

Determination and Validation of Method Based on Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry for Methimazole in Livestock and Fishery Product

Hyesu Lee , Jin Ha Sim , Ji Young Kim , Gui-Hyun Jang  and Miok Eom 

Pesticide and Veterinary Drug Residues Division, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Cheongju 28159, Korea

These authors contributed equally to this work.

* Correspondence: jkim98@korea.kr

Abstract: Carbimazole is used for the treatment of hyperthyroidism. Methimazole, which is produced after the rapid metabolism of carbimazole in the body, can adversely affect endocrine hormones and cause gastrointestinal problems. Methimazole is prescribed as a veterinary drug to treat thyroid disease in cats. However, there is suspicion that it is being used for rapid fattening of non-thyroidal livestock, such as cows and pigs, posing serious health risks to consumers. Currently, there are no analytical methods for the detection of methimazole in livestock and fishery products in Korea. Thus, in this study, we developed a method to efficiently analyze methimazole in livestock and fishery products through liquid chromatography with tandem mass spectrometry. Methimazole was extracted with 80% acetonitrile and purified using C₁₈ powder, followed by fat removal using acetonitrile-saturated hexane. The coefficient of linearity, average recovery, and coefficient of variation of the analytical method were verified according to the CODEX guidelines. The developed method was employed to monitor methimazole residue levels in livestock and fishery products, but no residues were detected. The proposed method can serve as a foundation for improving the safety management of domestic- and imported-livestock and fishery products.

Keywords: Carbimazole, Food safety, Liquid chromatography-tandem mass spectrometry (LC-MS/MS), Methimazole, Veterinary drugs residue

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Introduction

Carbimazole, chemically known as thionamide, is a relatively simple drug that is widely used for the treatment of hyperthyroidism, including Graves' disease. It is also used as a thyreostatic and thyrotropic agent [1,2]. Carbimazole, when used to treat hyperthyroidism, acts as a substrate for thyroid peroxidase (TPO), an enzyme required for thyroid hormone synthesis. It reduces TPO production and interferes with the binding of iodide ions and molecular residues, ultimately suppressing the production of thyroid hormones, namely, triiodothyronine (T3) and

thyroxine (T4) [1]. After administration, carbimazole is quickly absorbed into the gastrointestinal tract, where it rapidly and completely metabolizes to methimazole, which then reaches the target organs. Carbimazole is a carboxyl analog of methimazole. Methimazole accumulates in the thyroid and adrenal glands and is distributed throughout the body [3]. Considering that methimazole is metabolized in the liver, liver dysfunction has been reported as a result of acute toxicity in the human body following the administration of carbimazole. Methimazole has also been reported to cause gastrointestinal problems and undesirable changes in endocrine hormones. Based on clinical studies, methimazole has been linked to serious health effects such as acute pancreatitis [4,5]. Methimazole has also been identified as a risk factor for congenital malformations in pregnant women [5]. Methimazole is licensed for use as a veterinary drug for companion animals, such as for the treatment of thyrostatic cats [6]. The use of methimazole without approval from higher authorities (extra-lab) is prohibited; thus, it is crucial to manage the administration of this drug at a reasonable level. However, it is suspected of being unethically used for fattening non-thyroidal livestock, such as cows and pigs [2,7]. This poses a serious risk to consumers [3].

The Korean food code currently lacks an analytical method for detecting methimazole in food samples. As Korea's Ministry of Food & Drug Safety (MFDS) has begun the full implementation of its Positive List System (PLS) for veterinary drug residues in six food categories since January 2024, an appropriate analytical method for detecting is urgently required. Previous studies have selected surface water [8] and human blood [9] to develop analytical methods for methimazole detection based on liquid chromatography with tandem mass spectrometry (LC-MS/MS). However, an appropriate analytical method has not been developed to determine the residual quantities of the drug in various matrices, such as livestock or fishery products. Considering the introduction of PLS in Korea and food safety management, it is important to develop an analytical method. To this end, in this study, we develop an efficient and accurate analytical method using LC-MS/MS to detect methimazole in livestock and fishery products. This method is intended for establishing a foundation for improving the safety management of domestic- and imported-livestock and fishery products.

Materials and Methods

Reagents, materials, and equipment

The methimazole standard (>98%) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The stock solution was dissolved in methanol to achieve a concentration of $1000 \mu\text{g} \cdot \text{mL}^{-1}$ and stored at -20°C . The working solution was diluted from the stock solution by using 50% methanol. High-performance liquid chromatography (HPLC) grade methanol, acetonitrile, hexane, and water were purchased from Merck (Darmstadt, Germany). Formic acid, ammonium formate, and sodium hydroxide were purchased from Sigma-Aldrich (MO, USA). Octadecylsilane (C_{18}) was purchased from Waters (MA, USA). Ethylenediaminetetra acetic acid disodium salt dihydrate ($\text{Na}_2\text{-EDTA}$) was purchased from Sigma-Aldrich (MO, USA). Polytetrafluoroethylene (PTFE) and nylon syringe filters (15 mm diameter, $0.2 \mu\text{m}$ pore size) were purchased from Teknokroma (Barcelona, Spain).

The following devices were used for extraction and cleanup: a mechanical shaker (MMV-1000W, Eyela, Tokyo, Japan), a centrifuge (Heraeus meagafuge 16R, Thermo Fisher Scientific Inc., Germany), and a nitrogen evaporator (EC-1648N pro, Goo-jung EnT Co., Ltd., Seoul, Korea).

Sample collection

The samples were collected from eight representative livestock and fishery products: beef, pork, chicken, eggs, milk, eels, flatfish, and shrimp. All the samples were purchased from a domestic market in Korea. A glove was worn to minimize variations in sample quality and composition. Each sample was individually packaged to prevent cross-contamination. The collection of samples was conducted in accordance with the 'Food code No. 7', and the sample collection and handling method described in food code [9] was used to demonstrate the representativeness of the samples. Before analysis, each sample (excluding milk)

was homogenized using a blender and stored in a ziplock bag. Milk samples were poured into a 50 mL centrifuge tube and stored at -20°C . For each sample type, samples that tested negative for methimazole were used to check for contamination during sample preparation and instrument analysis.

LC-MS/MS

Methimazole detection was conducted on a Shimadzu LC MS-8060 system (Kyoto, Japan) equipped with Unison UK-C₈ column (Kyoto, Japan, 150×3 mm; $3.0 \mu\text{m}$, Imtakt, Kyoto, Japan). The injection volume was $10 \mu\text{L}$. To enhance sensitivity, peak shape, and resolution, 5 mM ammonium formate in water (A) and 0.1% formic acid in acetonitrile (B) were used. The LC gradient conditions were as follows: 10% B (0 min), increased to 60% B (5.50 min), increased to 100% B (6.00 min), held at 100% B (10.00 min), rapidly decreased to 10% (10.20 min), and maintained for stabilization (12.00 min). The flow rate and temperature were 0.3 mL/min and 40°C , respectively. The analytes were protonated using the electrospray ionization (ESI) mode. The capillary temperature was 300°C , the capillary voltage was 4.0 kV in the positive mode, and the collision gas was argon. The multiple-reaction monitoring conditions for methimazole are listed in Table 1.

Table 1. Experimental conditions for LC-MS/MS in the multiple-reaction monitoring mode

Compound	Retention time (min)	Ionization	Precursor ion (m/z)	Product ion (m/z)	Collision voltage
Methimazole	4.5	$[\text{M}+\text{H}]^{+}$	115.05	81.05	29
				88.30	17

Sample preparation

Homogenized samples (2 g) were prepared in 50 mL centrifuge tubes and 10 mL of 80% acetonitrile was added to the samples. Additionally, 1 mL of 0.1 M Na₂-EDTA was added for analyzing the egg and fishery samples. After shaking for 10 min, the samples were centrifuged at 4,800 G for 10 min at 4°C . Next, all supernatants were transferred to new 50 mL centrifuge tubes, and 500 mg of C₁₈ was added along with 10 mL of acetonitrile-saturated hexane. Again, each mixture was shaken for 10 min and centrifuged at 4,800 G for 10 min at 4°C . The lower layer (5 mL each) was transferred to 15 mL centrifuge tubes and evaporated to dryness under a nitrogen stream at 40°C . The residues were dissolved in 1 mL solution of 50% methanol, and centrifuged at 4,800 G for 3 min at 4°C . The final solutions were analyzed via LC-MS/MS after filtering with a $0.2 \mu\text{m}$ nylon syringe filter.

Method validation

The specificity, calibration curve linearity, accuracy, precision, and limits of quantification (LOQ) and detection (LOD) of the analytical method were examined in accordance with the CODEX guidelines (CAC/GL 71-2009) [11]. To confirm the specificity, the peaks of methimazole were uniquely identified from other substances in the chromatogram. The LOD and LOQ of the analytical method were statistically calculated by averaging the results for seven calibration curves.

$$\text{LOD} = 3.3 \times \frac{\sigma}{S} \text{ and } \text{LOQ} = 10 \times \frac{\sigma}{S}$$

Where σ and S are the standard deviation of the y-intercept and average slope, respectively, of the new calibration curve constructed using the three lowest concentrations on the calibration curve. The linearity of the calibration curves was indicated by the coefficient of determination (R^2). The calibration curve included five points: $1/2 \times \text{LOQ}$, LOQ , $2 \times \text{LOQ}$, $4 \times \text{LOQ}$ and $20 \times$

LOQ. Accuracy and precision were confirmed by repeating the experiment five times at three different concentrations (LOQ, 2×LOQ and 10×LOQ). Accuracy and precision are represented by the recovery and coefficient of variation (CV), respectively. The sample that did not contain any of the target compounds was used as the control. Method validation was performed in three different laboratories to ensure inter-laboratory reproducibility.

Results and Discussion

LC-MS/MS condition optimization

In this study, the methimazole retention time was determined using a C₈ column to develop an analytical method with optimized sensitivity and quantification accuracy. Methimazole has a Log P value of −0.3, indicating that it is very polar [12]. When a C₁₈ column, known for its nonpolar characteristics, was used to analyze methimazole, the compound was not retained by the column, resulting in no change in retention time despite variations in the gradient conditions. Using a C₁₈ column, the retention time of methimazole was analyzed to be less than 1 min, but using a C₈ column, it was detected in 4.5 min. The retention time in a C₁₈ column can be improved by adding an HFBA buffer, which is an ion-pair reagent; however, the HFBA buffer has the disadvantage of requiring a long time to stabilize the column and is strongly absorbed by the surface of the column filler, impeding its removal [13-15].

The organic solvent for the mobile phase was selected acetonitrile. It generally has a lower viscosity and results in a better peak shape in LC-MS/MS chromatograms. Several buffers and acids were tested and reviewed to optimize the experimental conditions [16]. The following two mobile phases were compared: (1) 5 mM ammonium formate in water and 0.1% formic acid in acetonitrile, and (2) 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The first mobile phase showed a better peak shape and higher sensitivity and resolution than the second mobile phase. Because methimazole is an alkaline and polar compound, pH balance is important. Ammonium formate should be added to the mobile phase to maintain the pH and stabilize it rather than making it too acidic [17]. Therefore, methimazole was analyzed under the optimal gradient conditions with 5 mM ammonium formate in water and 0.1% formic acid in acetonitrile.

To select the precursor and product ions of methimazole, the analyte was directly infused at a concentration of 0.01 mg/L. The precursor ion of the major compound was determined to be protonated [M+H]⁺ because of its high intensity. After selecting the precursor ion, a product scan was performed to test the different values of the product ions and their collision energies. In the obtained LC-MS/MS chromatogram, the transition at 115.05 was used for the quantitation and confirmation of the precursor, while the transitions at 81.05 and 88.30 were used for those of the product ions. Generally, protonated [M+H]⁺ molecular ions can be ionized by simply adding formic acid to the mobile phase [18,19].

Sample preparation method optimization

During experiments, when a blank sample was spiked with carbimazole, it immediately metabolized to methimazole, making it impossible to develop a quantitative analytical method. LC-MS/MS analysis of the carbimazole-spiked sample showed that presence of both the metabolized methimazole as well as carbimazole (Figs. 1, 2). However, carbimazole was not detected in the methimazole-spiked samples.

The 8.3.1 method, which is a simultaneous multiresidue analytical method for veterinary drugs according to the food code [20], was used for extraction and cleanup to obtain sufficient quantities of methimazole. This analysis method improves accuracy and precision by allowing the use of a small amount of sample, extracting methimazole with 80% acetonitrile, dispersing it with C₁₈ powder, and removing fat with acetonitrile-saturated hexane. This method was successfully employed for extracting methimazole from beef, pork, chicken, and milk. However, eggs and fishery products showed low recovery and reproducibility, possibly because of the presence of metal ions in these products [21]. During the extraction process, methimazole exhibits



Fig. 1. Chemical structures of carbimazole and methimazole.

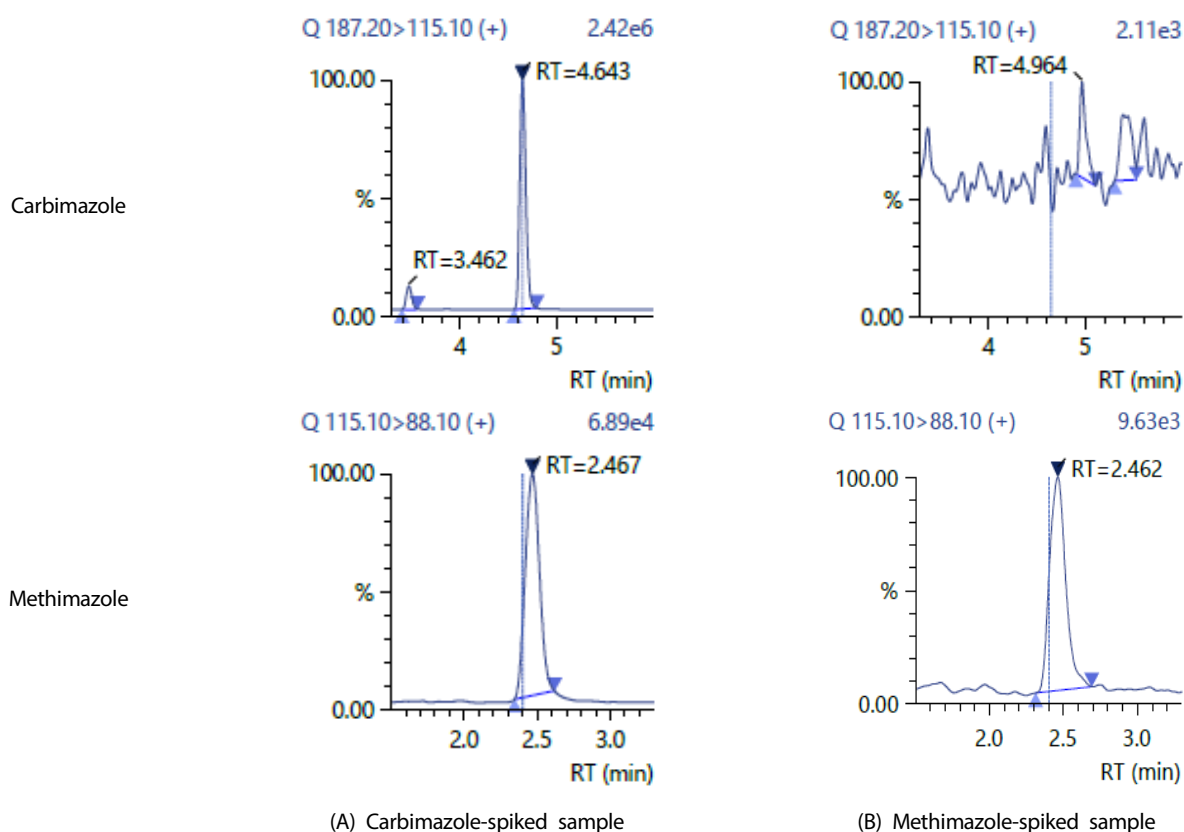


Fig. 2. Chromatograms of the (A) carbimazole- and (B) methimazole-spiked samples.

a strong tendency to form complexes with metal ions in matrices, resulting in low extraction efficiencies. Therefore, it is necessary to add $\text{Na}_2\text{-EDTA}$ to the extract for improving the recovery of methimazole by masking the interfering metal ions [22].

The performance of PTFE and nylon filters was evaluated both in the absence of a food matrix and in the presence of methimazole (20 ng) added to 10 mL of 80% acetonitrile. Recovered methimazole was sufficient when a nylon filter was used after cleanup with C_{18} . Additionally, the nylon filter showed a higher recovery of methimazole than the PTFE filter. By contrast, the nylon filter showed low recoveries for some matrix, such as beef, milk, and eel. Various types of syringe filters are widely used to develop analytical methods for sample preparation; however, incorrect filter selection can result in analyte loss by surface absorption. This loss is related to the chemical properties of the analyte, such as its acidity [23].

Matrix effect for methimazole

In LC-MS/MS, sample matrices, such as lipids or proteins, are known to affect the response and performance of the target

analyte, resulting in signal suppression or enhancement. The matrix effect (ME) was calculated as follows:

$$ME(\%) = \left(\frac{Slope_{spiked}}{Slope_{standard}} - 1 \right) \times 100$$

where and are the mean values of the slopes with the standard sample and sample spiked with the tissue standard, respectively. For an ME value of less than 0, there is signal suppression, and over than 0, there is signal enhancement [24].

As shown in Table 2, the MEs were significant, as indicated by the calculated values for methimazole: 133.1% for beef; 96.0% for pork; 56.1% for chicken; 209.8% for milk, 169.1% for egg; 171.7% for fat; 157.0% for flatfish; 571.5% for eel; and 268.8% for shrimp. The eel matrix exhibited the highest signal enhancement. The best way to eliminate MEs is the use of tissue standard curves. Therefore, tissue standards were used to construct the calibration curves to reduce matrix effects.

Table 2. Matrix effect for methimazole in the livestock and fishery products

Sample	Matrix effect (%)
Beef	133.1
Pork	96.0
Chicken	56.1
Milk	209.8
Egg	169.1
Fat	171.7
Flatfish	157.0
Eel	571.5
Shrimp	268.8

Method validation results

Linearity was represented as the coefficient of determination (R^2) with matrix-matched standards, which was greater than 0.98 in methimazole (Table 3). The obtained R^2 values of the method were as follows: 0.9993 for beef; 0.9990 for pork; 0.9994 for chicken; 0.9977 for egg; 0.9950 for milk; 0.9984 for fat; 0.9988 for flatfish; 0.9972 for eel; and 0.9981 for shrimp. The obtained linearity values ($R^2 > 0.99$) were in accordance with the criteria of the CODEX guidelines (CAC/GL 71-2009) [11]. The qualitative and quantitative ions of methimazole were sufficiently separated for identification from other substances in the samples on the chromatogram (Fig. 3), confirming the specificity of the method. The ions were completely separated, with a retention time of 4.5 min. The LOD and LOQ values of the method were 0.0001 and 0.005 mg/kg, respectively, for all samples. These limits were considerably low, indicating that the method is sufficiently sensitive to detect the measured analytes at concentrations of <0.01 mg/kg. Consequently, the linearity, specificity, and LOQ of the method were verified in accordance with the CODEX guidelines (CAC/GL-71-2009).

This method has been tested in different laboratories to confirm its reproducibility (Table 3). The recovery of the method was 96.3~97.9% for beef, 96.3~100.2% for pork, 95.3~96.9% for chicken, 89.4~90.7% for egg, 87.8~96.7% for milk, 94.4~104.1% for fat (pork), 98.4~104.5% for flatfish, 96.3~106.6% for eel, and 84.8~94.7% for shrimp. The CV of the method was 5.6~9.1% for beef, 10.1~12.1% for pork, 8.3~14.6% for chicken, 7.5~10.9% for egg, 7.6~11.2% for milk, 11.3~12.8% for fat (pork), 6.9~12.8% for flatfish, 10.7~12.5% for eel, and 5.7~12.2% for shrimp. The recovery and CV of the method were in the ranges of 87.8~106.6% and 5.6~14.6%, respectively. The obtained results were within the range of values required for each spiking concentration in accordance with the CODEX guidelines (CAC/GL 71-2009).

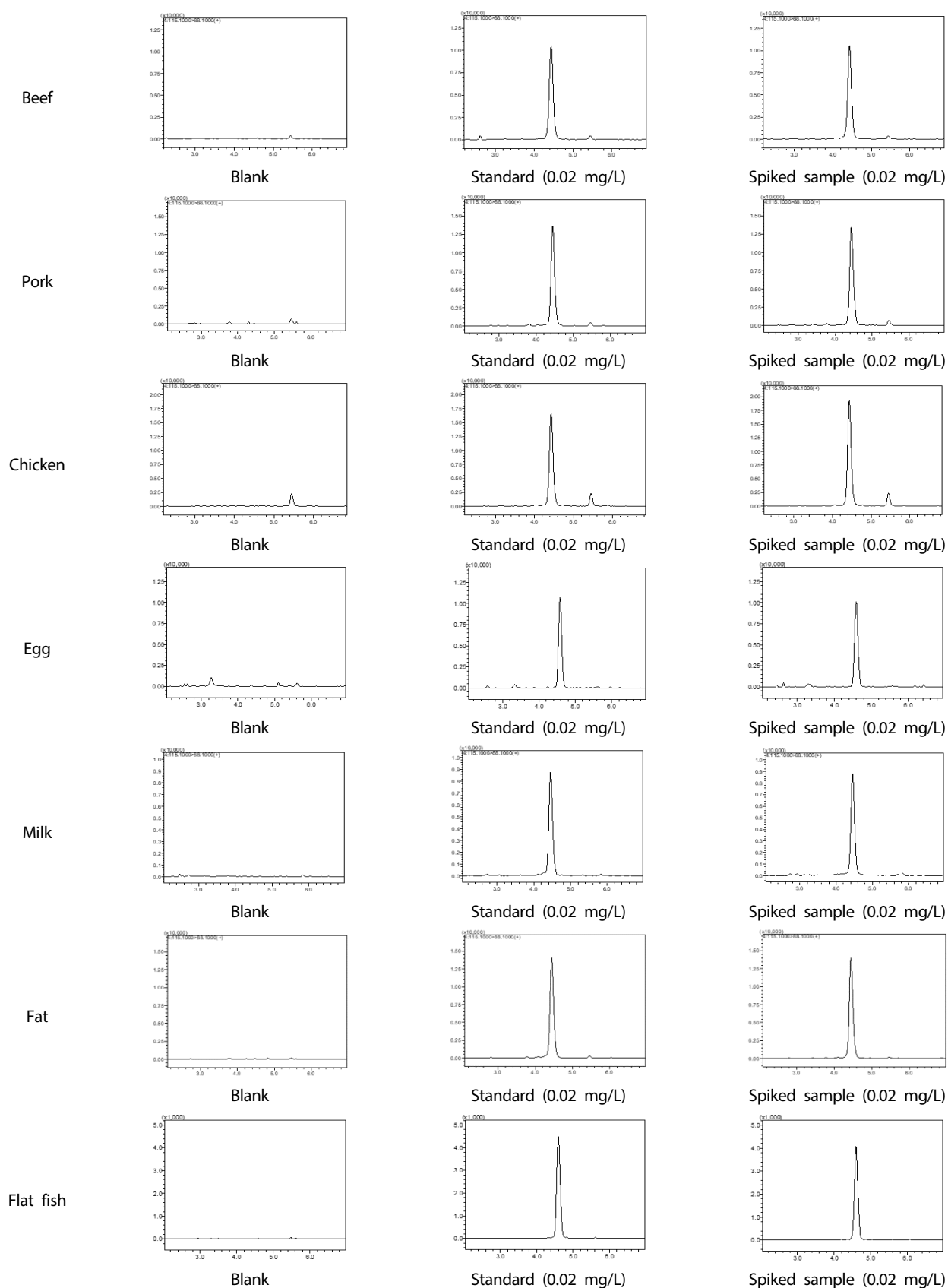
Table 3. Method validation of Inter-lab for methimazole in samples

Sample	Linearity (R ²)	Concentration (mg/kg)	Recovery (%)				CV ^a (%)
			Lab A (n=5)	Lab B (n=3)	Lac C (n=3)	Average	
Beef	0.9993	0.01	97.8	97.5	91.7	96.3	5.9
		0.02	99.5	97.9	91.7	97.1	5.6
		0.1	92.2	107.1	92.0	97.9	9.1
Pork	0.9990	0.01	100.1	102.1	80.2	96.3	10.4
		0.02	113.3	94.3	88.2	100.2	12.1
		0.1	103.9	100.6	85.6	98.4	10.1
Chicken	0.9994	0.01	112.7	83.6	92.8	96.9	14.6
		0.02	110.5	86.1	86.7	95.6	12.9
		0.1	103.4	93.5	84.7	95.3	8.3
Egg	0.9977	0.01	83.6	103.3	85.4	89.4	10.9
		0.02	93.7	95.1	81.4	90.7	9.0
		0.1	84.0	97.1	92.9	90.0	7.5
Milk	0.9950	0.01	86.2	83.5	97.8	87.8	10.5
		0.02	96.4	98.6	94.0	96.7	7.6
		0.1	101.0	98.4	79.1	95.0	11.2
Fat	0.9984	0.01	84.2	108.6	87.7	94.4	12.8
		0.02	115.5	102.0	88.5	104.1	12.0
		0.1	113.9	100.9	76.0	100.1	11.3
Flatfish	0.9988	0.01	96.2	108.7	98.0	100.1	6.9
		0.02	101.4	107.3	84.5	98.4	11.3
		0.1	107.8	115.8	87.7	104.5	12.8
Eel	0.9972	0.01	114.1	113.2	87.5	106.6	12.5
		0.02	96.0	105.5	87.7	96.3	10.7
		0.1	98.6	110.4	87.1	98.7	11.0
Shrimp	0.9981	0.01	88.6	78.8	84.4	84.8	5.7
		0.02	95.5	83.7	81.1	88.3	8.8
		0.1	108.7	87.1	79.1	94.7	12.2

^a CV: coefficient of variation.

Application to real samples

In this study, 60 samples, including both livestock (beef, pork, pork fat, chicken, milk and egg) and fishery products (flatfish, catfish, eel, mudfish, shrimp, and abalone), were obtained from the domestic market and analyzed using the method described. All samples were below the LODs for methimazole, indicating that they were safe for consumption. This is thought to be because methimazole is only approved for use as a thyroid treatment in cats and is not approved for use in livestock or aquatic products. However, there are suspicions that it is being used unethically to fatten livestock without thyroid glands, such as cows and pigs, so an analytical method was developed. This method is expected to help improve the efficiency of domestic food safety and analysis for methimazole that cannot be quantified using domestic analytical methods.



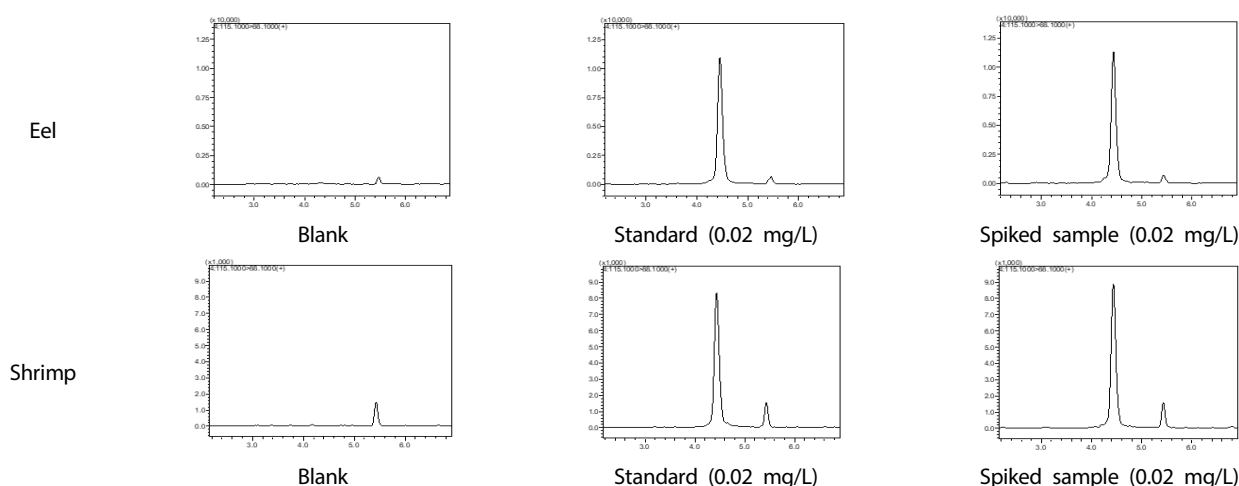


Fig 3. LC-MS/MS chromatograms of methimazole.

Conclusion

An analytical method was developed for the detection of methimazole in livestock and fishery products. The method was validated using three concentrations of the analytes. After treatment at three concentrations, methimazole was extracted with 80% acetonitrile, purified with C_{18} , fat-removed with acetonitrile-saturated hexane, and analyzed by LC-MS/MS. According to the CODEX guidelines (CAC/GL 71-2009), the intra- and inter-day accuracy, precision, specificity, calibration curve linearity, and LOQ of the analytical method were calculated. The chromatogram of the blank sample spiked with the methimazole standard confirmed that there was no peak near the retention time of the target compound. The coefficient of determination was >0.98 for all sample types, confirming linearity. At all concentrations, the accuracy and precision of the analytical method were verified according to the CODEX guidelines (CAC/GL 71-2009) requirements. Considering the effectiveness of this analytical method, it can be applied for ensuring food safety.

Data Availability: All data are available in the main text or in the Supplementary Information.

Author Contributions: H.L. and J.H.S. methodology, validation writing-original draft; J.Y.K. methodology, review and editing; G.H.J. and M.E. Project administration, review and revising manuscript.

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Additional Information:

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Correspondence and requests for materials should be addressed to Ji Young Kim.

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