

Determination of Coccidiostats (Amprolium and Decoquinatate) in Cattle and Chicken's Muscle using High Performance Liquid Chromatography

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An analytical method for the simultaneous determination of veterinary medicines (amprolium and decoquinatate) in cattle and chicken's muscle by HPLC/UV-vis was established. Samples were extracted by a HLB (Hydrophilic-Liphophilic Balance) cartridge with acetonitrile and methanol. Prior to HPLC injection, a mixture solvent (Water:MeOH, 1:1) was utilized as a reconstitution solvent. Chromatographic separation was achieved with a C18 column (250 × 4.6 mm, 5 μm) using gradient elution with 20 mM HFBA and MeOH:ACN (1:1.8). The calibration curves from the spiked blank matrix showed good linearity (above $r^2=0.997$) in the concentration range of 0.13-12.0 mg kg⁻¹. The relative recovery (accuracy) and limit of quantitation (LOQ) were in the range of 78.5-107.1% and 0.13-0.42 mg kg⁻¹, respectively. The developed method can be used to determine under the MRL (Maximum Residue Limits) levels of veterinary medicines in animal tissues.

Key Words : Coccidiostats, Amprolium, Decoquinatate, HPLC, Simultaneous determination

Introduction

Coccidiosis is an infectious disease caused by several species of *Eimeria* protozoa, which cause intestinal cell disruption, resulting in weight loss or poor weight gain.¹ Therefore, attention has been paid to the prevention and treatment of this disease, especially in large intensive farms where economical losses are the most substantial. Coccidiostats, such as amprolium and decoquinatate, are registered as veterinary drugs for the treatment of the clinical form of coccidiosis.² Amprolium and decoquinatate stop the growth of new protozoa and kills them as well.³ These coccidiostats are an anticoccidial feed additive approved for use in feeds for broiler chickens, cattle and calves, sheep, and goats, and they are approved for use in the prevention of coccidiosis in ruminating and nonruminating calves, including veal calves and cattle, caused by *Eimeria* protozoa.⁴

However, continuous administration of coccidiostats often leads to the accumulation of veterinary drug residues in food products for human consumption.^{5,6} The presence of residue of veterinary drugs has received much attention because of the growing concern for safety by consumers. The Korea Food and Drug Administration established maximum residue limits (MRLs) in chicken and cattle's muscle for the amprolium (0.5 mg/kg) and decoquinatate (2.0 mg/kg).

Therefore, a sensitive and selective analytical methodology is required to detect such low concentrations of these compounds in cow and chicken matrices. Several methods based on fluorescence or UV-detection with post-column derivatization have been reported for analysis of the drugs. Most of the methods available for determining amprolium are based on ion-pair liquid chromatography, mainly using heptafluorobutyric acid and ultraviolet detection.⁷⁻¹⁰ And the recent published methods for the determination of residue

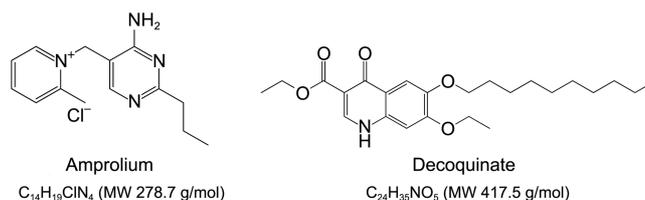


Figure 1. Chemical structures of amprolium and decoquinatate.

levels of coccidiostats employ the LC-MS/MS technique, because using mass spectrometry for detection allows better sensitivity and covers a wider range of analytes. However, at the same time, conventional HPLC has advantages, such as fitness for the intended application, low cost, and suitability for routine laboratories not equipped with sophisticated instruments. Notably, because of the different physico-chemical properties (Fig. 1), the opposite solubility of amprolium and decoquinatate in water and organic solvents, such as methanol and acetonitrile, enabled the determination of a single class of compounds or only single analytes.

Thus, a sensitive and reliable simultaneous HPLC method for amprolium and decoquinatate determination in animal muscle has not been described yet. Therefore, this study was designed to develop and validate an analytical technique and sample preparation using SPE for the simultaneous analysis of residual amprolium and decoquinatate in chicken and cattle's muscle. Additionally, the effective separation technique of thiamine, which is an endogenous interference for amprolium analysis in animal tissue, is described.

Experimental

Reagents, Materials, and Instruments. Amprolium and decoquinatate were prepared with high purity reagents pur-

chased from Sigma-Aldrich (St Louis, MO, USA). Organic solvents, such as methanol, acetonitrile, chloroform, and acetone, were obtained from J. T. Baker (Phillipsburg, NJ, USA) and those reagents were HPLC grade. Heptafluorobutyric acid (HFBA) that obtained from Sigma-Aldrich (St Louis, MO, USA) was of a high purity reagent. Amprolium and decoquinatate working standard solutions were prepared by dissolving them in distilled water and chloroform ($1000 \mu\text{g mL}^{-1}$ and $200 \mu\text{g mL}^{-1}$, respectively), respectively, and they were stored in a refrigerator (-20°C). Before use, these working standard solutions were intentionally diluted with water and methanol.

The glassware used for this experiment was washed by washing solvent, methanol, and a mixture of acetone and distilled water, and then dried. Polypropylene conical tubes were obtained from Falcon (Austin, Texas, USA). The supra 22 K centrifuge was obtained from Han-Il Scientific Co. Ltd. (Seoul, Korea) and centrifuge tube (50 mL) was obtained from Nalgene (New York, USA). GF/B glass filter paper was obtained from Whatman (Maidstone, UK), and syringe disc filter ($0.45 \mu\text{m}$ and 13 mm) was obtained from Life Sciences (Watford, UK). The SPE cartridge for the extraction of the analyte was an Oasis HLB (Hydrophilic-Lipophilic Balance, 200 mg, 6 cc) which was purchased from Waters (Milford, Massachusetts, USA) and the vacuum manifold was obtained from Supelco (Bellefonte, PA, USA). The concentrator was a TurboVap LV nitrogen concentrator which was obtained from Caliper Lifescience (Seattle, WA, USA), and the vortex mixer was obtained from Vision Scientific (Bucheon, Korea).

An Agilent 1100 series HPLC system (Palo Alto, CA, USA) equipped with a binary pump was connected to an Agilent G1313A autosampler and G1313A UV-Vis detector. The analytical column was a reverse-phase CAPCELL PAK C18 column ($250 \text{ mm} \times 4.6 \text{ mm}$, $5 \mu\text{m}$ particle size). Mobile phase A (20 mM HFBA in water) and phase B (methanol: acetonitrile = 1:1.8) were used with a flow rate of 1.0 mL/min. The separation was achieved with the following linear gradient system: at 0 min A:B=55:45 (v/v), 5 min A:B=55:45, 9 min A:B=5:95, 18 min A:B=5:95, and 18.5 min A:B=5:95, A 10 min post-time allowed for re-equilibration of the column. The injection volume was $20 \mu\text{L}$. Analytes were detected with the DAD set at 262 nm.

Sample Preparation. Samples, 10 g of cattle or chicken's muscle, were prepared in Polypropylene conical tubes (50 mL) and then homogenized using a mechanical homogenizer. When the solutes (amprolium and decoquinatate) were spiked, the $1000 \mu\text{g kg}^{-1}$ of standard solution (in water and chloroform) was spiked in this step. To extract the analyte, 10 mL of acetonitrile was added to each homogenized sample and was shaken for 30 min at 250 rpm. Then, centrifugal separation was used for 15 min at 3500 rpm in order to collect the upper layer. The supernatant was mixed with 1 mL of distilled water. The mixture solvent was vaporized down to 1 mL by using a nitrogen evaporator at 60°C and then 4 mL of distilled water was added. An Oasis HLB (200 mg, 6 cc) cartridge was connected to a vacuum manifold and

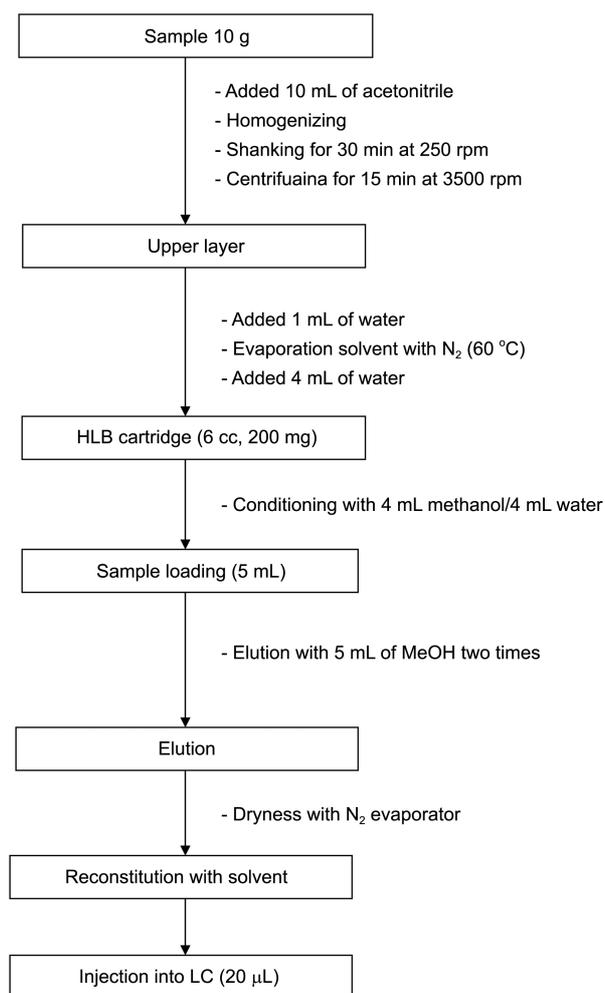


Figure 2. Schematic flow diagram of the sample preparation for the analysis of amprolium and decoquinatate from chicken and cattle's muscle.

conditioned by a solution of 4 mL methanol and 4 mL distilled water and then 5 mL of the sample was loaded into the cartridge. The sample was eluted with 5 mL methanol (two times) and the eluent was completely vaporized by nitrogen evaporator at 60°C . The residue was dissolved with $200 \mu\text{L}$ of solvent and then filtrated through a disc filter of $0.45 \mu\text{m}$ to be analyzed by LC/UV-vis (Fig. 2).

Results and Discussion

Chromatographic Separation. It is difficult to retain amprolium and decoquinatate, which have different physico-chemical properties, in reversed-phase mode simultaneously. Therefore, a chromatographic mode based on the ion pair mode was performed. Heptafluorobutyric acid was used as an ion-pair reagent to facilitate the retention of both drugs in the reversed-phase mode.

After mixed standard solution was injected into HPLC system, chromatogram was obtained. As shown in Figure 3, amprolium was eluted within the first 6.93 min, and decoquinatate was detected at 16.42 min, so the analytes were effectively separated simultaneously using the reversed-phase

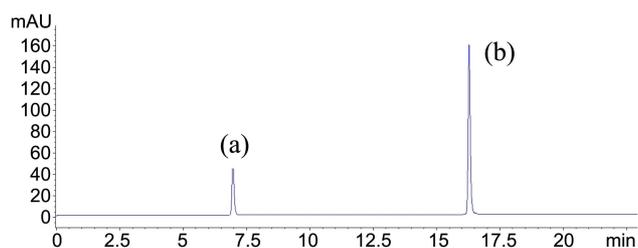


Figure 3. HPLC chromatogram of (a) amprolium and (b) decoquinatate standard mixture.

liquid chromatographic system without any derivatization procedure. Concentration of sample in Figure 3 was 1 mg kg^{-1} and chromatographic conditions are described in the experimental section.

Optimization of Volume of Extraction Solvent and Shaking Time. At first, the effects of the solvent volume and shaking time were investigated. The choice of extraction solvent volume and shaking time were performed by extracting a spiked sample. From the previous experiences and SPE compatibility, acetonitrile was chosen as an extraction solvent. Because of the distribution of analyte between the solvent and sample, the volume of the extraction solvent and shaking time utilized to extract analytes from samples affect the extraction yield. The volume of the solvent (shaking time was 30 min and acetonitrile was used as a solvent) was varied among 5, 10, 15, and 20 mL and the shaking time varied among 10, 20, 30, and 40 min. It was found that the recovery of the amprolium was affected little by the solvent volume, but decoquinatate's recovery increased up to 86.4% when 10 mL was used, and the recovery was not changed above 10 mL (Fig. 4). As the shaking time (at 10 mL of

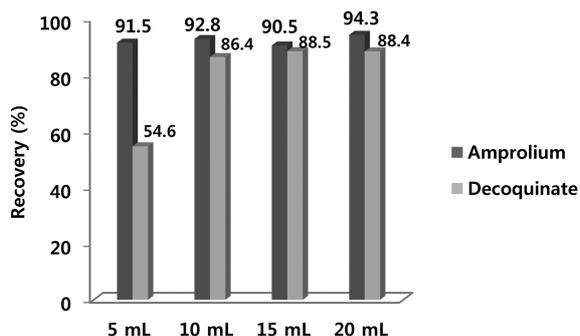


Figure 4. Effect of extraction solvent volume.

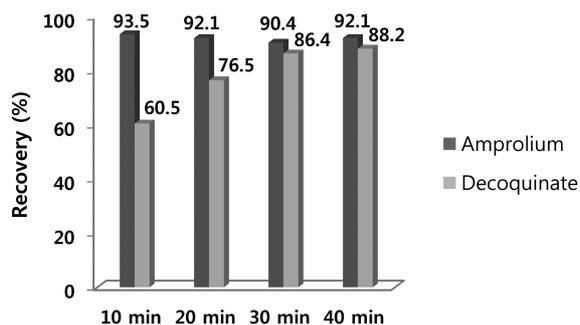


Figure 5. Effect of shaking time of amprolium and decoquinatate.

solvent volume) increased, the amprolium recovery was not changed, but the extraction yield of decoquinatate increased (Fig. 5). These could be explained by the differences of the solubility and distribution for the solvent. Because decoquinatate has a hydrophobic nature, it was difficult to extract from muscle and required more solvent. Consequently, 10 mL of extraction solvent volume and 30 minute of shaking time were chosen as optimized parameters.

Optimization of Elution Solvent Volume. Generally, the extracted solvent from a sample contains matrix components, causing interference in recovery and chromatographic separation. Methanol was chosen as a satisfactory solvent for the elution and cleanup of the loaded sample on an Oasis HLB cartridge. The volume of elution solvent was tested to show its efficiency in the elution of amprolium and decoquinatate from muscle samples. The criteria for a successful method are better recovery. The good recovery of analytes from exhausted extract, depending on the solubility of the targeted compound in the solvent, was used to evaluate the extractive process. Data from Figure 6 shows that the recovery of amprolium and decoquinatate were 77.4% and 42.5% in 3 mL of elution solvent, respectively. The recoveries increased as a function of the volume of elution solvent, but recovery of both drugs from 10 mL to 15 mL shows only a small increment (93.4% to 94.1% and 88.3% to 90.2%), and the 10 mL solvent run shows better precision than the 15 mL run. And then 10 mL of the elution solvent was selected as a criterion for further experimentation.

Optimization of Reconstitution Solvent and pH. After extraction and cleanup, the dried residue should be re-

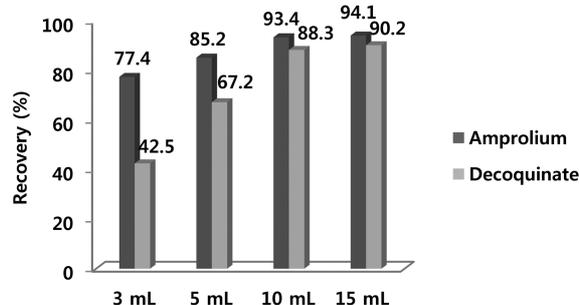


Figure 6. Effect of volume of elution solvent.

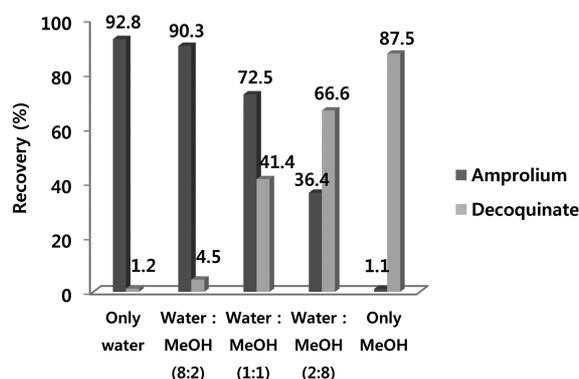


Figure 7. Effect of reconstitution solvent.

constituted with a suitable solvent prior to injection into the LC system. The reconstitution solvent affects the recovery from the HPLC column and consequently shows the results of the overall recovery. Amprolium is very soluble in water and slightly soluble in methanol, but decoquinatate is the opposite (Fig. 7). From the results, amprolium exhibited the highest recovery in water and the lowest recovery in methanol, and in contrast decoquinatate exhibited the opposite results. This may be explained by the fact that the solubility of analytes in the reconstitution solvent affects the behavior of amprolium and decoquinatate in the HPLC chromatographic system. The equal mixed solution of methanol and water (1:1) exhibited appropriate recoveries for both drugs (72.5% for amprolium and 41.4% for decoquinatate). Despite the losses, this solvent mixture was chosen for the simultaneous determination testing (sample preparation and instrumental analysis). The recoveries were sufficient to detect up to the maximum residual limits (MRLs) levels from the chicken and cattle's muscle. Although the mixture solvent (MeOH:water = 1:1) may be used as a reconstitution solvent for screening, water and methanol are appropriate for the effective quantification of amprolium and decoquinatate, respectively.

Also, the effect of pH was investigated, but the behavior of both drugs was nearly independent of pH the of the reconstitution solvent (Fig. 8).

Effective Separation of Amprolium and Thiamine. Thiamine or thiamin, also known as vitamin B1, has a similar chemical structure with amprolium and plays an important role in helping the body metabolize carbohydrates and fat to produce energy. Thiamine is concentrated in muscle tissue and it may interfere with the analysis of amprolium from cattle and chicken's muscle. Thiamine could not be removed during the extraction and purification process and was eluted closely in the HPLC analysis.¹¹ For the effective separation from amprolium in chromatography, several organic phases were investigated and the MeOH:ACN (1:1.8) mixture yielded a well-resolved chromatogram (Fig. 9).

Method Validation. Sample preparation was performed for amprolium and decoquinatate by an established solid phase extraction. Limits of Detection (LOD) and Limits of Quantification (LOQ) for spiked blank muscle were measured based on the LC/UV-vis analysis.

The measurement method for LOD and LOQ are dis-

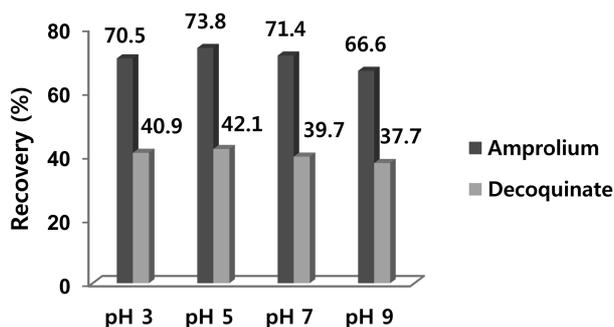


Figure 8. Effect of reconstitution solvent pH.

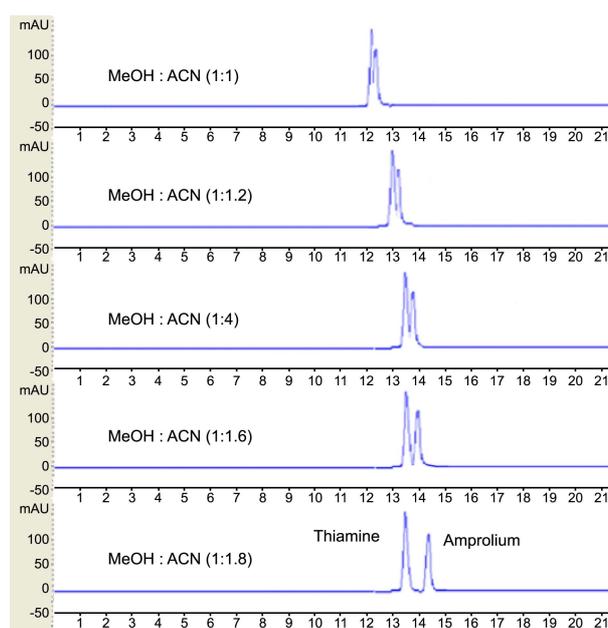


Figure 9. HPLC chromatograms for separation of amprolium and thiamine.

cussed below. The LOD was assumed from the instrumental analysis process and the sample ($n=7$) was prepared at a concentration within 1-5 times of the LOD. After the standard deviation (σ) was calculated using the results from 7 replicate experiments and the slope (m) of the linear calibration curve was calculated, the LOD and LOQ were set at $3\sigma/m$ and $10\sigma/m$, respectively. As a result, the LOD and LOQ of amprolium in cattle and chicken's muscle were in the concentration range of $0.04\text{--}0.05\text{ mg kg}^{-1}$ and $0.13\text{--}0.18\text{ mg kg}^{-1}$, respectively. The LOD and LOQ of decoquinatate in cattle and chicken's muscle were in the concentration range of $0.12\text{--}0.13\text{ mg kg}^{-1}$ and $0.38\text{--}0.42\text{ mg kg}^{-1}$ respectively. The LOQs were above the MRL in chicken and cattle's muscle.

The survey was performed in the concentration level of $\text{LOQ} \sim 6$ times of MRLs. The accuracy and precision (as a RSD%) of amprolium in the concentration range of $0.13\text{--}3.0\text{ mg kg}^{-1}$ from the spiked muscles was 96.6-102.8% and 2.2-7.9%, respectively. The accuracy and precision (as a RSD%) of decoquinatate in the concentration range of $0.38\text{--}12.0\text{ mg kg}^{-1}$ from the spiked muscles was 78.5-107.1% and 4.7-10.9%, respectively. The results were well within the recommended acceptable values of -30% to $+20\%$ at each

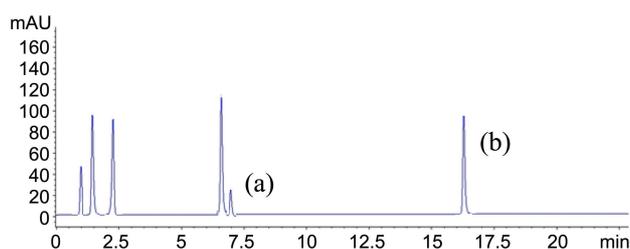


Figure 10. HPLC chromatogram of (a) amprolium and (b) decoquinatate in real sample.

Table 1. LODs, LOQs, accuracy, and precision for the amprolium and decoquinatate

Compound	Matrix	LODs ^a (mg/kg)	LOQs ^b (mg/kg)	Conc. (mg/kg)	Accuracy ^c (%)	Precision (%) (n=5)
Amprolium	Cattle	0.04	0.13	0.13	99.7	3.8
				0.25	101.8	3.1
				0.5	98.8	3.5
				1.0	101.9	3.1
				2.0	98.3	3.8
	Chicken	0.05	0.18	0.18	90.0	7.9
				0.25	96.6	5.4
				0.5	102.8	7.1
				1.0	101.1	7.0
				2.0	100.8	2.2
Decoquinatate	Cattle	0.12	0.38	0.38	78.5	4.7
				1.0	106.0	9.9
				2.0	98.6	8.8
				4.0	107.1	8.2
				8.0	94.7	5.7
	Chicken	0.13	0.42	0.42	98.6	10.5
				1.0	94.5	9.4
				2.0	101.9	8.9
				4.0	101.5	8.3
				8.0	102.0	6.1
			12.0	98.8	7.6	

^aLODs (Limits of Detection): $3 \sigma/m$. ^bLOQs (Limits of Quantitation): $20\% < RSD\%$ and $10 \sigma/m$. ^cAccuracy: (Measured value/Calculated value) $\times 100$

Table 2. Working range, linear equation, and R² for amprolium and decoquinatate

Compounds	Matrix	Working range (mg/kg)	Linear equation	R ²
Amprolium	Cattle	0.13-3.0	$y = 413.2126x - 15.8344$	0.9997
	Chicken	0.18-3.0	$y = 376.8236x + 14.1688$	0.9998
Decoquinatate	Cattle	0.38-12.0	$y = 444.9281x + 39.1446$	0.9970
	Chicken	0.42-12.0	$y = 602.2585x + 27.9470$	0.9993

concentration level (Table 1).

For analyzing the residual analytes in samples, the samples were spiked with standard solutions of 0.13 (or

0.18, in chicken), 0.25, 0.5, 1.0, 2.0 and 3.0 mg kg⁻¹ for amprolium, 0.38 (or 0.42 in chicken), 1.0, 2.0, 4.0, 8.0 and 12.0 mg kg⁻¹ for decoquinatate and then the analytes were extracted by the established analytical condition in order to be analyzed by LC/UV-vis. The calibration curves were obtained from the external standard method and the corresponding correlation coefficients for both drugs and matrices were reasonable because they were above $r^2=0.997$ (Table 2).

Conclusion

A sensitive, reliable, and reproducible simultaneous analytical method has allowed the detection of amprolium and decoquinatate in cattle and chicken's muscle using HPLC/UV-vis.

In cattle and chicken, the LOD and LOQ of amprolium were in the concentration range of 0.04-0.05 mg kg⁻¹ and 0.13-0.18 mg kg⁻¹, respectively, and the intraday precision and accuracy for the spiked samples were 2.2-7.9 RSD% and 90.0-102.8%, respectively. The LOD and LOQ of decoquinatate from cattle and chicken samples were 0.12-0.13 mg kg⁻¹ and 0.38-0.42 mg kg⁻¹, respectively. The established method satisfied the maximum residue limits (MRLs) of the Korean Food Code and it has been applied to the analysis of the veterinary drugs by government and private laboratories.

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