.9992 for pork, 0.9946 – 0.9979 for chicken, 0.9962 – 0.9998 for egg, 0.9976 – 0.9994 for milk, 0.9921 – 0.9998 for fat,

0.9957 – 0.9998 for flatfish, 0.9985 – 0.9994 for eel, and 0.9981 – 0.9995 for shrimp. All results were within the range of values required for each sample, in accordance with the CODEX guidelines.[19]

The qualitative and quantitative ions of the β-agonists were sufficiently separated from other substances on the chromatogram demonstrating specificity (Supplementary *Figs*. S1 – S5). The detection limits, represented as the LOQ, were determined as follows: zilpaterol, 0.0005 mg/kg (0.00025 mg/kg for beef); salbutamol, 0.0002 mg/kg; cimaterol, 0.0002 mg/kg; ractopamine, 0.0005 mg/kg; and clenbuterol, 0.0002 mg/kg across all nine types of samples.

3.4.2. Accuracy and precision

The accuracy and precision of the method of the five β-agonists are represented as percentage recovery and CV, respectively (Lab A, *Table* 4). The recovery of the method was 78.9 – 113.5 % for beef, 71.8–113.4 % for pork, 71.7 – 111.9 % for chicken, 86.2 – 116.1 % for egg, 88.2 – 107.0 % for milk, 84.2 – 105.2 % for fat, 81.5 – 110.4 % for flatfish, 70.9 – 111.4 % for eel, and 90.1 – 107.8 % for shrimp. The CV of the method was 2.4 – 11.6 % for beef, 3.0 – 11.1 % for pork, 1.7 – 24.0 % for chicken, 2.3 – 10.6 % for egg, 2.9 – 16.6 % for milk, 2.7 – 20.0 % for fat, 1.6 – 19.4 % for flatfish, 7.0 – 23.2 % for eel, and 3.0 – 15.5% for shrimp. Overall, the recovery and CV of the method fell within the ranges of 70.9 – 116.1 % and 1.6 – 24.0 %, respectively, across the nine types of samples. All results were within the required range for each spiking concentration, in accordance with CODEX guidelines.[19]

This method was also tested in two additional laboratories (Labs B and C, *Table* 4) to confirm its reproducibility. The recovery of the method was 87.2 – 107.2 % for beef, 85.1 – 106.4 % for pork, 83.3 – 108.3 % for chicken, 91.3 – 110.1 % for egg, 88.9 – 103.5 % for milk, 92.1 – 108.5 % for fat, 93.3 – 107.8 % for flatfish, 81.0 – 106.2 % for eel, and 93.7 – 103.6 % for shrimp. The CV of the method was 5.2 – 11.3 % for beef, 2.0 – 15.9 % for pork, 7.6 – 19.1 % for chicken, 4.2 – 8.7 % for egg, 5.0 – 14.6 % for milk, 3.3 – 15.2 % for fat, 5.8 – 13.7 % for flatfish, 6.5 – 17.3 % for eel, and 4.6 – 11.9 % for shrimp. Overall, the recovery and CV of the method were 81.0 – 110.1 % and 2.0 – 19.1 %, respectively.

The experiments were conducted in three different laboratories, each using its own equipment and reagents, without standardizing the conditions across laboratories. Despite these differences, key analytical parameters, such as recovery rates and CV values, remained consistent and within the acceptable ranges defined by the CODEX guidelines.[19] This consistency underscores the robustness and reliability of the method. The results from these laboratories show that the method ensures accurate and reproducible outcomes, regardless of the laboratory conditions.

To evaluate the analytical performance of the newly developed HCl–based method, its accuracy and precision were compared with those of a previously reported method that used 0.4 N perchloric acid for extraction.[32] In the present study, the extraction solvent was modified to 0.01 N HCl, and the redissolution solvent was changed to 0.1 % formic acid in DW, replacing the previous 0.01 N HCl. In the previous study, matrix-matched calibration curves showed correlation coefficients (R^2) greater than 0.98, recoveries ranged from 62.0 % to 109.8 %, and CVs were ≤ 20.1 %. In contrast, the present study achieved correlation coefficients exceeding 0.99, recoveries ranging from 70.9 % to 116.1 %, and CV values below 24 %, all of which meet the acceptance criteria outlined by the CODEX guidelines.[19] Notably, while the previous study compared results from two institutions, the current study involved cross-validation across three different institutions, further reinforcing the robustness and reliability of the method. The LOQ was generally consistent with the previous method, but was further improved for zilpaterol in beef, where the LOQ was lowered to 0.00025 mg/kg to reflect the MRL. These results indicate that the HCl–based method offers comparable or enhanced performance in terms of accuracy and precision, while providing a safer alternative to the perchloric acid–based protocol.

Unlike previous studies, this new method provides a more versatile approach by offering enhanced efficiency for β-agonist residue monitoring and

Table 4. Validation of the method across nine livestock and fishery samples from three laboratories

Compounds	Sample	Concentration (mg/kg)	Recovery (%)				CV (%)
			Lab A (n = 5)	Lab B (n = 3)	Lab C (n = 3)	Average	
Zilpaterol	Beef	0.00025	96.6	80.2	105.0	94.4	11.2
		0.0005	105.2	92.9	107.1	102.3	9.8
		0.001	112.4	99.2	89.0	102.4	10.4
	Pork	0.0005	109.1	92.0	109.8	104.6	9.1
		0.001	101.7	97.3	107.4	102.1	7.6
		0.005	71.8	97.8	94.5	85.1	15.9
	Chicken	0.0005	87.6	100.3	105.4	95.9	14.2
		0.001	91.5	101.6	104.2	97.7	11.5
		0.005	109.6	84.3	86.5	96.4	13.5
	Egg	0.0005	112.1	112.7	100.7	109.2	6.1
		0.001	105.7	98.4	103.8	103.2	5.0
		0.005	86.2	100.0	91.1	91.3	6.9
	Milk	0.0005	88.2	85.1	103.9	91.6	11.2
		0.001	101.4	87.4	103.2	98.1	13.8
		0.005	94.5	115.4	86.9	98.1	13.4
	Fat	0.0005	104.5	89.8	103.2	100.1	9.1
		0.001	93.7	108.9	106.1	101.2	8.7
		0.005	90.2	101.1	88.8	92.8	6.7
	Flatfish	0.0005	106.5	109.8	98.2	105.2	6.2
		0.001	110.4	105.1	97.8	105.5	6.1
		0.005	100.5	98.5	89.2	96.9	5.8
	Eel	0.0005	79.5	83.0	99.4	85.9	17.3
		0.001	94.6	86.5	102.8	94.6	12.4
		0.005	80.5	92.9	96.2	88.2	10.6
	Shrimp	0.0005	107.8	100.9	99.2	103.6	5.7
		0.001	97.5	98.6	97.7	97.9	5.8
		0.005	92.9	100.3	88.6	93.7	6.3
Salbutamol	Beef	0.0002	109.3	93.4	96.5	101.5	10.2
		0.0004	113.5	94.9	102.6	105.5	10.1
		0.002	100.6	94.3	85.3	94.7	9.6
	Pork	0.0002	106.4	106.5	98.6	104.3	8.1
		0.0004	101.3	90.3	102.9	98.7	10.1
		0.002	89.8	108.2	87.7	94.2	10.4
	Chicken	0.0002	109.4	116.6	94.1	107.2	11.3
		0.0004	89.2	110.8	98.4	97.6	11.8
		0.002	71.7	101.4	84.5	83.3	18.2
	Egg	0.0002	105.4	101.8	96.0	101.8	7.6
		0.0004	104.3	102.5	99.7	102.5	4.9
		0.002	91.1	106.2	90.2	95.0	8.7
	Milk	0.0002	98.7	95.3	94.1	96.5	9.2
		0.0004	91.8	94.5	91.4	92.4	9.7
		0.002	105.9	101.1	85.8	99.1	14.6
	Fat	0.0002	94.3	100.2	94.8	96.0	10.9
		0.0004	84.2	96.5	105.3	93.3	15.2
		0.002	94.9	96.0	89.5	93.7	4.7
	Flatfish	0.0002	103.9	108.1	104.4	105.2	12.4
		0.0004	96.9	102.2	98.8	98.9	13.3
		0.002	100.9	105.9	88.5	98.9	12.3
	Eel	0.0002	98.9	88.4	98.2	95.8	11.5
		0.0004	94.2	101.8	96.2	96.8	8.3
		0.002	87.4	92.8	91.7	81.0	17.0
	Shrimp	0.0002	90.1	97.6	98.0	94.3	6.4
		0.0004	98.4	107.8	95.0	100.0	7.0
		0.002	95.0	102.0	86.0	94.5	8.3

Table 4. Continued

Compounds	Sample	Concentration (mg/kg)	Recovery (%)				CV (%)
			Lab A (n = 5)	Lab B (n = 3)	Lab C (n = 3)	Average	
Cimaterol	Beef	0.0002	108.3	99.7	90.4	101.0	9.0
		0.0004	103.9	89.7	100.5	99.1	9.1
		0.002	103.3	94.2	89.9	97.2	7.6
	Pork	0.0002	113.4	107.3	90.4	105.5	11.5
		0.0004	105.3	100.3	101.5	102.9	6.3
		0.002	100.1	108.0	93.8	100.5	7.2
	Chicken	0.0002	81.6	110.2	93.6	92.7	19.1
		0.0004	103.0	75.3	99.5	94.5	16.0
		0.002	110.3	99.8	89.2	101.7	12.0
	Egg	0.0002	116.1	111.9	98.4	110.1	7.7
		0.0004	109.2	98.5	98.0	103.2	7.9
		0.002	92.8	104.9	94.2	96.5	7.4
	Milk	0.0002	94.6	79.8	88.6	88.9	11.8
		0.0004	97.9	102.6	94.8	98.3	8.3
		0.002	96.5	102.1	89.4	96.1	9.3
	Fat	0.0002	97.7	111.3	88.9	99.0	10.7
		0.0004	95.0	105.6	105.5	100.7	8.6
		0.002	91.1	103.1	96.4	95.8	8.9
	Flatfish	0.0002	87.4	101.5	95.0	93.3	9.3
		0.0004	101.4	107.4	96.0	101.6	7.7
		0.002	106.2	101.8	91.0	100.9	7.6
	Eel	0.0002	103.5	103.3	100.8	102.7	10.3
		0.0004	92.8	87.6	95.5	92.1	12.7
		0.002	89.0	103.9	93.4	94.2	9.4
	Shrimp	0.0002	98.4	89.9	104.0	97.6	8.5
		0.0004	102.7	107.2	99.3	103.0	5.6
		0.002	95.0	108.7	92.7	98.1	9.4
Ractopamine	Beef	0.0005	110.3	102.1	107.3	107.2	5.8
		0.01	107.4	98.1	105.8	104.4	8.2
		0.02	92.7	89.5	91.0	91.4	5.2
	Pork	0.0005	109.5	97.5	109.9	106.4	7.0
		0.01	106.6	91.1	107.5	102.6	10.4
		0.02	89.3	106.3	95.5	95.6	8.1
	Chicken	0.0005	80.2	86.9	104.6	88.7	14.0
		0.001	111.9	105.5	105.0	108.3	7.8
		0.005	106.7	81.2	91.1	95.5	13.3
	Egg	0.0005	114.5	108.2	100.9	109.1	6.5
		0.001	102.6	107.7	104.1	104.4	4.2
		0.005	104.2	97.5	96.8	100.4	4.2
	Milk	0.0005	105.1	104.1	100.1	103.5	5.7
		0.001	105.5	91.2	95.9	99.0	11.9
		0.005	100.7	106.0	92.6	100.0	8.9
	Fat	0.0005	84.7	88.3	108.3	92.1	11.9
		0.01	93.2	91.5	110.1	97.4	9.6
		0.02	85.8	106.6	97.4	94.7	12.6
	Flatfish	0.0005	109.1	100.6	105.7	105.9	6.2
		0.001	108.9	115.7	98.1	107.8	9.9
		0.005	106.6	95.6	94.5	100.3	7.9
	Eel	0.0005	105.3	111.4	102.2	106.1	6.5
		0.001	92.2	89.6	101.7	94.1	8.0
		0.005	70.9	97.3	97.3	85.3	16.7
	Shrimp	0.0005	93.9	109.0	96.9	98.8	11.9
		0.001	101.4	105.3	94.8	100.7	6.2
		0.005	105.2	95.4	93.2	99.2	8.0

Table 4. Continued

Compounds	Sample	Concentration (mg/kg)	Recovery (%)				CV (%)
			Lab A (n = 5)	Lab B (n = 3)	Lab C (n = 3)	Average	
Clenbuterol	Beef	0.0002	78.9	90.7	97.4	87.2	11.3
		0.0004	96.8	90.3	106.4	97.6	7.0
		0.002	101.6	106.9	89.7	99.8	7.4
	Pork	0.0002	105.5	93.3	100.1	100.7	8.4
		0.0004	105.7	105.8	105.0	105.5	2.0
		0.002	101.0	115.6	93.4	102.9	9.0
	Chicken	0.0002	82.6	107.1	97.5	93.3	15.7
		0.0004	96.4	109.5	103.8	102.0	7.6
		0.002	103.1	97.7	90.3	98.1	8.5
	Egg	0.0002	108.3	107.4	103.8	106.8	7.4
		0.0004	105.1	103.3	105.4	104.7	4.6
		0.002	104.1	112.9	94.8	104.0	7.0
	Milk	0.0002	107.0	88.1	101.8	100.4	10.4
		0.0004	101.5	91.4	99.4	98.2	6.0
		0.002	97.1	97.1	91.8	95.7	5.0
	Fat	0.0002	101.9	97.2	98.9	99.8	3.3
		0.0004	105.2	114.5	108.2	108.5	5.3
		0.002	96.2	104.6	94.1	97.9	5.3
	Flatfish	0.0002	81.5	107.4	103.3	94.5	13.7
		0.0004	93.1	107.5	101.1	99.2	7.1
		0.002	95.7	105.6	95.3	98.3	6.2
	Eel	0.0002	111.4	96.9	106.8	106.2	7.8
		0.0004	97.3	89.2	103.7	96.8	15.8
		0.002	108.2	106.7	97.0	104.7	7.6
	Shrimp	0.0002	104.3	94.7	101.4	100.9	8.0
		0.0004	97.9	101.0	98.0	98.8	4.6
		0.002	100.4	101.0	91.7	98.2	5.1

Table 5. Results of monitoring five β-agonist residues

Samples	Zilpaterol	Salbutamol	Cimaterol	Ractopamine	Clenbuterol
Beef	-	-	-	Trace[a)] (1)	-
Pork	-	-	-	Trace (3)	-
Chicken	-	-	-	-	-
Egg	-	-	-	-	-
Milk	-	-	-	-	-
Flatfish	-	-	-	-	-
Catfish	-	-	-	-	-
Eel	-	-	-	-	-
Mudfish	-	-	-	-	-
Shrimp	-	-	-	-	-
Abalone	-	-	-	-	-

[a)]Trace: <LOQ

management. These improvements, including the modified extraction and redissolution procedures, position the method as a reliable and safer option for analyzing a wide range of livestock and fishery products.

3.5. Application to real samples

To confirm its applicability to livestock products, five samples—beef, pork, chicken, eggs, and milk—were purchased. Additionally, flatfish, catfish, eel, mudfish, shrimp, and abalone were acquired to

confirm the applicability of this method to fishery products. A total of 60 samples were obtained from a domestic market (Osong, Republic of Korea) and analyzed using the developed method (*Table* 5). None of the target analytes were detected in the samples.

4. Conclusions

In this study, a method for the simultaneous quantitative analysis of five β-agonists was developed. To improve the safety of the analytical procedure, perchloric acid—specified in the existing Food Code—was replaced with HCl. Additionally, 0.1 % formic acid was used as the redissolution solvent to reduce the burden on both the analyst and the analytical equipment. The developed method was validated in accordance with the CODEX guidelines and showed reliable results across various food matrices. In particular, the method achieved a lower LOQ for zilpaterol in beef samples, demonstrating improved sensitivity over the previous domestic method. Furthermore, the inclusion of cimaterol, a compound for which no domestic standard currently exists, further broadens the method's applicability, making it relevant for imported food products as well. By enabling the simultaneous analysis of β-agonists in both livestock and fishery products, this method contributes to the broader applicability and standardization of food safety monitoring.

Conflict of Interest Statement

None of the authors of this study have any financial interest or conflict with industries or parties.

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