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Research Article Research Article

Development of an Improved HPLC-PDA Method for the Determination of Tolfenamic Acid in Meat and Milk

Ji Sun Lee[1], Soo Hee Cho[2], Chae Mi Lim[3], Moon Ik Chang[4] and Hojae Bae[5]*

[1]*Imported Food Analysis Division, Seoul Regional Food and Drug Administration, 212 Mokdongjungang-ro, Yangcheon-Gu, Seoul, 07978, Republic of Korea*

[2]*Korea Health Supplements Association Sub., Korea Health Supplements Institute, A-dong 101, 700 D, Republic of Korea*

[3]*Ministry of Food and Drug Safety, 187 Osongsaengmyeong2(i)-ro, Osong-eup, Heungdeok-gu, Chungju-si, Chungcheonbuk-do 28159, Republic of Korea*

[4]*Pesticide and Veterinary Drug Residues Division, Food Safety Evaluation Department, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, Cheongju 28159, Republic of Korea*

[5]*KU Convergence Science and Technology Institute, Department of Stem Cell and Regenerative Biotechnology, Konkuk University, Hwayang-dong, Kwangjin-gu, Seoul 05029, Republic of Korea*

Abstract

Tolfenamic acid (TA) is one of the non-steroidal anti-inflammatory drugs (NSAIDs) which are widely used as analgesic, anti-inflammatory and antipyretic drugs. In this study, we developed an improved detection method focusing on the validation of the TA residue using high performance liquid chromatography (HPLC) and a photodiode array (PDA) detector. TA in bovine, swine muscles and milk were analyzed using a C_{18} column (5 µm, 250 mm × 4.6 mm i.d.) and the mobile phase consisted of water with 0.1% phosphoric acid (eluent A) and 0.1% phosphoric acid in acetonitrile (ACN) (eluent B). Additionally, to optimize TA detection, liquid-liquid extraction (LLE) and additional purification steps were established to minimize the endogenous peaks and interferences. The developed method was validated through the limit of detection (LOD), the limit of quantification (LOQ), linearity, accuracy, and precision testing. TA in food samples were successfully detected after 20 min in chromatography. The LOQ was evaluated at 0.01 mg/kg and coefficient of determination of the linear regression (r^2) was well over 0.99. The recovery rates ranged from 81.9-90.8%, 74.2-92.8% and 74.5-80.7% with relative standard deviations lower than 20% for bovine, swine muscles and milk, respectively, which corresponds to the CODEX International Food Standards guideline. Finally, the developed method has been applied for monitoring samples collected from the markets in major cities and proven great potential to be used as the confirmatory method to analyze and monitor TA residue in animal-based food products.

Keywords: Tolfenamic acid; HPLC-PDA; Pork muscle; Cattle muscle; Milk; Method validation; Monitoring

Introduction

As people's diet has developed recently in Korea, the consumption of high-protein foods including meat has significantly increased, expanding the size of the domestic livestock and fishery industries [1]. With the increasing livestock consumption, the usage of veterinary drugs to prevent animal diseases and to improve productivity has been also on the rise [2-4].

Given that the use of antibiotics compared to the annual volume of livestock production in Korea is greater than any other leading livestock countries such as the United States, Japan, Denmark, New Zealand and Sweden, the abuse of antibiotics in livestock production in Korea has reached a serious level [5]. Meanwhile, countries around the world allow the proper use of veterinary drugs to improve the productivity of livestock production, and the maximum residue limits (MRLs) for veterinary drugs in foods are regulated by the Council of the European Union and the U.S. Food and Drug Administration (FDA) [6].

The MRLs of about 147 veterinary drugs including antibiotics are also set and managed in Korea [7]. The MRLs and analysis methods for veterinary drugs are set under the Korea Food Code and the Manual for Analyzing Hazardous Substances in Livestock and Fishery Products. Since the Animal and Plant Quarantine Agency first implemented the National Residue Analysis Program in 1991, the government has continued to conduct tests to monitor residues annually, strengthening the management of veterinary drug residues in animal feeds and meat [8].

If veterinary drugs that are administered to livestock are transmitted and remain in foods, end consumers may be exposed to those residues, which, in turn, can develop into serious hazards to health such as resistant bacteria and cancer. Against this backdrop, the demand for the development of methods to analyze, verify, and monitor the results is ever growing [9].

Tolfenamic acid (TA), a substance within the class of NSAIDs, has been widely administered to both humans and animals for the purpose of reducing inflammation (Figure 1) [10,11]. Currently, it is used as an antibiotic only for veterinary purposes to treat musculoskeletal disorders, mastitis, lung disease, and enteritis. These types of veterinary drugs have a low toxicity, but its relative risk is high as it may cause resistant bacteria. For this reason, it is categorized as Grade 3 in the priority-based assessment of veterinary drugs such as in the assessment

***Corresponding author:** Hojae Bae, Associate Professor, KU Convergence Science and Technology Institute, Department of Stem Cell and Regenerative Biotechnology, Konkuk University, Hwayang-dong, Kwangjin-gu, Seoul 05029, Republic of Korea, Tel: +8224500525; E-mail: hojaebae@konkuk.ac.kr

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reports of JECFA (Joint FAO/WHO Expert Committee on Food Additives), EMA (European Medicines Agency) and CVM (Center for Veterinary Medicine). In addition, if accumulated in the body, their toxicity may increase, causing aplastic anemia or stomach and intestine diseases [12].

Against this backdrop, the Ministry of Food and Drug Safety added methods to analyze TA to the Korean Food Code in 2012 [13]. The current MRLs of TA in Korea are 0.05 mg/kg for the muscle of cattle and pork; 0.4 mg/kg for the liver of cattle and pork; 0.1 mg/kg for the kidney of cattle and pork; and 0.05 mg/kg for the dairy products (Table 1). After comprehensive review of the analysis method regarding TA in Korean Food Code, specific analytical method for TA could not be found for meat and dairy products [13].

Various determination methods for NSAIDs in foods have been developed and described in the previous studies. Most of them use HPLC with ultraviolet (UV) [14,15] or fluorescence [16] detection. Fluorescence has the advantage of being more sensitive and selective, but does not have the same range of applicability as UV making UV the most widely used detection method [17]. In addition, LLE with additional clean-up and solid phase extraction (SPE) are often used for sample preparation in food-producing animals [18]. The SPE is generally applied to clean animal products but does not provide sufficient extraction efficiency [19]. To sum up, methods capable for simultaneous determination of TA in cattle, pork, and milk by HPLC-PDA with LLE have not been developed.

In this study, our aim was to reestablish the analysis method by optimizing the composition of the mobile phase with HPLC and PDA detector for the determination of TA residues in livestock products. In addition, LLE and additional purification steps were established to minimize the endogenous peaks and interferences. Finally, newly established confirmatory method was validated according to the CODEX guidelines and was applied to monitor the TA residues in cattle, pork and milk collected from the markets in major cities [20].

Materials and Methods

Materials

Analytical standards of TA were purchased from Fluka (St. Louis, Missouri, USA). The molecular structure of this substance is shown in Figure 1. Phosphoric acid (85%, w/v), ammonium formate (≥ 99.995%), and octadecyl-functionalized silica gel (200-400 mesh) for the sample pretreatment and mobile phase for HPLC were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Sodium chloride (99%) was supplied by Daejung chemical and metals Co., Ltd. (Siheung, Gyeonggi, South Korea). Acetonitrile (ACN), methanol, and hexane were supplied by J. T. Baker (Phillipsburg, New Jersey, USA). Water was purified by using ELGA system. All the solvents used were filtered using 0.22 μm JG type filter (Millipore, Billerica, Massachusetts, USA) prior to injection.

Standard solution

Stock standard solutions (1000 μg/mL) were prepared by dissolving 10 mg of each compound in 10 mL of methanol and stored in glass-stopper bottles at 4°C. Working standard solutions of various concentrations were prepared by appropriate dilution with the stock solutions aliquoted with methanol.

Sample preparation

Meat samples: For the analysis, muscle samples of cattle and pork were collected from the markets located in major cities of Korea (Seoul, Busan, Incheon, Daegu, Gwangju, and Ulsan). Samples were ground using a high-speed food blender and stored at -20°C before the analysis (Figure 2A). Each ground sample (5 g) was put into a 50-mL centrifuge tube and then 200 mM ammonium formate solution (0.2 mL) and water (4 mL) were added to the tube. Analyte was extracted with ACN (16 mL) on a shaker for 15 min. After separation by centrifugation at 4500 g for 10 min, the supernatant was transferred into a separate centrifuge tube. Then C_{18} powder (1 g) was dispersed in the tube and ACN-saturated hexane (20 mL) was added. The tube was then shaken for 10 min and centrifuged at 4,000 g for 10 min followed by the removal of hexane layer. After repeating the extraction procedure twice, the ACN extract layer was moved to a rotary evaporator and concentrated at low pressure in water (40°C). The sample was finally reconstituted in methanol (1 mL) and used as a test solution after filtering through a 0.2 μm membrane filter (Waters, Milford, Massachusetts, USA) (Figure 2B).

Milk samples: Milk sample (5 g) was weighted into 50 mL polypropylene tubes. ACN (20 mL) was added to each tube followed by addition of sodium chloride (4 g). The sample was then shaken vigorously for 30 s and centrifuged at 4500 g for 10 min. The ACN (upper layer) was transferred into 50 ml polypropylene tubes. ACN-saturated hexane (8 mL) was added and the tube was shaken for 30 s followed by centrifugation (4500 g × 10 min). The hexane layer was then removed and discarded to waste. ACN extract layer was moved to a rotary evaporator and concentrated at low pressure in water (40°C). The sample was finally reconstituted in ACN: water (28:72, v/v) (1 mL) and used as a test solution after filtering through a 0.2 μm membrane filter (Waters, USA) (Figure 2C).

LC conditions

The liquid chromatography system (Shiseido Co., Ltd., Tokyo, Japan) was equipped with a solvent delivery pump (SI-2/3101), an auto sampler (SI-2/3133), a column oven (SI-2/3004), and a PDA detector (Accela ChemBio Inc., USA). An analyte was separated with a Capcell Pak UG C_{18} column (5 μm, 250 mm × 4.6 mm i.d., Shiseido Co., Ltd., Tokyo, Japan). The temperature of the column oven was set at 40°C. The gradient elution program was applied with 0.1% phosphoric acid in water (phase A) and 0.1% phosphoric acid in ACN (phase B). The flow rate, injection volume, and total runtime were 1.0 mL/ min, 20 μL, and 40 min, respectively. The UV detector was set at a wavelength of 218 nm for TA. The gradient profile is shown in Table 2.

Method validation

The developed method was validated according to the CODEX

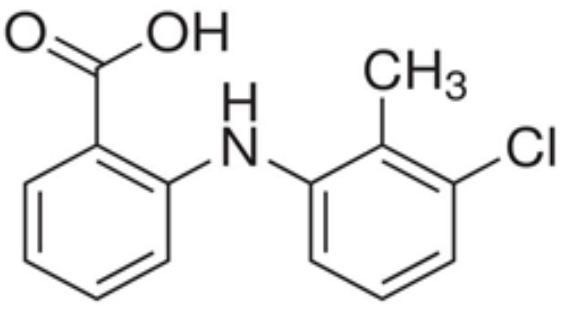


Figure 1: Molecular structure of examined TA.

Sample 5 g

↓

ACN (20 mL)

↓**Mix for 20 min**

Centrifuge at 4,000 rpm for 10 min

↓

Pour supernatant into 50 mL tube and add Hexane (20 mL)

↓**Vortexing**

Lower layer evaporation using steam nitrogen (50°C)

↓

Dissolve the residues in ether (4 mL)

↓**Mix for 10 min**

2 mM NaOH solution (10 mL), NaCl soultion (750 µL)

↓**Mix for 20 min**

Centrifuge for 10 min

↓

Transfer the aqueous lower phase into new tube

↓

Add 2% acetic acid (600 µL) and ethyl acetate (40 mL)

↓**Vortexing for 20 min**

Evaporation using steam nitrogen (50°C)

↓

Dissolve the residues with 500 µL methanol

↓**Vortexing for 10 min**

Centrifuge for 2 min

↓

Filtered with 0.45 µm filter

Sample 5 g

↓

Add 200 mM ammonium formate (0.2 mL)

↓

DW (4 mL) and ACN (16 mL)

↓Mix for 15 min

Centrifuge at 4,000 g for 10 min

↓

Pour supernatant into 50 mL tube and add C_{18} powder (1 g)

↓

Saturate ACN with hexane (20 mL)

↓Mix for 10 min

Centrifuge at 4,000 g for 10 min

↓Remove supernatant

Saturate ACN with hexane (20 mL) and mix for 10 min

↓

Centrifuge at 4,000 g for 10 min

↓Remove supernatant

Transfer lower layer into pear shape flask

↓

Evaporate to dry at 40°C

↓

Dissolve the residues with methanol (1 mL)

↓

Filter & Injection (HPLC)

Sample 5 g

↓

Add ACN (20 mL) and sodium chloride (4 g)

↓

Mix for 30 s

↓

Centrifuge at 4,500 g for 10 min

↓

Pour supernatant into 50 mL tube

↓

Add ACN saturated of hexane 8 mL and mix it for 30 s

↓

Centrifuge it at 4500 g for 10 min

↓

Evaporate to dry at 40°C

↓

Dissolve in methanol (1 mL)

Figure 2: Analytical procedure for TA residues in (A) Korean Food Code, (B) newly improved analysis method for cattle, pork, and (C) for milk.

Cattle	mg/kg	Pork	mg/kg	Milk	mg/kg
Cattle muscle	0.05	Pork muscle	0.05	Milk	0.05
Cattle liver	0.4	Pork liver	0.4		
Cattle kidney	0.1	Pork kidney	0.1		

*Not for use in animals from which milk is produced for human consumption.

Table 1: MRLs of TA residues.

Time (min)	A (%)[a]	B (%)[b]
0	50	50
30	50	50
31	0	100
36	0	100
37	50	50
40	50	50

[a]Mobile phase A: 0.1% phosphoric acid in water; [b]Mobile phase B: 0.1% phosphoric acid in ACN

Table 2: Gradient elution profile.

Mobile phase	Retention time	mAU	Peak area
A	8.33	33.06	342694
B	7.54	60.68	596246
C	7.95	370.37	3578147

Phase A: 0.1% Acetic acid/ACN (50:50) (pH 3.3); Phase B: 0.1% Formic acid/ACN (50:50); Phase C: 0.1% Phosphoric acid/ACN (50:50)

Table 3: Result of TA chromatogram.

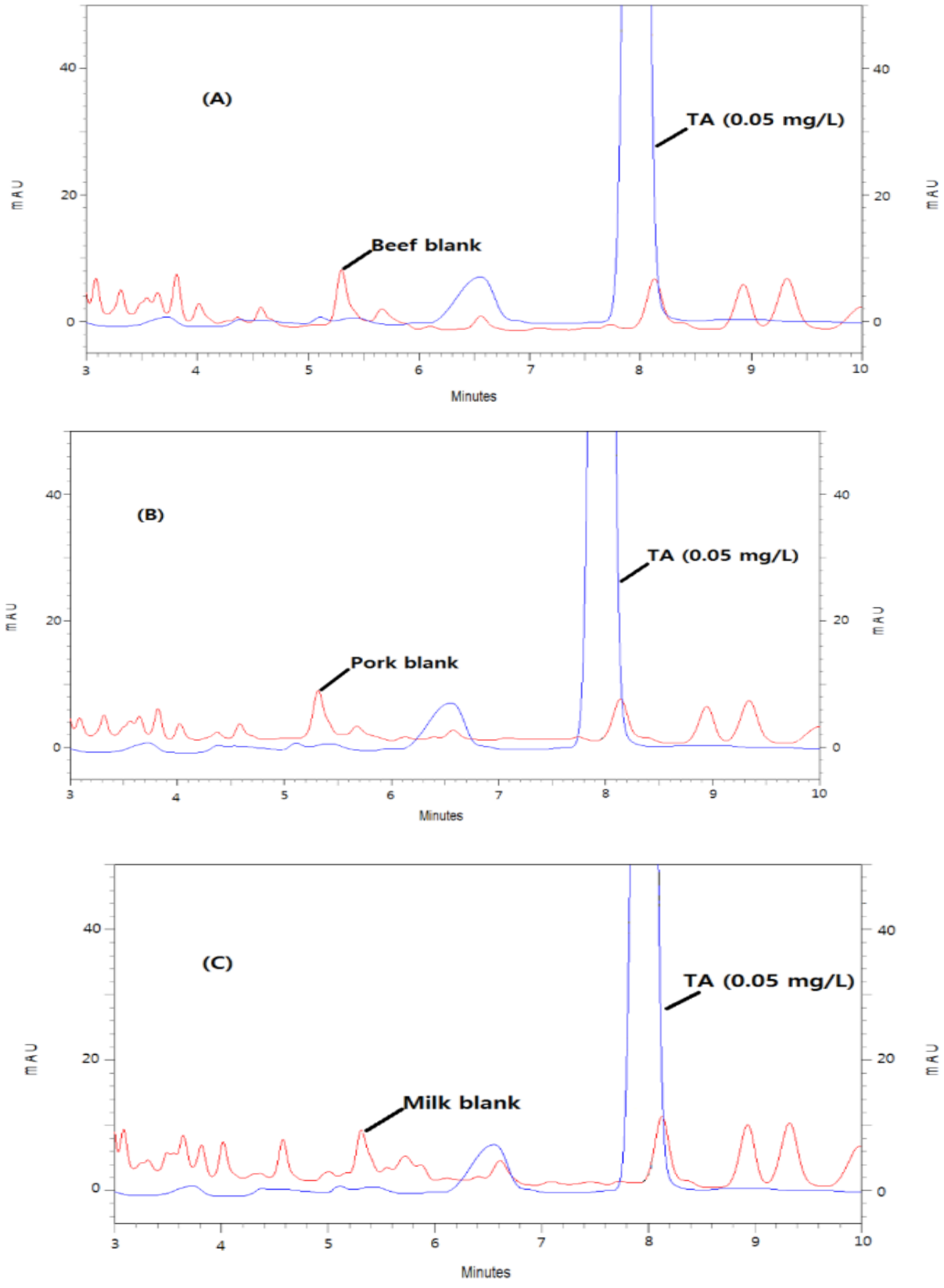


Figure 3: Analysis of TA by the analysis method in Korean Food Code. (A) Cattle (B) Pork (C) Milk.

guidelines [20]. For the validation, following performance parameters were determined: specificity, LOD and LOQ, linearity, recovery, and the precision (repeatability and reproducibility). The LOD and LOQ were calculated based on the signal to noise ratio (S/N=3 for LOD and S/N=10 for LOQ) of the chromatograms. The signal to noise ratio was measured using the Fine Chemicals System (Shiseido, Japan). To determine the specificity, interferences were checked by analyzing 20 blank cattle, pork, and milk samples, respectively. These samples were prepared according to the described procedure and analyzed. The linearity determining TA residues in the cattle, pork and milk samples was tested at the concentrations of 0.0625, 0.125, 0.25, 0.5, and 1 mg/L. Calibration curves were prepared by plotting the peak area against analyte concentrations expressed as mg/L. The linear regression equations and correlation coefficients were calculated for the curves and used for calculating the concentrations of analytes. The accuracy and precision of the method were investigated by analyzing the fortified samples (n=6) at the concentration levels of 1/2 × MRL (Maximum Residue Limits), MRL, and 2 × MRL. Six replicates at each level were analyzed to calculate the recovery (expressed as percentile) and the coefficient of variation (CV%).

Results and Discussion

Optimization of sample preparation step

The purification procedure in analysis method for TA stipulated in the current Korean Food Code is based on LLE. To reduce the HPLC runtime of with adequate peak separation, Capcell Core C_{18} S2.7 (2.7 μm, 250 mm × 4.6 mm i.d., Shiseido Co., Japan) was used. When tests were analyzed using the HPLC method with LLE in current Korean Food Code as shown in Figure 2A, specificity for TA could not be found (Figure 3). Therefore, the purification method was chosen after comparing the chromatograms of a HLB cartridge in SPE and LLE and additional purification step with C_{18} powder from the livestock hazardous materials analysis handbook [21].

The SPE is one of the analysis methods that are widely used recently to increase the selectivity of target substances compared to LLE [22,23]. For instance, Gentili et al. [24] have reported that the recovery rate of TA in Cattle was high (97~99%) by LC-MS in combination with SPE [24]. However, peak values of TA were found in the same time range both in standard solutions and the blank samples, showing no specificity when TA, as a single substance, was analyzed using a HLB cartridge in SPE with this analysis method.

With the method using C_{18} powder to eliminate impurities, peak values of blank samples were not detected in the same time range of the standard. Also, TA was separated with a Capcell Pack UG C_{18} column (5 μm, 250 mm × 4.6 mm i.d.) instead of Capcell Core C_{18} S2.7 column (2.7 μm, 250 mm × 4.6 mm i.d.). Therefore, we confirmed that the TA was isolated after 20 min from the Capcell Pack UG C_{18} column, while the TA was isolated after 7 min using the Capcell Core C_{18} S2.7 column. However, in the case of the Capcell Core C_{18} S2.7 column, sufficient washing procedure was required to remove the diverse peaks using organic solvent. Adequate washing procedure was essential to avoid accumulation of impurities in the column ends that eventually results in peak interference that are detected within the TA retention time. Therefore, we decided to use the Capcell Pack UG C_{18} column which is more cost effective than the Capcell Core C_{18} S2.7 column with similar separation performance. TA was well separated by the sample preparation using C_{18} powder, however there was no specificity for the analysis of TA in milk, which is due to the emulsification of milk protein that has not been sufficiently removed [15,25]. Therefore, milk samples were extracted with ACN and sodium chloride was added to aid partition of the milk and ACN mixture [25]. TA was then identified by matching retention times of peak to the values of the corresponding standard analyzed under the same experimental conditions in all samples (Figure 4).

Optimization of the HPLC mobile phase

In the case of using the analysis method stipulated in the Korean Food Code, acetic acid and ACN caused the analysis equipment to malfunction and columns as they remained within the equipment and are extracted slightly. To find the optimal solvent condition for the HPLC analysis method, three different mobile phase compositions were tested as follows: (1) 0.1% acetic acid:ACN (50:50) (pH 3.3) [5]; (2) 0.1% formic acid:ACN (50:50); and (3) 0.1% phosphoric acid:ACN (50:50). From the study of Park (2009) [5], the composition of the mobile phase was acidified to analyze TA by HPLC [5]. The highest peak areas of TA were found using Mobile Phase A (0.1% Phosphoric acid in water), and Mobile Phase B (0.1% Phosphoric acid in ACN) as shown in the chromatograms in Figure 5 and Table 3.

Validation of the analytical method

The specificity of the method was demonstrated by checking interfering peaks at the retention time of target analytes. The results showed that there were no interfering peaks co-eluted with target analytes in a wide range of food matrices. The HPLC-PDA method is capable of separating TA under the given gradient condition within 40 min, demonstrating that the method could be applied to monitor the residues in different livestock samples. Representative chromatograms of each fortified samples are shown in Figure 4.

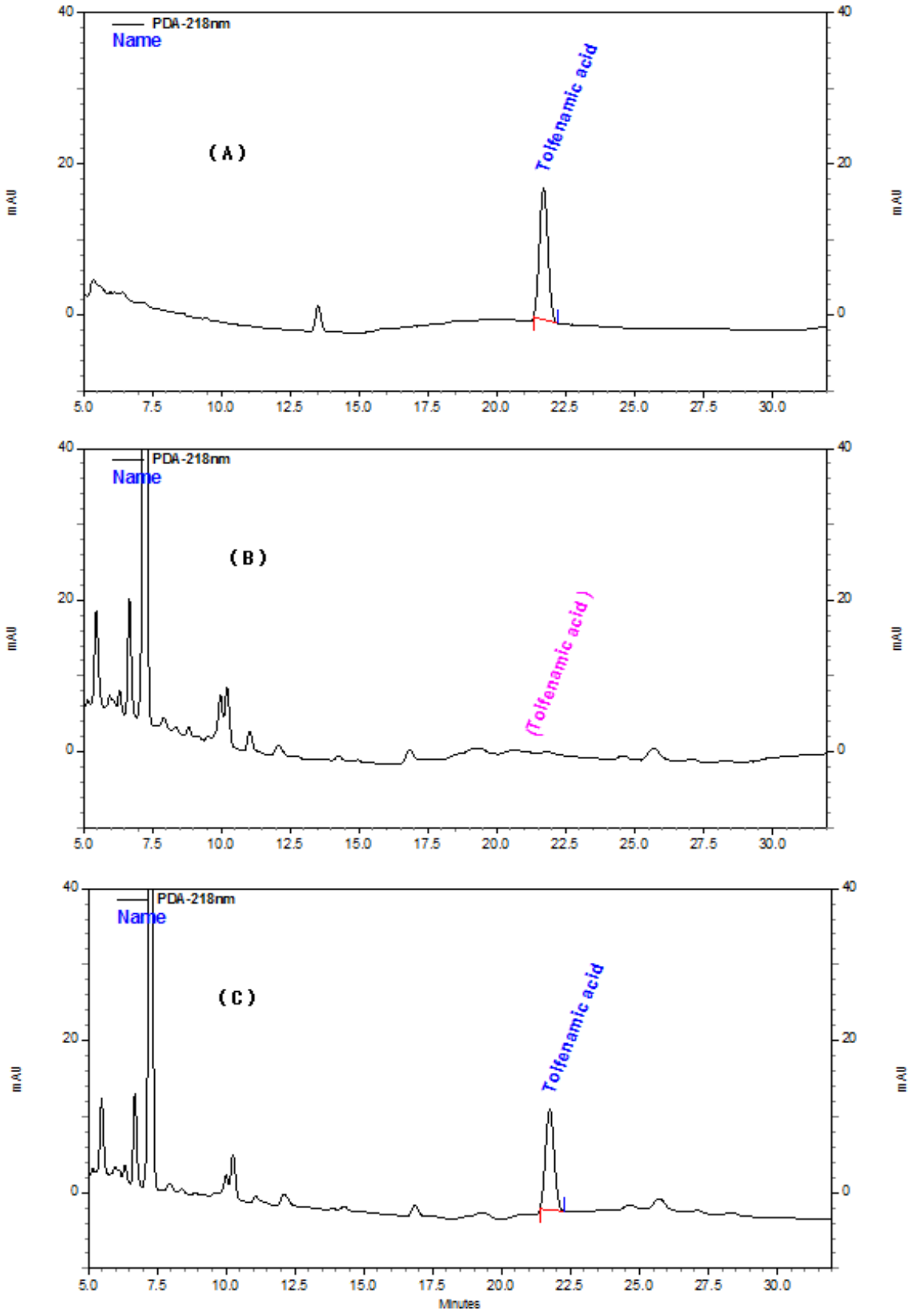
To compensate for compound loss during sample preparation, measurements of linearity were carried out with standard solutions at the concentration of 0.0625-1 mg/L. Satisfactory linearity with correlation coefficients greater than 0.99 was achieved with the LOD of 0.003 mg/kg and the LOQ of 0.01 mg/kg (Figure 6).

The recovery rates of the procedure were determined by spiking blank samples with three different levels (0.025, 0.05 and 0.1 mg/kg) of TA (six replicates). As a result, the recovery rates of Cattle, pork, and milk were 81.9~90.8%, 74.2~92.8% and 74.5~80.7% respectively. Accuracy was assessed as the ability to measure the amount of the target substance added into the actual samples at certain concentration levels, and precision was assessed by the coefficient of variation of analysis results that were obtained by repeating sample tests. The coefficient of variation of Cattle, pork and milk were 2.1~10.0%, 6.2~18.2%, and 6.0~6.8%, respectively (Table 4).

According to the CODEX guidelines for the attributes of analytical methods for residues of veterinary drugs in foods, average recoveries of 80 to 110% should be achieved when the spiked level is 100 μg/kg or greater and coefficient of variation should be less than 15%. Recommended acceptable recoveries are 70 to 110% when the spiked level is 10 μg/kg to 100 μg/kg and coefficient of variation is less than 20% [20]. Both recovery rates and accuracy are in compliance with the criterion of the codex.

Application of the developed method

The developed method was applied for the analysis of 125 commercially available animal based foods such as Cattle, pork, and milk collected from the markets located in major cities (Table 5). The collected food samples consisted of 47 Cattle (38%), 45 pork (36%), and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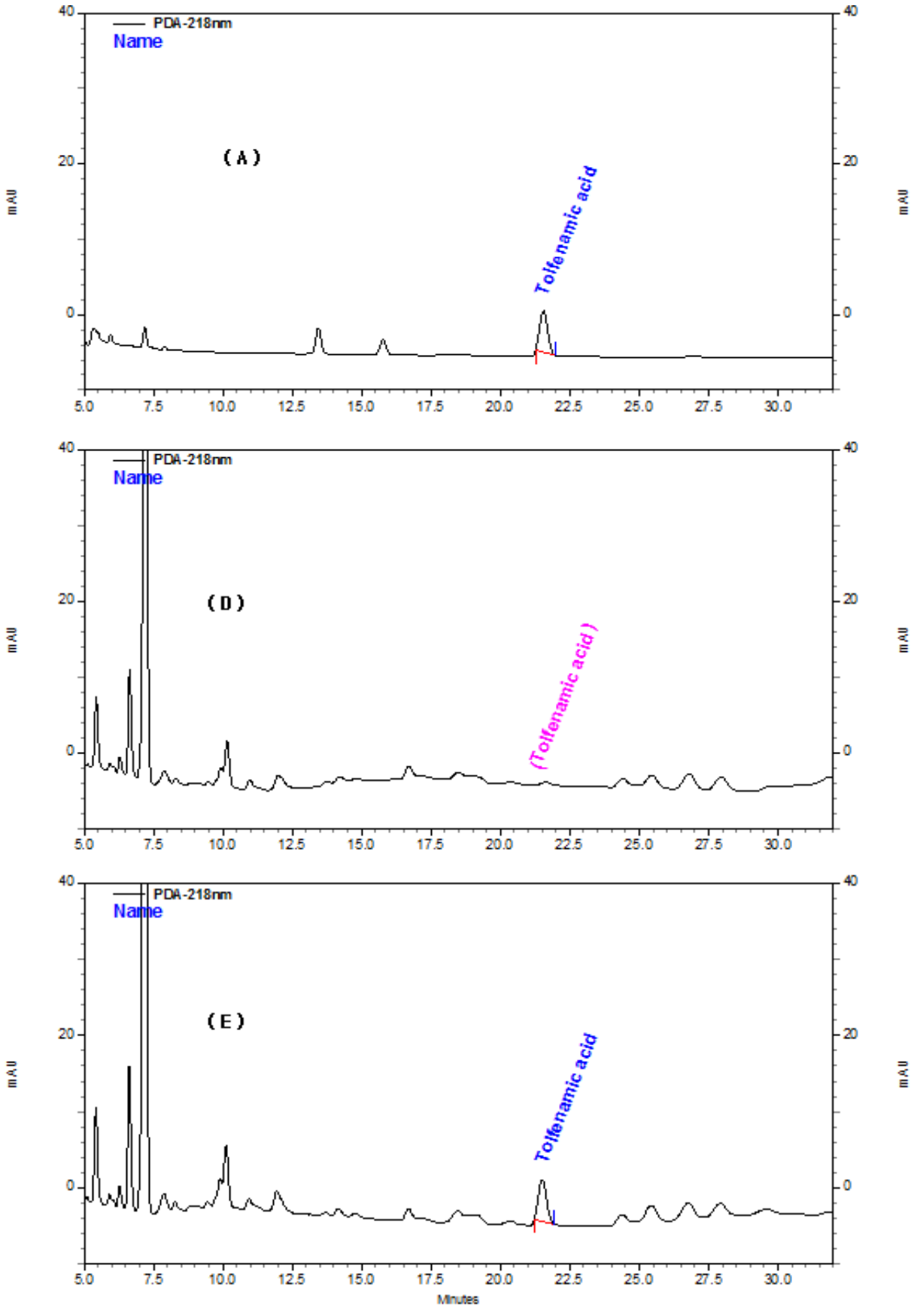
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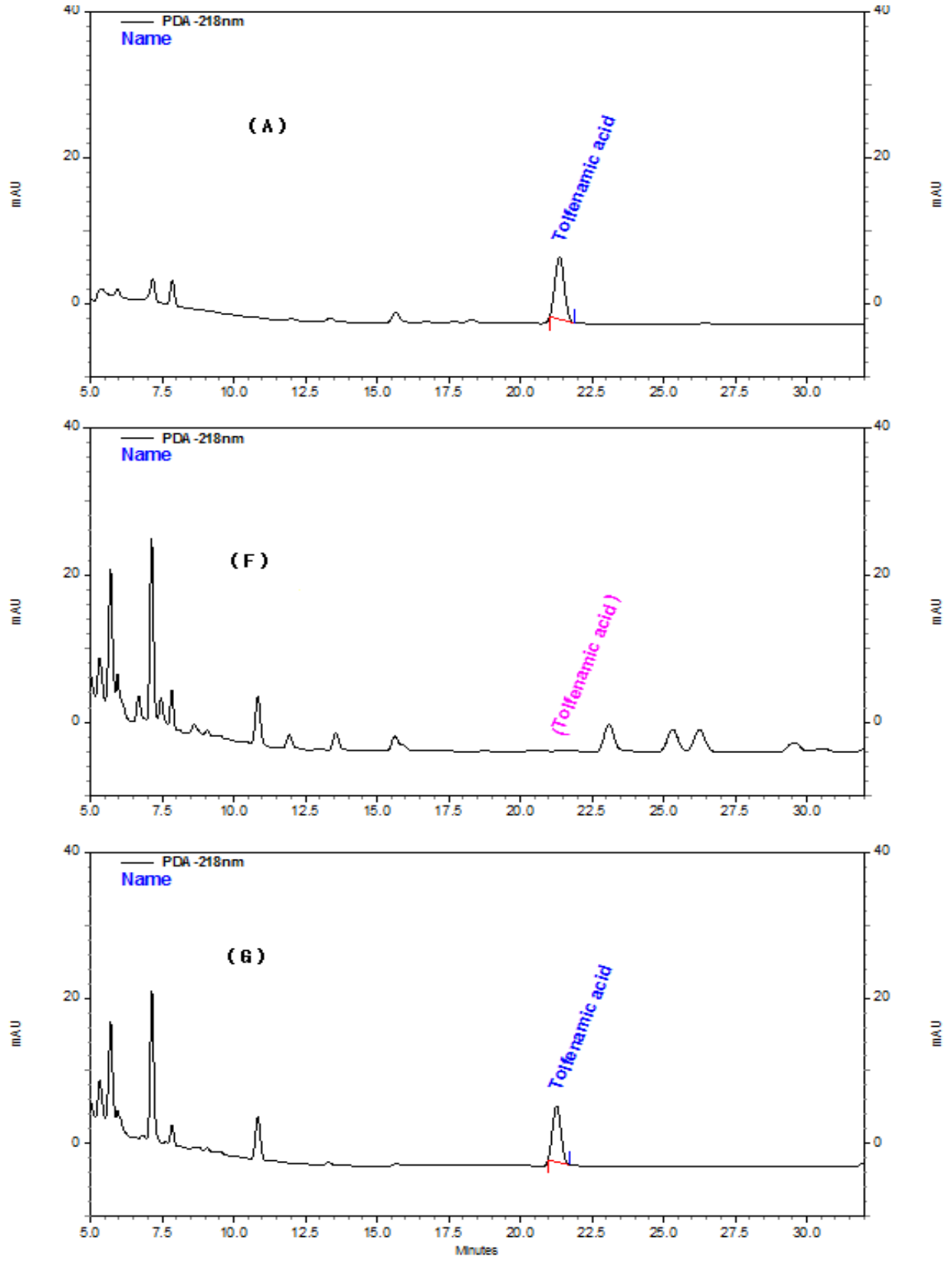

Figure 4: HPLC-PDA Chromatogram of TA. (A) Standard, (B) Cattle blank, (C) spiked Cattle at 0.05 mg/kg of TA, (D) pork blank, (E) spiked pork at 0.05 mg/kg of TA, (F) milk blank and (G) spiked milk at 0.05 mg/kg of TA.

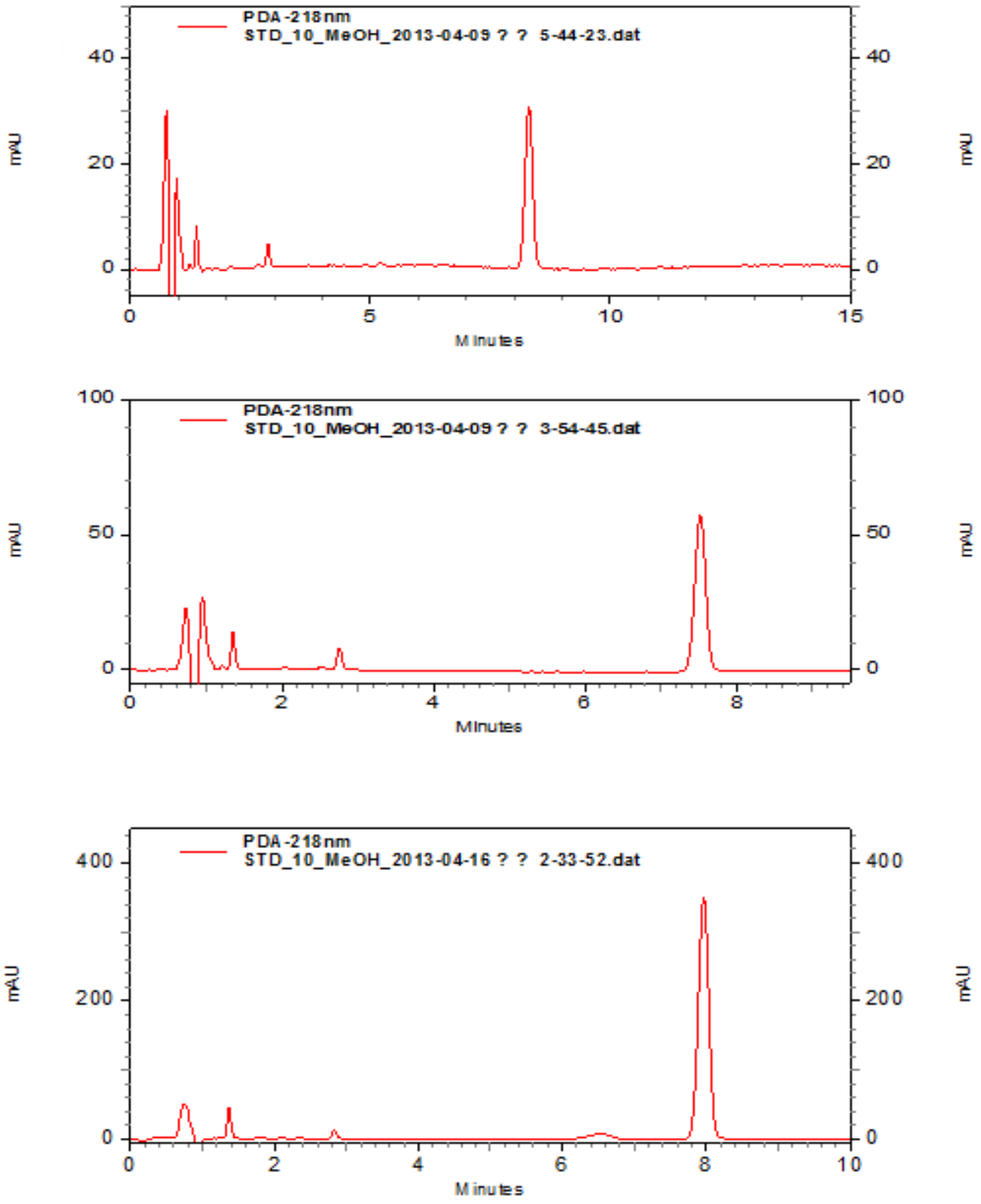

Figure 5: Mobile phase test and chromatogram of TA. (A) 0.1% acetic acid:ACN (50:50) (pH 3.3), (B) 0.1% formic acid:ACN (50:50), (C) 0.1% phosphoric acid:ACN (50:50).

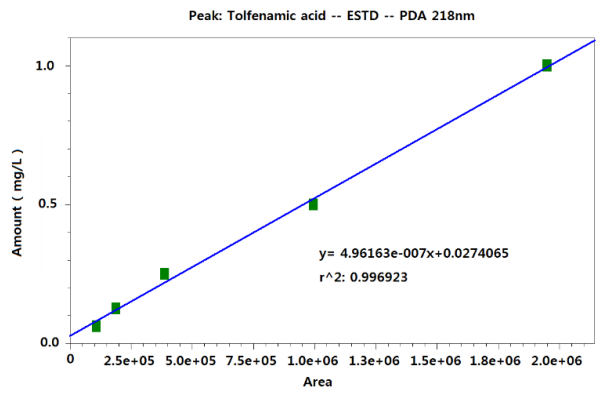

Figure 6: Calibration curve for TA.

Sample	Spiked Conc. (mg/kg)	Recovery (%)[1]	CV (%)[2]
Cattle	0.025	90.8	5.1
	0.05	85.3	10
	0.1	81.9	2.1
Pork	0.025	92.8	9.5
	0.05	74.2	18.2
	0.1	84.7	6.2
Milk	0.025	74.5	6
	0.05	80.7	6.8
	0.1	80.2	6.1

[1]Values of six replicates with standard deviations; [2]Relative standard deviation

Table 4: The average recovery rate and RSD of TA in samples.

Region	Cattle	Pork	Milk	Total
Seoul	18(ND[1])	19(ND)	14(ND)	51(ND)
Busan	7(ND)	7(ND)	4(ND)	18(ND)
Incheon	5(ND)	5(ND)	5(ND)	15(ND)
Daegu	7(ND)	5(ND)	4(ND)	16(ND)
Gwangju	2(ND)	3(ND)	3(ND)	8(ND)
Daejeon	4(ND)	3(ND)	1(ND)	8(ND)
Ulsan	4(ND)	3(ND)	2(ND)	9(ND)
Total	47(ND)	45(ND)	33(ND)	125(ND)

(Unit: case); [1]Not detectable

Table 5: Summary of purchased region and TA residue results.

33 milk (26%) products. All samples were processed according to the method described above and the analytical results showed that none of the sample contained any detectable amount of TA.

Conclusion

A simple and effective HPLC-PDA method was developed for the determination of TA in Cattle, pork, and milk. TA residues were successfully separated by HPLC-PDA. The proposed method showed satisfactory validation characteristics with respect to specificity, accuracy, precision, analytical limits, and applicability. Therefore, it can be concluded that the proposed method has great potential to be used as the confirmatory method to analyze and monitor TA residues in animal-based food products.

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