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Simultaneous Determination of Albendazole, Thiabendazole, Mebendazole and Their Metabolites in Livestock by High Performance Liquid Chromatography

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ABSTRACT

An analytical method using high performance liquid chromatography (HPLC) was developed for the simultaneous determination of thiabendazole, 5-hydroxythiabendazole, albendazole sulfoxide, albendazole sulfone, albendazole 2-aminosulfone and mebendazole in livestock. These six benzimidazoles were extracted with acetonitrile under basic conditions, followed by a Sep-Pak C18 cartridge cleaning procedure. The HPLC separation was achieved on a Cosmosil 5C18-MS-II column (5 μm, 4.6 mm i.d. × 250 mm) using gradient elution with acetonitrile and 0.02M sodium dihydrogen phosphate (pH 3.3), and detection was performed with photodiode array and fluorescence detector. Good linearity was observed from the calibration plot at concentrations from 0.05 to 2.50 μg/mL. Recovery studies of the analytes were performed at 0.02, 0.10 and 1.00 ppm spiked levels. The average recovery of 5-hydroxythiabendazole and mebendazole at low concentrations ranged from 71.4 to 87.1% and ranged from 81.6 to 104.3% at other concentrations, with coefficient of variation less than 5.9%. The detection limit for benzimidazoles was between 0.005~0.030 ppm under photodiode array detection and 0.004~0.020 ppm under fluorescence detection. The coefficient of variation of intra-day and inter-day assays was lower than 1.92% and 7.22%, respectively. These results indicated that the developed method had an acceptable precision. This method was applied to detect benzimidazoles in samples of pork, swine liver, beef, lamb, bovine milk and goat milk purchased from various markets in Taipei. The results showed that none of the six benzimidazoles were detected in the thirty samples.

Key words: veterinary drug, albendazole, thiabendazole, mebendazole, high performance liquid chromatography

INTRODUCTION

Benzimidazoles anthelmintics are commonly used in veterinary industry for the prevention and control of internal worm parasites in animals. The most widely used benzimidazoles in farm animals are albendazole, thiabendazole, oxfendazole, and fenbendazole[1,2]. As some members of this class of anthelmintics showed teratogenic and embryotoxic effects in a variety of animal species[3], concerns for the risks posed by residues in food of drug treated animals were thus arisen[4,5].

Albendazole, methyl (5-(propylthio)-1*H*-benzimidazol-2-yl) carbamate, is a benzimidazole carbamate. Its mode of action is by binding strongly with the tubulin in the intestinal cells of nematodes causing them to starve to death[3]. Following dosing to farm animals, albendazole is readily absorbed by the gut and rapidly metabolized by oxidation of its sulfide group to form albendazole sufoxide, with further oxidation to form albendazole sulfone, and deacetylation of the carbamate group to form albendazole 2-aminosulfone[4]. Toxicological studies in mice, rats, rabbits, and sheep have shown albendazole to be teratogenic[6]. Thiabendazole (2-(4-thiazolyl)-1*H*-benzimidazole) is widely used for the prevention and control of animal parasites and as a pre- or post- harvest fungicide for vegetables and fruits[7]. Thiabendazole is metabolized in farm animals via hydroxylation of the benzimidazole ring at the 5-position to form 5-hydroxythiabendazole with subsequent glucuronide or sulfate conjugation[8]. Mebendazole (methyl-5-benzoyl-2-benzimidazole carbamate) is the first benzimidazole anthelmintic used to treat echinococcosis. However, it has the disadvantage of low bioavailability owing to its extremely poor solubility[9]. Mebendazole is used to be an antiparasitic agent in Taiwan[10].

The Department of Health announced the revised "Tolerances for Residues of Veterinary Drugs" in January 2001[11]. According to this regulation, maximum residue limits of albendazole, thiabendazole, and mebendazole were set. So far, no analytical method has been promulgated by the Department of Health. Therefore, it is an important issue to establish a standard analytical method for monitoring the residual benzimidazoles in livestock.

Methods for analyzing benzimidazoles include high performance liquid chromatography (HPLC) coupled with ultraviolet (UV)[1,12-25] or fluorescence (FL) detection[26-29], HPLC/mass spectrometry (HPLC/MS)[30,31], and gas chromatography/mass spectrometry (GC/MS)[15,22,32]. HPLC is commonly applied to the determination of benzimidazoles. Benzimidazoles in samples were extracted with solvent, followed by single[16,28] or double cleanup steps[1,15,27]. The

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mobile phase elution can be isocratic[1,12,27], gradient[31], or several mobile phase systems[15,16,28]. Among these methods, the adequate methodology for thiaextraction, separation and quantitation of albendazole, thiabendazole, mebendazole and their metabolites in tissue and milk samples has not yet been described.

According to the regulation of the Codex, marker residue of albendazole has been defined as albendazole 2-aminosulfone. While the marker residue in milk has not yet been defined[33], the marker residue of thiabendazole has been defined as the sum of thiabendazole and 5-hydroxythiabendazole[34], whereas mebendazole is not approved by the Codex for use. The aim of this study was to develop an HPLC analytical method to simultaneously determine six benzimidazoles including albendazole sulfoxide, albendazole sulfone, albendazole 2-aminosulfone, thiabendazole, 5-hydroxythiabendazole, and mebendazole in livestock using photodiode array and fluorescence detection. Specificity, sensitivity, and accuracy of this method were evaluated.

MATERIALS AND METHODS

I. *Samples*

A total of thirty samples, including five samples each of pork, swine liver, beef, lamb, bovine milk, and goat milk, were purchased from various markets in Taipei in October 2002. All samples were stored at -20°C until analyzed.

II. *Chemicals*

(I) *Standards*

Thiabendazole (TBZ, 100%) and mebendazole (MBZ, 100%) were purchased from Sigma Chem. Co. (St. Louis, MO, U.S.A.), 5-hydroxythiabendazole (TBZ-OH, 98.9%) and albendazole 2-aminosulfone ($ABZSO_2$-NH_2, 98.2%) were purchased from Hayashi Pure Chemical Industries Ltd. (Osaka, Japan), albendazole sulfoxide (ABZ-SO, 97.0%) and albendazole sulfone (ABZ-SO_2, 98.8%) were kindly provided by GlaxoSmithKline (Worthing, West Sussex, U.K.). The chemical structures of six benzimidazoles are shown in Figure 1.

(II) *Solvents and other reagents*

LC grade acetonitrile (CH_3CN) was purchased from DBH Laboratory Supplies (Poole, England) and ethyl acetate, methanol (MeOH) and n-hexane were purchased from Labscan Co., Ltd. (Bankok, Thailand). GR grade dibutyl hydroxytoluene (BHT), dimethyl sulfoxide (DMSO), 1-propanol, ammonium dihydrogen phosphate, sodium dihydrogen phosphate (NaH_2PO_4), sodium hydrogen carbonate ($NaHCO_3$) and sodium carbonate (Na_2CO_3) were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Sep-Pak C18 cartridge was purchased from Waters Corporation (Milford, MA, U.S.A.).

III. *Equipment*

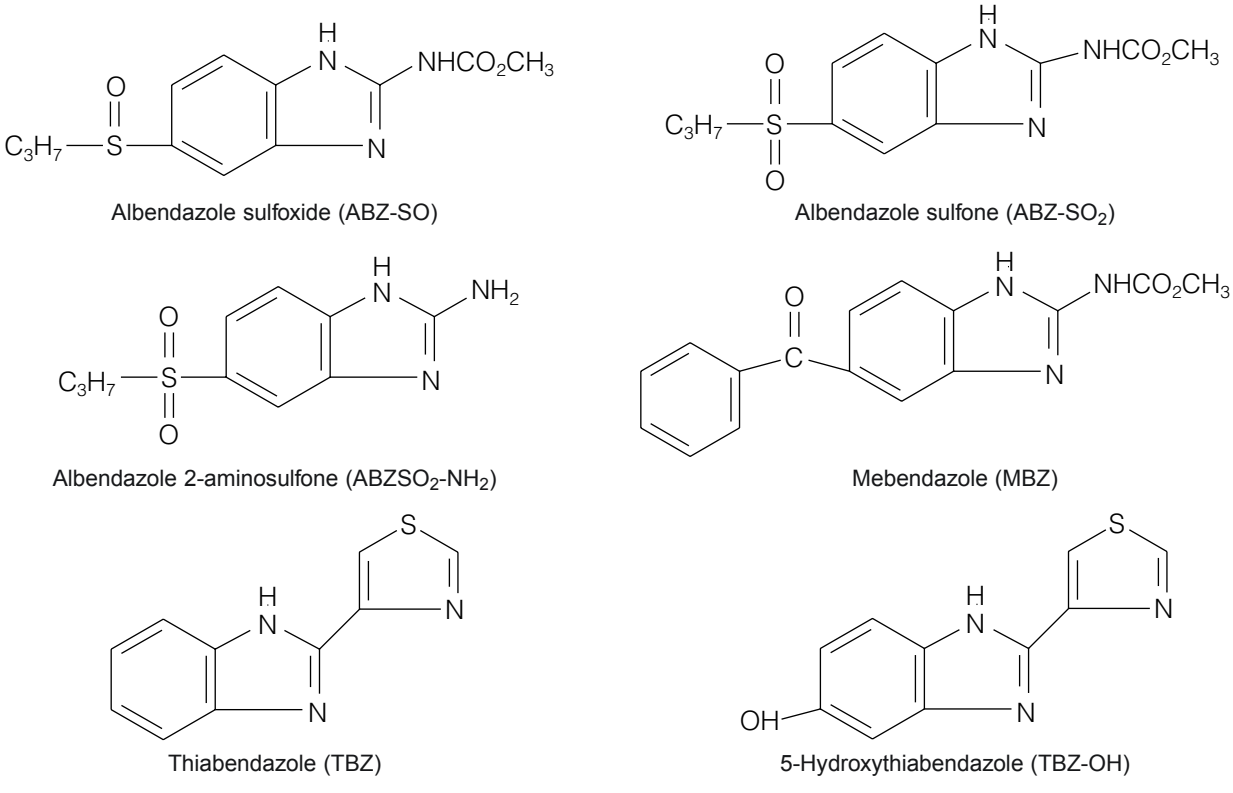

Figure 1. Chemical structures of six benzimidazoles.

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Samples were minced and ground with a homogenizer (Nihonseiki Kaisha Ltd., Tokyo, Japan). Rotary evaporator (Buchi, Labortechnic AG, Flawil, Switzerland), centrifuge (Labofuge 400, Heraeus Instruments GmbH, Hanau, Germany), and vortex mixer (Type 37600 Mixer, Thermolyne Corporation, Iowa, U.S.A.) were used during the sample preparation. Fluorescence spectra were obtained with a fluorescence spectrophotometer (F-4500, Hitachi Ltd., Tokyo, Japan). A high performance liquid chromatographic system consisted of a Shimadzu LC-10AT pump, a CBM-10A interface controller, a SIL-10A auto injector, a SPD-M6A photodiode array (PDA) detector, and a RF-551 fluorescence detector (Shimadzu Corporation, Kyoto, Japan) was used to analyze the samples.

IV. *Preparation of Buffer Solution for Extraction*

Saturated sodium hydrogen carbonate was adjusted to pH 10 with saturated sodium carbonate.

V. *Preparation of Standard Solution*

(I) *Stock standard solution*

ABZ-SO, ABZ-SO_2, ABZSO_2-NH_2, TBZ and TBZ-OH (5 mg of each) were separately weighed into a 100 mL volumetric flask, dissolved and diluted to volume with methanol. MBZ (5 mg) was accurately weighed into a 100 mL volumetric flask, dissolved and diluted to volume with DMSO. All stock standard solutions were stored in a refrigerator. Because DMSO was solidified under this condition, standard stock solution of MBZ must be reliquified by placing volumetric flask in a beaker of lukewarm water and vortex mixing solutions before use.

(II) *Mixed standard solution*

The above stock solutions were mixed and diluted with acetonitrile: methanol: 0.02M sodium dihydrogen phosphate (2:2:6, v/v/v) solution to a series of concentrations ranged from 0.05 to 2.50 μg/mL.

VI. *Analytical Methods*

(I) *Extraction*

Test samples of pork, swine liver, beef and lamb were ground. Ground sample (5 g) was weighed into a homogenizer cup, 1 mL of the buffer solution, 1 mL of BHT solution (1 g in 100 mL of acetonitrile) and 30 mL of acetonitrile were added followed by homogenizing for 2 min. After filtration, the residue was homogenized with another 30 mL of acetonitrile. The homogenizing and filtration procedures were repeated. The pooled filtrate was transferred into a separation funnel containing 50 mL of acetonitrile-saturated n-hexane and shaken for 5 min. The acetonitrile layer was collected into a concentration bottle. 5 mL of 1-propanol was added to reduce 'bumping' and evaporated to dryness at 40°C using a rotary evaporator.

Five mL of milk was placed into a centrifuge tube. 1 mL of the buffer solution, 1 mL of BHT solution and 15 mL of acetonitrile were added followed by shaking for 5 min and centrifuging at 3500 rpm for 5 min. The residue was shaken with another 15 mL of acetonitrile. The shaking and filtration procedures were repeated. The pooled supernatant was transferred into a separation funnel containing 30 mL of acetonitrile-saturated n-hexane and shaken for 5 min. The acetonitrile layer was collected into a concentration bottle. 5 mL of 1-propanol was added and evaporated to dryness at 40°C using a rotary evaporator.

(II) *Cleanup*

The above dry residue was reconstituted with 5 mL of 10% methanol and applied onto a Sep-Pak C18 cartridge, which was preconditioned with 5 mL of methanol and 5 ml of 10% methanol. The concentration bottle was washed with 5 mL of 10% methanol, which was then applied onto the same cartridge. The eluate was discarded. The same concentration bottle was then washed twice with 4 ml of acetonitrile and the resulting solution was passed through the same cartridge. The eluate was collected and evaporated to dryness at 40°C using a rotary evaporator. The residue was reconstituted with 2 mL of acetonitrile: methanol: 0.02M sodium dihydrogen phosphate (2:2:6, v/v/v) solution, mixed on a vortex mixer to dissolve residue, then filtered through a 0.2 mm membrane (nylon, Micron Separations Inc., West Borough, MA, U.S.A.) prior to HPLC analysis. The operation procedure for the analysis of six benzimidazoles is demonstrated in Figure 2.

VII. *HPLC Analysis*

(I) *Analytical conditions*

The column for separating benzimidazoles was Cosmosil 5C18 MS-II (5 μm, 4.6 mm i.d. × 250 mm; Nacali Tesque Inc., Kyoto, Japan). The detectors were PDA and fluorescence detector. The scan range of PDA was 200~400 nm, and UV detection were set at 290 and 320 nm. The excitation (Ex) and emission (Em) wavelengths of fluorescence detector were set to time program. The wavelengths for ABZSO_2-NH_2 were Ex 280/Em 310, and the wavelengths for TBZ, ABZ-SO and ABZ-SO_2 were Ex 290/Em 320. Sensitivity was set at low and the gain was set at ×4. Acetonitrile (A) and 0.02M sodium dihydrogen phosphate (B) were used as the mobile phase, and the gradient profile was shown in Table 1. Flow rate was 1 mL/min. The injection volume of samples was 100 μL.

(II) *Standard curves*

Four concentrations (0.02, 0.25, 1.25, and 2.50 μg/mL) of mixed standard solutions were prepared as described.

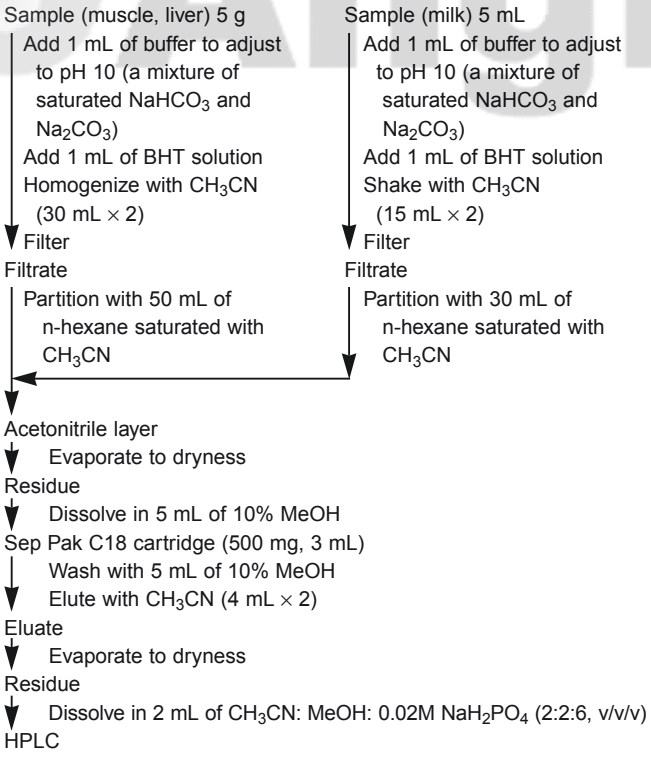

Figure 2. Analytical procedure for six benzimidazole residues.

Table 1. Gradient elution profile

Time (min)	A (%)[a]	B (%)[a]
0	10	90
10	40	60
19	70	30
21	10	90

[a]Mobile phase A: acetonitrile; mobile phase B: 0.02M sodium dihydrogen phosphate (pH 3.3).

These solutions were analyzed three times by HPLC-PDA and fluorescence detector, respectively. Standard curves were plotted according to the peak areas versus concentrations.

(III) *Identification and quantitative analysis*

Benzimidazoles were identified by: (1) comparing the peak retention times of samples with those of standards from HPLC, (2) comparing the spectra of analytes against the spectra from PDA of those standards. The following formula was used to calculate the amounts of veterinary drugs in test samples:

Amount of veterinary drug (ppm) = C × V / W

Where C is the drug concentration (μg/mL) calculated from standard curve, V is the volume of sample solution (mL), and W is the weight (or volume) of sample (g or mL).

VIII. *Recovery Test*

Recovery test was performed in triplicate by spiking standards at 3 levels (0.02, 0.10, and 1.00 ppm) into samples. The spiked samples and blank samples without standard were then analyzed by HPLC. Recovery was determined by comparison of the amount of benzimidazole added with the amount of benzimidazole found.

IX. *Limits of Detection*

Samples, spiked with various concentrations of standard solutions, were analyzed. Limits of detection were based on a signal to noise (S/N) ratio with 3:1 as the minium.

RESULTS AND DISCUSSION

I. *Study on the HPLC Conditions*

(I) *The optimal wavelength of detection*

HPLC with PDA and fluorescence detections has been used for benzimidazoles analysis in many cases. Except MBZ (has no fluorescent characteristic) and TBZ-OH (has very weak fluorescent characteristic), the rest all have fluorescent characteristic. In this study, PDA detector and fluorescence detector were therefore used. After spectrum scanning on benzimidazoles by PDA detector and fluorescence spectrophotometer, the maximum absorption wavelengths of UV, as well as the excitation and emission wavelengths of fluorescence, are shown in Table 2. UV detections of the benzimidazoles at 290 and 320 nm were used. The more intense absorption wavelengths at 220 nm ($ABZSO_2$-NH_2, ABZ-SO, ABZ-SO_2) and 250 nm (MBZ) were not suitable for detection because of self-absorption of the mobile phase. The fluorescence wavelength was set to time program in two Ex/Em detection wavelengths, Ex 280/Em 310 nm for $ABZSO_2$-NH_2 and Ex 290/Em 320 nm for the rest of analytes.

(II) *Mobile phase selection*

Although some chemical similarity exists among the six benzimidazoles, there is a significant difference of polarity between the first and last eluting compounds. In

Table 2. Optimum detection wavelengths of six benzimidazoles for UV and fluorescence detection

Benzimidazole	Peak	UV (nm)	Fluorescence (nm)	
			Excitation	Emission
TBZ-OH	1	320	*	*
$ABZSO_2$-NH_2	2	290	280	310
TBZ	3	290	290	320
ABZ-SO	4	290	290	320
ABZ-SO_2	5	290	290	320
MBZ	6	320	*	*

*TBZ-OH and MBZ have fluorescence quantum yield too low to be detected.

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our preliminary study, when acetonitrile was used in the mobile phase, the peak shape of TBZ and TBZ-OH was sharp, while those of ABZ metabolites and MBZ were broad and tailing. Replacing acetonitrile by methanol resulted in the opposite situation. The HPLC chromatograms of the benzimidazoles using acetonitrile: methanol: 0.02M sodium dihydrogen phosphate (2:2:6, v/v/v) solution as the mobile phase for isocratic elution are shown in Figure 3. It is shown that ABZ-SO_2NH_2 and TBZ-OH (capacity factor, K' 0.16) eluted overlapped near the solvent front. The last-eluted MBZ (K' 4.81), which is more hydrophobic than the other benzimidazoles, appeared as a broad and badly tailed peak, and so its quantification would be more difficult. Therefore, it was not appropriate to determine all benzimidazoles with isocratic elution because of the difference in hydrophobic character. Therefore, a gradient elution was applied.

A mobile phase consisted of acetonitrile and 0.02M sodium dihydrogen phosphate was selected and the gradient elution was performed as described in Table 1. The resolution of the benzimidazoles was affected by the pH of 0.02M sodium dihydrogen phosphate. The effect of pH, ranging from 3.0 to 5.0 adjusted by phosphoric acid, on the retention times of the benzimidazoles is shown in Figure 4. The K' of TBZ and TBZ-OH increased along with the pH, while the other four benzimidazoles were not affected by the pH of the mobile phase. The peaks of $ABZSO_2$-NH_2 and TBZ eluted closely each other at pH 3.0, showing an overlap between TBZ-OH and $ABZSO_2$-NH_2, as well as between TBZ and $ABZSO_2$ at both pH 4.3 and 5.0. A mobile phase containing 0.02M sodium dihydrogen phosphate adjusted to pH 3.3 was thus used in this study. Figure 5 shows the HPLC chromatograms of a mixed standard solution of six benzimidazoles using a gradient elution of acetonitrile and 0.02M sodium dihydrogen phosphate (pH 3.3) detected by a PDA detector followed with a fluorescence detector. All six benzimidazoles were well separated within 19 min with good peak resolution, sharpness and symmetry.

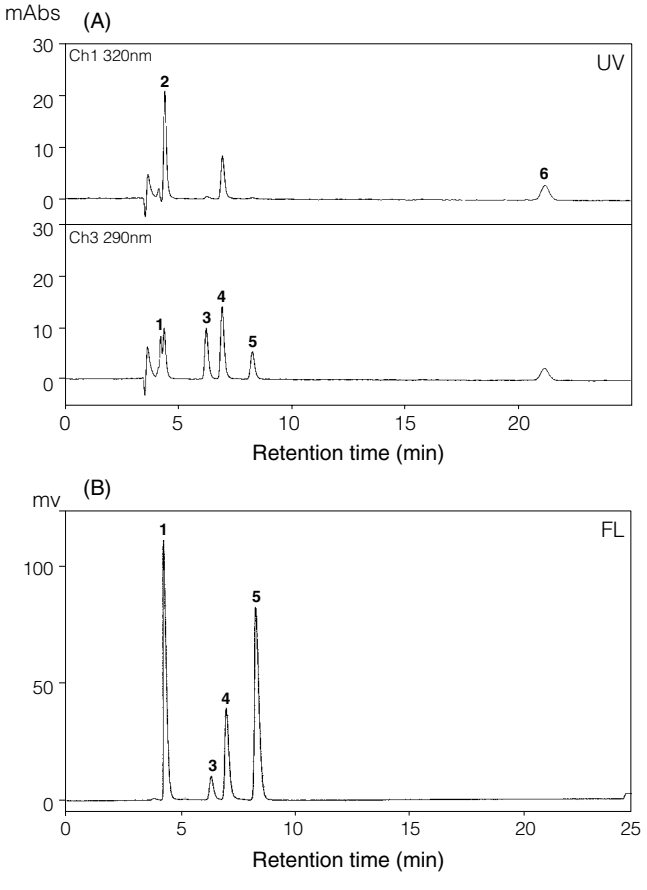

Figure 3. HPLC chromatograms of six benzimidazoles by (A) photodiode array detector and (B) fluorescence detector using isocratic elution.
HPLC conditions: Cosmosil 5C18-MS-II; mobile phase, acetonitrile: methanol: 0.02M NaH_2PO_4 (2:2:6, v/v/v); flow rate, 1.0 mL/min; injected amounts: 25 ng for each substance. Peak identification: 1, $ABZSO_2$-NH_2; 2, TBZ-OH; 3. ABZ-SO; 4. TBZ; 5. ABZ-SO_2; 6, MBZ.

II. *Sample Preparation*

(I) *Solvent extraction*

Polarity is the most important factor in choosing the extraction solvent. The closer the solvent polarity to the analyte polarity, the higher the extraction efficiency may be[24]. Acetonitrile[14,15,18,23] or ethyl acetate[12,18,22,23,28,29] is widely used as extraction solvent for the benzimidazoles in animal products. Ethyl acetate was chosen as the extraction solvent under alkaline condition because of its high solvating power for these weakly basic compounds[12,16,22,29]. Because the extraction of milk with ethyl acetate caused persistent emulsion, acetonitrile was used as an extracting solvent to eliminate this problem[18]. Kawasaki *et al.*[23] demonstrated that the suitable solvents for extraction were ethyl acetate for swine muscle and liver, and acetonitrile for bovine milk in the presence of a buffer (a mixture of saturated $NaHCO_3$ and Na_2CO_3) for the

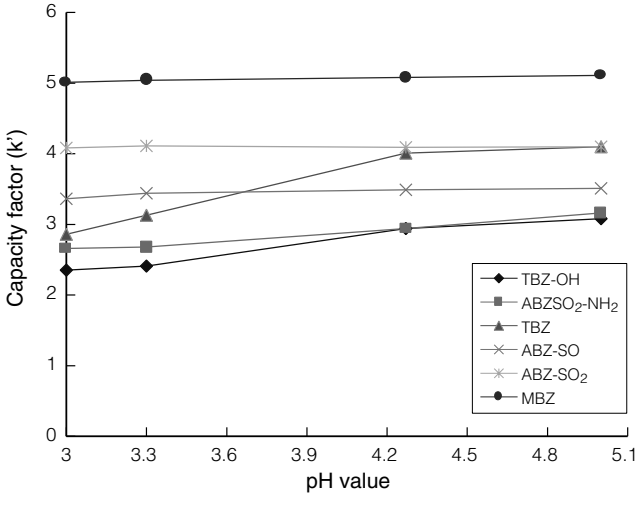

Figure 4. Effect of pH value in 0.02M sodium dihydrogen phosphate solution on the retention times of HPLC for six benzimidazoles.

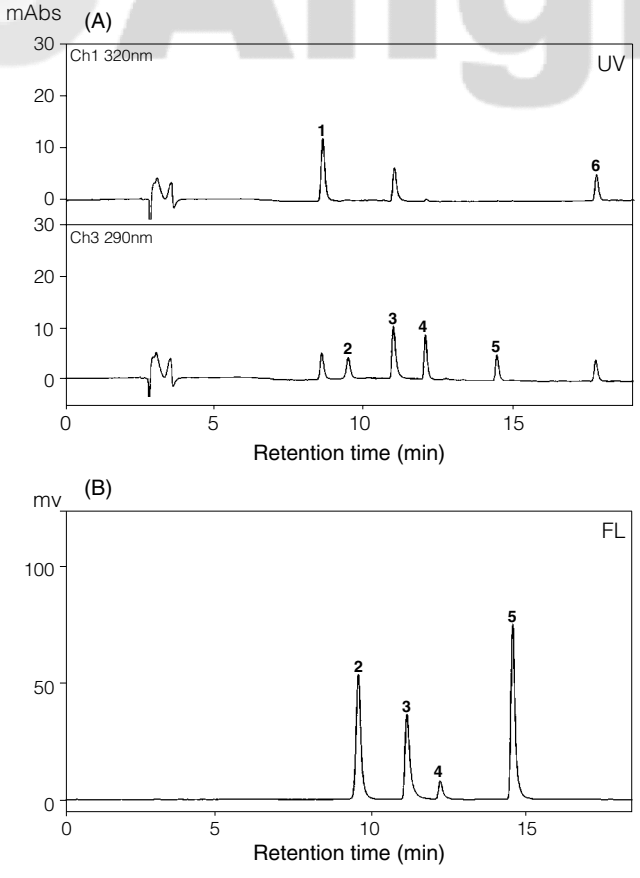

Figure 5. HPLC chromatograms of a mixed standard solution of six benzimidazoles detected by (A) photodiode array detector and (B) fluorescence detector.
HPLC conditions were described in the text. Injected amounts: 25 ng for each substance. Peak identification: 1, TBZ-OH; 2, $ABZSO_2$-NH_2; 3. TBZ; 4. ABZ-SO; 5. ABZ-SO_2; 6, MBZ.

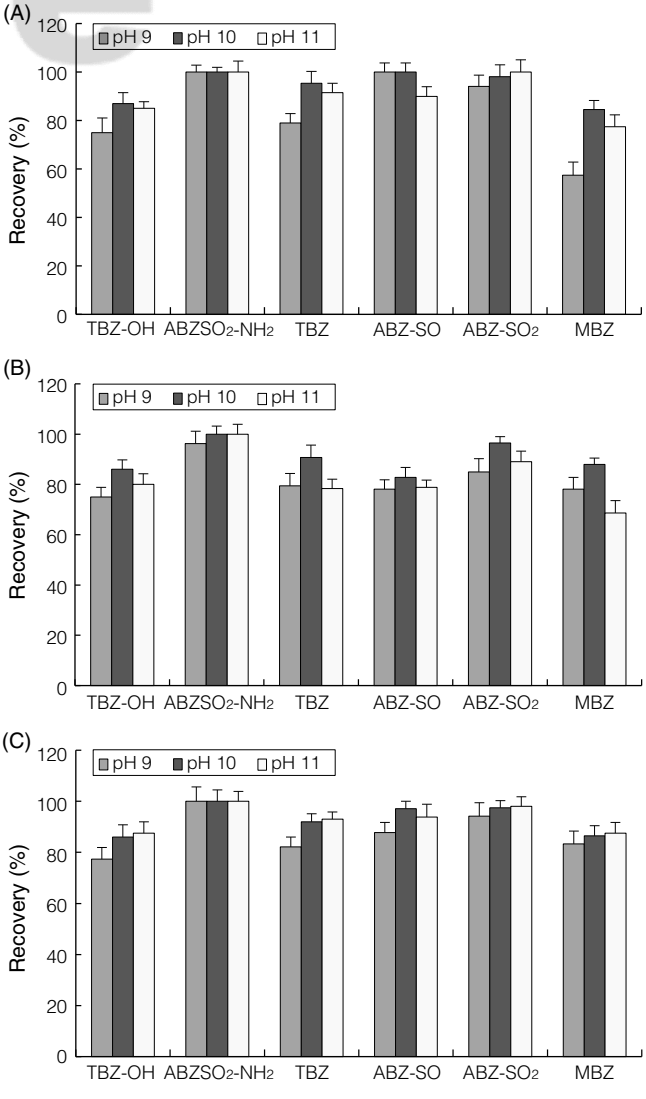

Figure 6. Recoveries of six benzimidazoles from spiked (0.1 ppm each) samples of (A) beef, (B) swine liver and (C) bovine milk by extraction with acetonitrile in the presence of buffer at various pH values.

salting-out effect. BHT solution was added to the ethyl acetate extracts of milk to increase the recovery of TBZ-OH[13].

Our preliminary study showed that extraction of milk with ethyl acetate caused persistent emulsion, and the recoveries of the benzimidazoles were low (40~50%). According to Kawasaki *et al.*[23], acetonitrile was selected as the extraction solvent in the presence of a buffer and BHT solution[13], and the suitable pH of the buffer was investigated. Recoveries of the six benzimidazoles by extracting with acetonitrile in the presence of buffer ranging from 9 to 11 are shown in Figure 6. The average recoveries (85.2~100.3%) of the benzimidazoles in beef, swine liver, and bovine milk samples were the highest at pH 10, while they were lower at pH 9 and pH 11. Thus, pH 10 was the optimum buffer pH for extraction.

(II) *Cleanup conditions*

The cleanup procedure for removing the impurity can be achieved by partitioning with n-hexane followed by passing the extract through a cartridge. A cleanup procedure by two cartridges has been described[1,15]. A cleanup method for the analysis of eight benzimidazoles (not include metabolites) using a C18 and a florisil cartridge has been proposed by Marti *et al.*[15]. For muscle samples, cleanup on the florisil cartridge may be omitted. Using a silica gel and a C18 cartridge for the analysis of five benzimidazoles has been reported by Commission of the European Communities[1].

Based on the cleanup treatment reported by Marti *et al.*[15], the dried acetonitrile extract was dissolved in acetonitrile, which was applied onto the Sep-Pak C18 cartridge, and the benzimidazoles were eluted from the cartridge with acetonitrile followed by HPLC analysis. Results showed that many impurities appeared on the chromatogram and interfered with the quantification of the benzimidazoles, therefore Marti *et al.* used a florisil cartridge for further cleanup. In this study, the dried acetonitrile extract was dissolved in 10% methanol, loaded onto a Sep-

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Pak C18 cartridge, washed with 10% methanol, eluted with acetonitrile, and analyzed by HPLC. It was found that the interference peaks were significantly reduced, therefore, no need to pass through a florisil cartridge for further cleanup. Acetonitrile[1,15], methanol[25], or ethyl acetate[29] was examined subsequently for elution. The extraction efficiency of acetonitrile was better than methanol and ethyl acetate (Figure 7). In addition, the volume of acetonitrile needed for elution was also tested, and the eluent was collected in fractions and analyzed by HPLC. Almost all benzimidazoles were eluted completely within 6 mL (Figure 8), thus 8 mL of acetonitrile was used for elution.

In the preliminary study, the peak shape of the benzomidazoles of HPLC chromatograms was affected by the components of the dissolution solution. Therefore, the solution suitable for dissolution of the dried residue of benzimidazoles obtained from cleanup procedure was investigated by mixing acetonitrile, methanol, and 0.02M sodium dihydrogen phosphate in various proportions. The results showed that the peak shape of all benzimidazoles was sharp and symmetric when the residue was dissolved in 1 mL of the solution consisted of acetonitrile: methanol: 0.02M sodium dihydrogen phosphate (2:2:6, v/v/v). However, the recoveries of TBZ-OH and MBZ were low (about 46% and 60%, respectively). When 2 mL of the solution was added instead of 1 mL to dried residue, the recoveries of both were better than 81.6%. It may be due to the very low solubility of TBZ-OH and MBZ[9].

III. *Standard Curves, Recovery and Limits of Detection*

Linear regression equations of the standard curves for the benzimidazoles in the range of 0.05~2.50 μg/mL are shown in Table 3. Satisfactory linearity with correlation coefficients greater than 0.999 was achieved.

Accuracy is generally expressed as the percent recovery of the analyte of interest[35]. Recovery test was performed in triplicate by spiking 0 (as blank), 0.02, 0.10, and 1.00 ppm of the six benzimidazoles to 5 g ground samples (pork, swine liver, beef and lamb) or 5 mL milk samples (bovine milk and goat milk). Recoveries were calculated based on the best detection conditions, that is, UV 320 nm for TBZ-OH and MBZ, and fluorescence detection for the rest. The results are shown in Table 4, and their HPLC chromatograms are shown in Figure 9 and 10. Average recoveries of low concentration (0.02 ppm) for TBZ-OH and MBZ ranged from 71.4 to 87.1% and those of the rest ranged from 81.6 to 104.3% with coefficients of

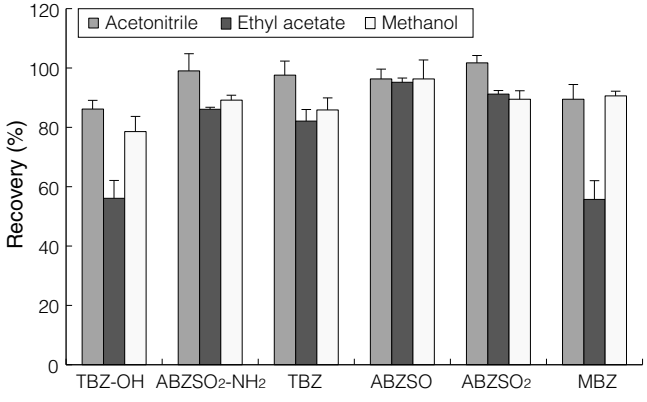

Figure 7. Comparison of the recoveries of benzimidazoles from Sep-Pak C18 cartridge eluted with various solvents.

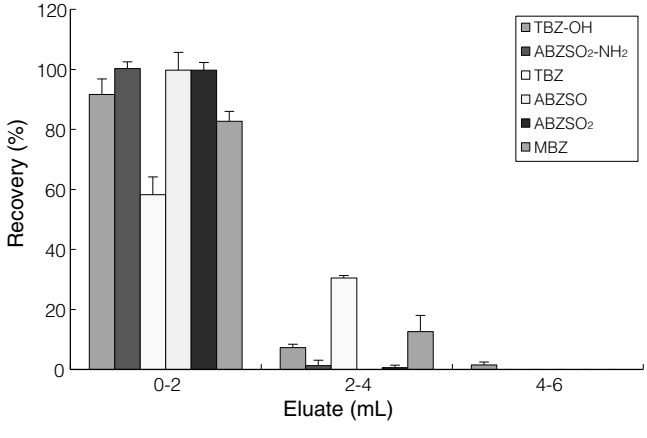

Figure 8. Distribution of the recoveries of benzimidazoles from the Sep-Pak C18 cartridge in the different fraction of eluate using acetonitrile as eluent.

Table 3. Linear regression equations and correlation coefficients of standard curves for benzimidazoles

Benzimidazole[a]	Detection	Linear equation[b]	Correlation coefficient
	Fluorescence		
$ABZSO_2$-NH_2	Ex 280/Em 310 nm	$Y = 1.94 \times 10^6 X + 182077$	0.9992
TBZ	Ex 290/Em 320 nm	$Y = 1.87 \times 10^6 X + 6815$	1.0000
ABZ-SO	Ex 290/Em 320 nm	$Y = 3.36 \times 10^5 X + 85$	0.9999
ABZ-SO_2	Ex 290/Em 320 nm	$Y = 2.56 \times 10^6 X + 197127$	0.9991
	Ultraviolet		
TBZ-OH	320 nm	$Y = 5.34 \times 10^5 X - 1743$	0.9999
$ABZSO_2$-NH_2	290 nm	$Y = 1.61 \times 10^5 X - 214$	0.9999
TBZ	290 nm	$Y = 4.90 \times 10^5 X - 614$	1.0000
ABZ-SO	290 nm	$Y = 3.20 \times 10^5 X - 698$	0.9999
ABZ-SO_2	290 nm	$Y = 1.94 \times 10^5 X - 1479$	0.9999
MBZ	320 nm	$Y = 2.30 \times 10^5 X - 2266$	1.0000

[a]The concentration ranges from 0.05 to 2.50 μg/mL.

[b]Y = AX + B, where Y is peak area, X is the concentration of the analyte.

Table 4. Recoveries[a] (%) of six benzimidazoles fortified into pork and swine liver at various spiked levels

Tissue source	Benzimidazole[b]	Spiked level		
		0.02 ppm	0.10 ppm	1.00 ppm
Pork				
	TBZ-OH	84.6 (3.3)[c]	88.2 (4.4)	84.8 (3.2)
	$ABZSO_2$-NH_2	83.8 (5.3)	92.4 (1.1)	99.9 (1.3)
	TBZ	84.5 (3.9)	90.9 (1.1)	87.9 (0.1)
	ABZ-SO	99.2 (4.5)	95.4 (5.0)	99.8 (1.1)
	ABZ-SO_2	89.6 (1.0)	93.6 (2.6)	91.8 (0.4)
	MBZ	71.4 (1.8)	85.2 (5.8)	85.6 (5.9)
Swine liver				
	TBZ-OH	78.3 (2.8)	86.2 (2.9)	87.0 (2.2)
	$ABZSO_2$-NH_2	91.4 (3.4)	99.0 (5.8)	94.5 (0.6)
	TBZ	91.7 (1.7)	97.6 (4.7)	90.4 (3.0)
	ABZ-SO	83.9 (1.8)	96.3 (3.3)	94.5 (1.0)
	ABZ-SO_2	98.3 (2.5)	99.9 (2.5)	96.2 (1.4)
	MBZ	83.4 (3.3)	89.5 (4.9)	85.6 (3.4)
Beef				
	TBZ-OH	74.9 (1.1)[c]	89.8 (2.0)	88.0 (2.7)
	$ABZSO_2$-NH_2	93.5 (5.2)	93.2 (1.1)	95.5 (1.9)
	TBZ	88.1 (0.9)	94.3 (3.6)	92.9 (0.5)
	ABZ-SO	99.8 (2.5)	95.2 (2.7)	95.4 (1.8)
	ABZ-SO_2	93.7 (2.6)	99.4 (1.0)	94.0 (1.8)
	MBZ	74.2 (5.1)	90.1 (4.7)	86.9 (2.1)
Lamb				
	TBZ-OH	77.8 (5.9)	81.6 (2.3)	91.6 (0.7)
	$ABZSO_2$-NH_2	98.4 (2.3)	87.6 (0.2)	102.5 (1.2)
	TBZ	87.7 (5.2)	86.5 (4.0)	92.9 (2.1)
	ABZ-SO	94.3 (3.5)	90.0 (1.0)	96.9 (1.0)
	ABZ-SO_2	94.9 (3.4)	87.8 (2.6)	93.3 (3.1)
	MBZ	85.8 (0.3)	85.1 (2.8)	83.1 (3.1)
Bovine milk				
	TBZ-OH	87.1 (2.0)[c]	86.8 (1.2)	95.4 (4.2)
	$ABZSO_2$-NH_2	91.0 (5.0)	94.3 (2.6)	104.3 (0.3)
	TBZc	90.3 (2.2)	89.1 (5.4)	90.4 (4.5)
	ABZ-SO	88.4 (2.3)	95.7 (2.2)	94.2 (4.5)
	ABZ-SO_2	88.9 (2.0)	94.6 (0.4)	92.6 (2.3)
	MBZ	86.5 (2.9)	87.0 (5.4)	85.1 (4.5)
Goat milk				
	TBZ-OH	84.6 (2.8)	87.4 (1.4)	91.5 (2.5)
	$ABZSO_2$-NH_2	94.2 (0.6)	92.5 (0.2)	101.1 (2.2)
	TBZ	94.1 (1.3)	93.3 (1.8)	91.7 (2.4)
	ABZ-SO	99.9 (3.8)	96.5 (1.8)	94.1 (0.9)
	ABZ-SO_2	97.5 (0.4)	91.9 (1.4)	93.0 (1.1)
	MBZ	86.7 (3.5)	83.7 (0.9)	86.3 (1.5)

[a]Average of triplicate.
[b]TBZ-OH and MBZ were determined by UV detection, and the rest was determined by fluorescence detection.
[c]Value in parenthesis is the coefficient of variation (CV, %).

variation less than 5.9%. 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 obtained 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%[35]. Even though the average recoveries of 0.02 ppm for MBZ in pork and beef samples and for TBZ-OH in beef and lamb samples were in the low range of 71.4 to 77.8%, they were still in compliance with the criterion of the Codex. The detection limits with UV detection were between 0.005 ppm (TBZ-OH) and 0.030 ppm (ABZ-SO_2), while the detection limits with fluorescence detection were between 0.004 ppm (ABZ-SO_2) and 0.020 ppm (ABZ-SO) (Table 5). All values are lower than the residue limits of veterinary drugs set by the Department of Health[11].

IV. *Intra-day and Inter-day Repeatability*

Three concentrations of mixed standard solutions of the six benzimidazoles were used for analyzing the intra-day and inter-day repeatability. Each concentration was

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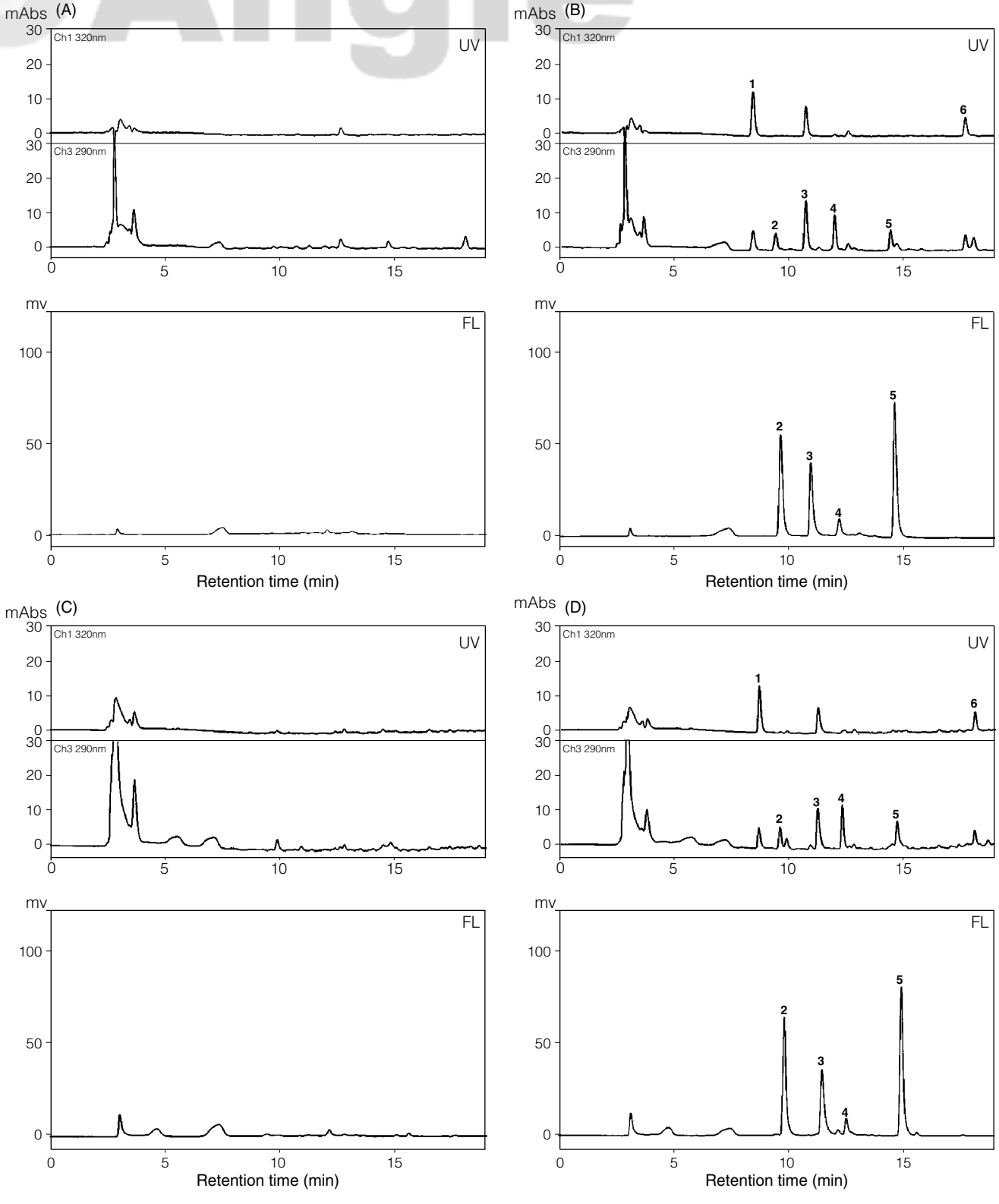

Figure 9. HPLC chromatograms of six benzimidazoles spiked into samples each at 0.1 ppm. (A) Blank pork, (B) spiked pork, (C) blank swine liver, (D) spiked swine liver. The performance conditions and peak identification are the same as Figure 5.

analyzed three times for intra-day repeatability. For inter-day repeatability, each concentration was analyzed three times for three days using daily prepared standard solutions and mobile phase, that is, each concentration was analyzed for nine times. The coefficients of variation of intra-day and inter-day assays were lower than 1.92% and 7.22%, respectively (Table 6). These results indicated that the developed method had an acceptable precision.

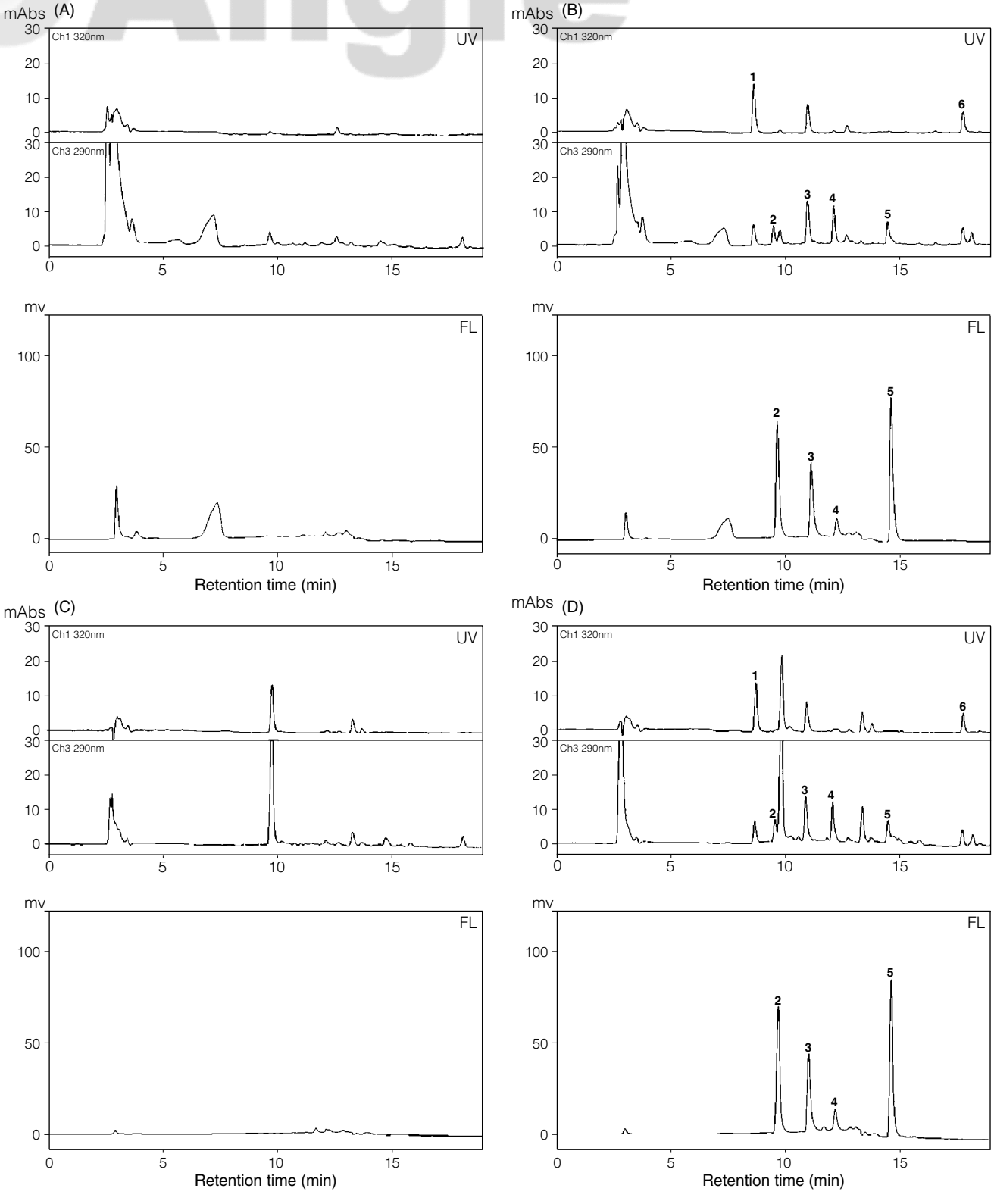

Figure 10. HPLC chromatograms of 6 benzimidazoles spiked into samples each at 0.1 ppm. (A) Blank beef, (B) spiked beef, (C) blank bovine milk, (D) spiked bovine milk. The performance conditions and peak identification are the same as Figure 5.

V. *Comparison of the Established Method in this Study and in Published Research*

Previous works[17,21] have developed a matrix solid phase dispersion technique for extraction and cleanup. Such procedure saves time and manipulation, but lacks the sensitivity required to monitor benzimidazoles residues at concentrations close to the maximum residue limits suggested

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Table 5. Detection limits of UV and fluorescence detection method of six benzimidazoles

Benzimidazole	UV		Programmable fluorescence		UV/FL ratio
	Wavelength (nm)	Detection limit (ppm)	Ex/Em (nm)	Detection limit (ppm)	
TBZ-OH	320	0.005	280/310	N.D.[a]	—
$ABZSO_2$-NH_2	290	0.020	280/310	0.005	4
TBZ	290	0.020	290/320	0.005	4
ABZ-SO	290	0.020	290/320	0.020	1
ABZ-SO_2	290	0.030	290/320	0.004	7.5
MBZ	320	0.010	290/320	N.D.	—

[a]Not detected.

by regulatory agencies[11,12]. Fletouris *et al.*[12] developed a liquid-liquid partition procedure, in which the extraction solution was partitioned with water and then analyzed by HPLC without passing through a cartridge for further cleanup. This method resulted in many interferences peaks in the beginning of HPLC chromatograms. Therefore, it is not suitable for a quantification purpose. Commission of the European Communities[1] and Marti *et al.*[15] used a two-cartridge for sample cleanup. This method is time-consuming and offers less recoveries for TBZ[1,15] and MBZ[15]. LeVan and Barnes[16] described a collaborative study of an HPLC method for the determination of five benzimidazoles. Tissues were made basic with Na_2CO_3, and homogenized in ethyl acetate, followed by a liquid-liquid partition and silica cartridge cleanup. With the exception of fenbendazole and MBZ in liver, the study data were not satisfactory. The method developed in this study used acetonitrile as the extraction solvent under alkaline condition, n-hexane for removing lipid as well as other co-extracts, and a Sep-Pak C18 cartridge for sample cleanup prior to HPLC-PDA and HPLC-fluorescence analysis. This method is simple in comparison with the method of Marti *et al.*[15], since a cartridge wash step was taken to reduce the interference peaks, without another florisil cartridge for further cleanup. The recoveries for all of the benzimidazoles tested were greater than 80% except for TBZ-OH and MBZ at low concentration (0.02 ppm).

VI. *Investigation of Benzimidazoles in Commercial Livestock Products*

This method was used to detect the benzimidazoles in five samples of pork, swine liver, beef, lamb, bovine milk, and goat milk (thirty samples in total) purchased from various markets in Taipei. The results showed that no benzimidazole residues were detected.

CONCLUSIONS

A method using HPLC with a PDA and fluorescence detector to simultaneously determine six benzimidazoles in livestock was developed. This method is easy to operate and capable of removing most of interferences after cleanup with a Sep-Pak C18 cartridge. All benzimidazoles were well separated with satisfactory peak resolution, sharpness, symmetry and repeatability. The average recoveries for all benzimidazoles were greater than 80% except for TBZ-OH and MBZ at low concentration (0.02 ppm). The detection limits of benzimidazoles were 0.005~0.030 ppm with UV detection and 0.004~0.020 ppm with florescence detection. PDA detector can be used to further confirm the compounds of interest by comparing the spectra of analyte with those of standards. The method developed is suggested to be a routine method for the analysis of the six benzimidazoles in livestock products.

Table 6. Intra-day and inter-day repeatability of six benzimidazoles

Benzimidazole	Concentration (μg/mL)	Inter-day (CV, %) (n=3)	Intra-day (CV, %)(n=9)
TBZ-OH	0.1	1.55	4.07
	0.5	1.01	2.53
	5.0	1.21	6.94
$ABZSO_2$-NH_2	0.1	0.60	2.76
	0.5	0.46	1.87
	5.0	0.61	4.55
TBZ	0.1	0.39	5.90
	0.5	1.16	6.86
	5.0	0.80	5.30
ABZ-SO	0.1	1.92	3.72
	0.5	0.19	1.69
	5.0	1.09	2.71
ABZ-SO_2	0.1	0.95	1.01
	0.5	0.41	1.29
	5.0	0.25	1.84
MBZ	0.1	0.94	4.28
	0.5	1.59	7.22
	5.0	1.65	1.88

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