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Detection of Residues of the Coccidiostat Diclazuril in Poultry Tissues by Liquid Chromatography−Tandem Mass Spectrometry after Withdrawal of Medicated Feed

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A liquid chromatography−tandem mass spectrometric (LC-MS/MS) method for the quantitative determination of diclazuril in poultry tissues and feed is presented. A simple clean up with an organic solvent was carried out. A reversed-phase C_{18} column was used for the high-performance liquid chromatography (HPLC) to separate the analyte with a gradient of acetonitrile and water as mobile phase. The precursor ions produced by electrospray negative ionization were selected for collisional dissociation. Validation of the methods was performed based on Commission Decision 2002/657/EC (*Off. J. Eur. Communities* **2002**, *L221*, 8−36). For the detection of diclazuril in poultry meat, the decision limit was found to be 0.5 μg/kg. An animal experiment was set up in which 70 chickens were held for 6 weeks. From day 22 until day 32, they were fed feed containing 730 μg/kg diclazuril. From day 33 until day 42, every day six chickens were slaughtered, and breast, thigh, and liver were analyzed. Average steady-state concentrations of 94, 135, and 722 μg/kg in breast, thigh, and liver were obtained, respectively. Nine days after withdrawal of the medicated feed, diclazuril was still present in the different sample types.

KEYWORDS: Coccidiostat; diclazuril; residues; LC-MS/MS

INTRODUCTION

Diclazuril (4-chlorophenyl [2,6-dichloro-4-(4,5-dihydro-3H-3,5-dioxo-1,2,4-triazin-2-yl)phenyl] acetonitrile) is a benzeneacetonitrile broad-spectrum anticoccidial (Janssen Research compound R 64433) with proven efficacy against intestinal *Eimeria* species in avian coccidiosis (*2*), intestinal and hepatic coccidiosis in rabbits (*3*), and toxoplasmosis in mice (*4*). It is commercially available as an oral suspension for ovine applications and as a premix for inclusion in poultry feed. Avian coccidiosis is a contagious amoebic disease carried by unicellular organisms belonging to the genus *Eimeria* (*Eimeria acervulina*, *E. maxima*, *E. necatrix*, *E. brunetti*, and *E. tenella)* in the class Sporozoa and is the most important parasitic disease of poultry (*5*). To treat and prevent avian coccidiosis, diclazuril is marketed under the trade name Clinacox and is commercially available as a 0.5% premix for inclusion in poultry feed at a concentration of 1 mg/kg. The withdrawal time is 5 days. The molecular structure of diclazuril is presented in **Figure 1**.

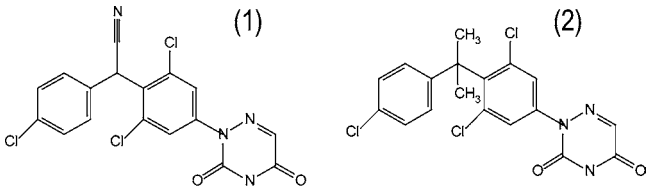

Figure 1. Molecular structure of diclazuril and the internal standard.

According to Regulation 1831/2003/EC, coccidiostats are at the moment licensed as feed additives, which puts them in an unclear position with regard to residue legislation (*6*). European regulations concerning residues of coccidiostats are at the moment difficult to interpret due to the absence of maximum residue levels (MRLs). They are under evaluation by the European Food Safety Authority (EFSA). However, Codex Alimentarius has set the following MRLs for poultry: 500 μg/kg for muscle, 1000 μg/kg for fat/skin, 2000 μg/kg for kidney, and 3000 μg/kg for liver (*7*).

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To our knowledge, no liquid chromatography−tandem mass spectrometric (LC-MS/MS) methods for the detection of diclazuril in poultry meat, liver, or feed have been published. De Kock et al. described a liquid chromatographic method with UV detection for the detection of diclazuril in feed suitable for concentrations ranging from 100 to 150 μg/kg (*8*). A gas chromatographic mass spectrometric method for the detection of diclazuril in avian feed and premixes with a detection limit of 20 μg/kg was described by Blanchflower et al. (*9*). Dirikolu et al. published a liquid chromatographic method with a diode array detector for the detection in horse plasma (*10*). They obtained a limit of detection of 10 ng/mL. Croubels et al. described a LC-MS/MS method for the detection of diclazuril in animal plasma with a limit of quantification of 1 ng/mL (*11*).

We developed a LC-MS/MS method for the detection of diclazuril in poultry feed, meat, and liver since tandem mass spectrometry offers the possibility to detect residues in a very specific way and at very low levels. Our laboratory already developed and validated a LC-MS/MS method for the simultaneous detection of five coccidiostats, including diclazuril, in eggs (*12*). For the validation of the method in meat, blank tissue was needed. Since we assumed that home bred chicken meat certainly would be free of residues, this was used as blank, but analyzing this meat revealed that it contained 60 μg/kg diclazuril. After further research, it appeared that the private breeder had used feed containing 1 mg/kg diclazuril and had not respected the withdrawal time of 5 days, that is, only 3 days before slaughter, coccidiostat-free feed was given. This made us decide to set up an animal experiment to further investigate the effect of not respecting the withdrawal time.

MATERIALS AND METHODS

Reagents and Standards. Diclazuril and internal standard R062646 (**Figure 1**), a structural analogue of diclazuril, were gifts from Janssen Animal Health (Beerse, Belgium). According to the certificate of analysis, a total of 0.10% impurities and 1.6% achiral impurities were measured by LC for diclazuril and the internal standard, respectively. Acetonitrile and methanol (gradient grade Lichrosolv), formic acid (98−100%), dimethylformamide (pro analysi), and anhydrous sodium sulfate (pro analysi) were from Merck (Darmstadt, Germany). Water was HPLC grade (generated by an ELGA purification system). Filters for filtration of the extract were from Millipore (Millex GV, 0.22 μm).

Standard stock solutions of 1 mg/mL diclazuril and the internal standard were prepared in dimethylformamide and stored refrigerated at 4 °C. The diclazuril standard stock solution is stable for at least 8 months when stored at 4 °C. Working standard solutions of 100, 10, 1, and 0.1 ng/μL were prepared daily by diluting the stock solutions in a mixture of acetonitrile−water (50/50, v/v). Tuning solutions of 0.05 ng/μL were made by diluting the working solution of 10 ng/μL in water−acetonitrile (50/50,v/v) containing 0.1% formic acid.

Animal Treatment. Only male broiler chicks (Ross 308) were used in this trial. The animals were housed in the poultry experimental facility of CLO-DVV (Agricultural Research Centre, Department of animal nutrition and husbandry), poultry house number 12. The initial stocking density of the broiler chicks was 18 per square meter. Central water heating provided optimal house temperature. The lighting program was as follows: light/dark = 21/3 during the entire period. There was a dynamic ventilation with lateral air entrance (at both sides) and air extraction centrally at the top of the building. The ventilation rate depended on the measured temperature and age of the broilers, thereby keeping the temperature as close as possible to the optimal temperature schedule and minimizing the moisture, NH_3, and CO_2 content of the inside air. All birds received feed (meal) and water (one hanging drinker per pen) ad libitum. The broiler chicks were vaccinated the first day of age against Newcastle (NDW, spray) and Bronchitis (H120, spray). At 16 days of age, the vaccination against Newcastle was repeated with La Sota (Clone 30, drinking water). Twice daily, animals and housing facilities were inspected for the general health status, constant feed, and water supply as well as temperature and ventilation, dead birds, and unexpected events. A phase feeding program was used for protein with 20.8% during the starter period (1−21 days of age) and 18% during the subsequent period (22−42 days of age). From day 1 until day 21, a starter feed containing 100 mg/kg monensin was given to all birds. From day 22 to day 32, they were fed a commercially available feed theoretically containing 1 mg/kg diclazuril. From day 33 until day 42, every day six chickens were slaughtered. From day 33 on, after removal of the first six chickens, a feed without any coccidiostat was given. Three animals were housed separately from day 22 on a coccidiostat-free diet. These birds were slaughtered on day 33 and were used as negative controls. From each chicken, the same muscle group of breast and thigh and the liver were sampled. All samples were immediately minced and ground with a Knifetec sample mill (Tecator, Hoganas, Sweden) and stored at −18 °C until analysis.

Sample Preparation. *Poultry Muscle and Liver.* Two grams of minced liver or muscle was weighed in a 50 mL centrifuge tube, and the internal standard was added at a concentration of 50 μg/kg. The sample was vortex mixed and allowed to stand for 10 min. Then 6 g of anhydrous sodium sulfate was added, and with a spatula, the tissue was carefully mixed until a powdery mixture was obtained. Ten milliliters of acetonitrile was added, and the tube was vortex mixed to homogenize the material and solvent. The tube was then placed on a horizontal shaker (SM, Edmund Bühler) for 30 min. Afterward, the mixture was centrifuged (IEC, Centra MP4, VWR, Leuven, Belgium) during 15 min at 4000 × *g*. Five milliliters of supernatant was transferred into a tube and was evaporated to dryness under nitrogen in a water bath at 60 °C. The sample was then redissolved in 1 mL of a mixture of acetonitrile−water (50/50, v/v) containing 0.1% formic acid, sonicated, and finally filtered through a 0.22 μm filter.

Poultry Feed. The method for the analysis of poultry feed was based on the method described by Cannavan et al. for the detection of nicarbazin in feed (*13*). Five grams of feed was weighed in a 50 mL centrifuge tube. Internal standard was added at a concentration of 1000 μg/kg. The sample was vortex mixed, and after 10 min, 25 mL of methanol was added. The tubes were then shaken on a horizontal shaker (SM, Edmund Bühler) for 30 min and centrifuged (IEC, Centra MP4, VWR, Leuven, Belgium) during 10 min at 600 × *g*. One milliliter of supernatant was transferred in a tube and was evaporated to dryness under nitrogen in a water bath at 60 °C. The sample then was redissolved in 1 mL of a mixture of acetonitrile−water (50/50, v/v) and filtered through a 0.22 μm filter.

Liquid Chromatography−Tandem Mass Spectrometry. *Chromatography.* A model 2695 Alliance LC system (Waters, Milford, MA) was used. Separation was performed on a Waters Symmetry C_{18} column (150 mm × 2.1 mm) with 5 μm particle size protected with a guard column Alltima C_{18} 7.5 mm × 2.1 mm with 5 μm particle size (Alltech, Deerfield, IL). Column temperature was maintained at 35 °C using a column heater. HPLC eluent A was water, containing 0.1% formic acid; eluent B was pure acetonitrile. The gradient was initiated with 0% eluent B for 0.5 min, continued with a linear increase to 45% B in 0.1 min, followed by a linear increase to 65% B in 7.9 min, and finally a linear increase to 100% B in 0.1 min. This condition was maintained for 1 min. The system was returned to 100% A in 0.2 min and was reequilibrated for 10.2 min before the next injection. The flow rate was 0.25 mL/min, and the injection volume was 10 μL. No split was necessary to introduce the LC eluent in the mass spectrometer.

Mass Spectrometry. The MS equipment consisted of a Waters Micromass Quattro Ultima Pt (Altrincham, Cheshire, U.K.) equipped with a Z-spray system. The MS system was controlled by version 4.0 of the MassLynx software. Tuning was performed in electrospray negative ionization mode with solutions of 0.05 ng/μL. For both diclazuril and the internal standard, the $(M - H)^-$ ion was determined, and dissociation of the molecular ion with argon was induced. Cone voltage and collision energy were tuned to optimize the transition of the molecular precursor ion to the most abundant product ions. Nitrogen was used as cone gas and desolvation gas at flow rates of 60 and 700 L/h, respectively. The source block and desolvation temperature were

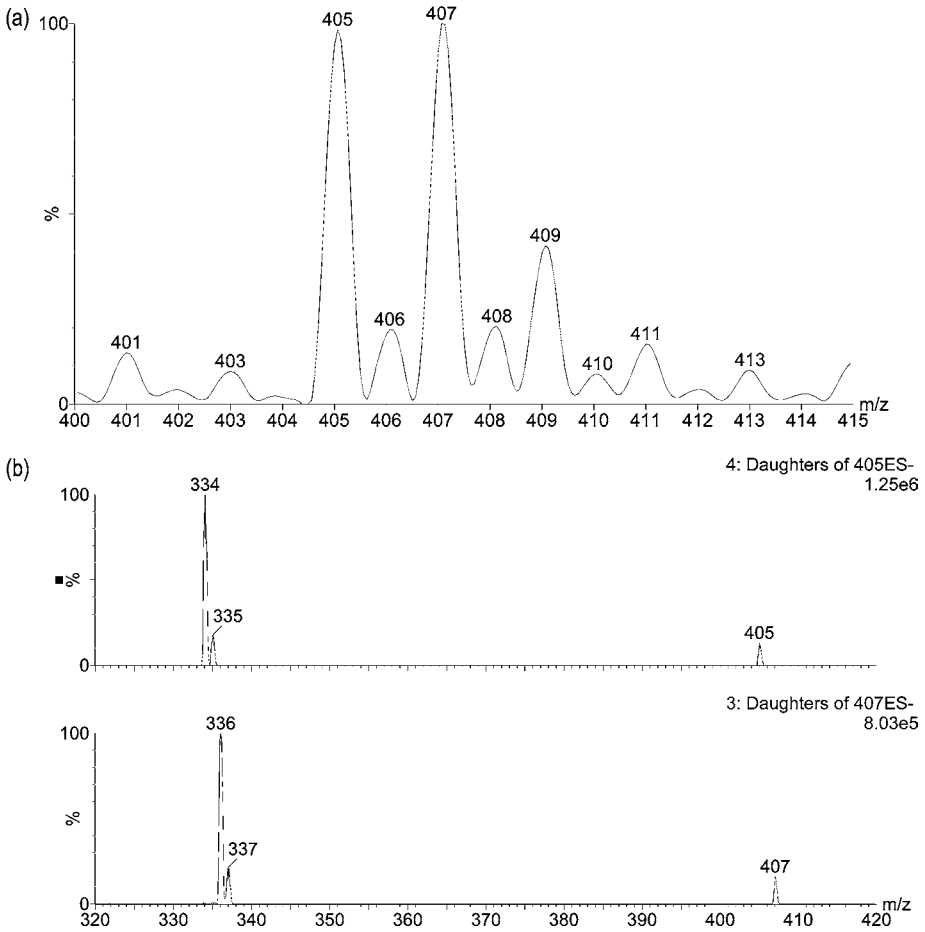

Figure 2. **Panel a** presents the MS spectrum of diclazuril. The ions at *m*/*z* 411, 409, 407, and 405 in the negative MS mode for diclazuril represent the chlorine isotopes, that is, $^{37}Cl_3$, $^{35}Cl^{37}Cl_2$, $^{35}Cl_2{}^{37}Cl$, and $^{35}Cl_3$ versions of diclazuril as a deprotonated molecule, respectively. **Panel b** presents the daughter spectra of *m*/*z* 407 and 405, representing $^{35}Cl_2{}^{37}Cl$ and $^{35}Cl_3$ versions of diclazuril minus a proton, respectively. Product ions in the MS/MS mode of *m*/*z* 407 and 405 are 336 and 334, respectively.

Table 1. Mass Spectrometric Conditions

compound	ionization mode	precursor ion (*m*/*z*)	cone voltage (V)	product ions (*m*/*z*)	collision energy (eV)
diclazuril	ES negative	405.2	50	334.2	15
		407.2	50	336.2	15
internal standard	ES negative	421.1	50	323.1	25

set at 120 and 300 °C, respectively. RF 1 and capillary voltage were set at 35 V and 3.0 kV, respectively. The optimized MS parameters are presented in **Table 1**. As stated in Commission Decision 2002/657/EC, a minimum of four identification points is required for forbidden substances. For LC-MS/MS, in which the transition of one precursor ion into two product ions is recorded, four identification points are provided. For diclazuril, only one major product ion could be generated, that is, *m*/*z* 334. The second product ion, *m*/*z* 335, differs only 1 amu from the first one and has a very low abundance. Therefore it is not very suitable for use in multiple reaction monitoring (MRM). The second product ion can be observed in the upper spectrum of **Figure 2b**. But since the diclazuril molecule contains three chlorine atoms and chlorine has two stable isotopes (^{35}Cl and ^{37}Cl), a second precursor ion can be used. The ions at *m*/*z* 411, 409, 407, and 405 in the negative MS mode for diclazuril represent the chlorine isotopes, that is, $^{37}Cl_3$, $^{35}Cl^{37}Cl_2$, $^{35}Cl_2{}^{37}Cl$, and $^{35}Cl_3$ versions of diclazuril as a deprotonated molecule, respectively. The occurrence of the natural isotopes of chlorine is 75.8% for ^{35}Cl and 24.2% for ^{37}Cl. By applying the formula $(a + b)^3$ with a the occurrence of ^{35}Cl and b the occurrence of ^{37}Cl, one can calculate that diclazuril contains 1.4% $^{37}Cl_3$, 13.3% $^{35}Cl^{37}Cl_2$, 41.7% $^{35}Cl_2{}^{37}Cl$, and 43.5% $^{35}Cl_3$. This pattern can be observed in the MS spectrum of diclazuril (**Figure 2a**). Product ions in the MS/MS mode of *m*/*z* 407 and 405 are 336 and 334, respectively. A solution of 1 ng/μL diclazuril in water−acetonitrile (50/50,v/v) was used to record a daughter spectrum. This spectrum is presented in **Figure 2b**. It shows the occurrence of a prominent product ion from the chlorine isotopic variants *m*/*z* 405 and 407 of diclazuril, in both cases representing the loss of 71 atomic mass units (*14*). The ionization and collisional induced dissociation of the $^{35}Cl_3$ version of diclazuril is schematically presented in **Figure 3** (*14*). Since the ions at *m*/*z* 407 and 405 have about the same abundance, both transitions 407 < 336 and 405 < 334 can be used. In this way, four identification points can be provided for diclazuril. The LC effluent was connected to the interface via a divert valve from 10 to 14 min after the injection of the extract into the LC system to avoid pollution of the mass spectrometer. The instrument was operated in MRM mode with a dwell time of 0.35 s, an interchannel delay of 0.01 s, and an interscan delay of 0.1 s.

Blank meat (breast and thigh) and liver for validation experiments and calibration curves were bought at an organic butcher's shop. The coccidiostat-free feed that the chickens received from day 33 on was used as blank feed. The most recent EU legislation concerning residue analysis was used as a guideline for the validation of the presented methods and analysis of the samples (*1*).

Specificity of the MS response was tested by injecting standard solutions of other coccidiostats, namely, dimetridazole, halofuginone, robenidine and dinitrocarbanilide, the marker analyte of nicarbazin, narasin, monensin, salinomycin, and lasalocid, and analyzing them on the presence of diclazuril. No interferences were detected.

Method of Quantification. A matrix calibration curve was made using the MRM-data of the transition of the precursor ion into the most abundant product ion, that is, 405.2 > 334.2. Quantification was conducted by internal calibration using a weighing factor of 1/*x*. The

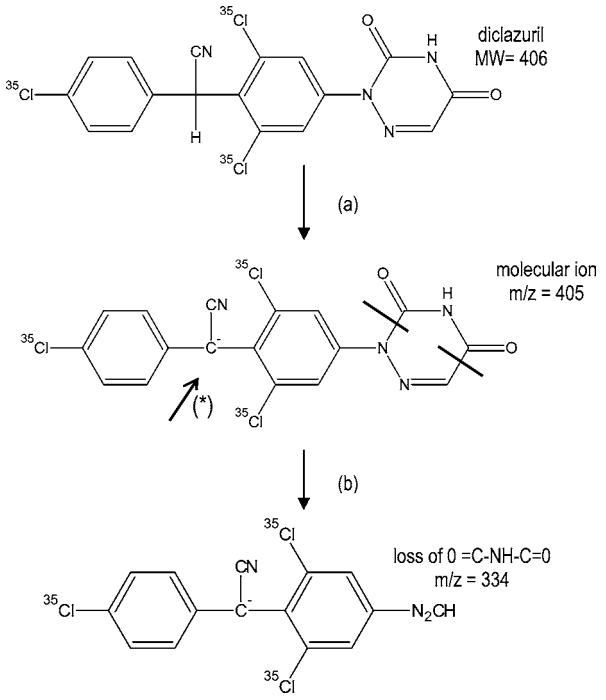

Figure 3. Ionization (**a**, at the position marked by (*)) and collisionally induced dissociation (CID, **b**) of diclazuril. Fragmentation of the triazidione ring and loss of O═C–NH–C═O is shown here for the $^{35}Cl_3$ anion (*m*/*z* = 405).

results were calculated by the TargetLynx software. For each series of samples, a calibration curve was made in a specific concentration range to make sure that the concentrations in the samples of that particular series were covered.

RESULTS AND DISCUSSION

Validation of the Method for Poultry Muscle. Three calibration graphs of spiked meat samples were made in the concentration range 0−600 μg/kg. The determination coefficients were at least 0.9969. Detailed results of the regression analysis are presented in **Table 2**. Since no certified reference material was available, trueness was determined with recovery. Precision was determined with repeatability. This was performed on three concentration levels at three different occasions. Six replicates of known diclazuril negative meat were spiked at 5, 50, and 500 μg/kg with diclazuril, extracted, and analyzed. Spiking of the meat was performed by adding an appropriate amount of one of the working standard solutions (as described in the Reagents and Standards section) to 2 g of meat, that is, 100 μL of a standard solution of 0.1 ng/μL, 100 μL of a standard solution of 1 ng/μL, and 1000 μL of a standard solution of 10 ng/μL for spiking at the 5, 50, and 500 μg/kg level, respectively. Quantification was performed with a matrix containing calibration curve. Detailed results are presented in **Table 3**. An overall recovery of 91.3%, 99.5%, and 96.5% for the concentration levels 5, 50, and 500 μg/kg was obtained. Commission Decision 2002/657/EC states that for concentrations between 1 and 10 μg/kg, recovery should fall within the range −30% to +10% and for concentrations higher than 10 μg/kg, within the range −20% and +10%. This criterion was fulfilled.

According to the Horwitz equation (*15*), the intralaboratory coefficient of variation (CV) should not exceed 23.7%, 16.7%, and 11.8% for the concentration levels of 5, 50, and 500 μg/kg, respectively. For the 500 μg/kg level, this criterion is satisfied. Commission Decision 2002/657/EC states that for mass fractions lower than 100 μg/kg the application of the Horwitz equation gives unacceptable high values so the CVs should be as low as possible. In our opinion, the values obtained here are very acceptable.

Table 2. Overview of the Regression Analysis Parameters of the Standard Calibration Curves and of the Calibration Curves in the Matrices of Meat, Liver, and Feed

	curve	slope	intercept	R^2	no. of data points	concn range (μg/kg)
meat	1	0.9101	15.8028	0.9969	10	0–1–2–5–10–20–50–100–200–400–600
	2	0.9327	13.7285	0.9993		
	3	0.9533	12.9593	0.9993		
		rsd (%): 2.3				
liver	1	0.114	–0.4413	0.9951	8	0–50–100–200–400–800–1000–1200
	2	0.1159	–0.1918	0.9999	8	0–50–75–100–150–200–300–400
	3	0.1237	–1.6410	0.9953	9	0–25–50–75–100–125–150–200–300
		rsd (%): 4.4				
feed	1	3.235	7.072	0.9969	11	0–10–20–50–100–250–500–750–1000–1500–2000
	2	3.178	–3.330	0.9934		
	3	3.455	–8.880	0.9953		
		rsd (%): 4.4				

Table 3. Detailed Results of the Inter- and Intraday Precision and Recovery Experiments for Meat

concn level		day 1	day 2	day 3	overall
5 μg/kg	no. of replicates	6	6	6	18
	mean (μg/kg)	4.7	4.4	4.5	4.6
	sd (μg/kg)	0.4	0.4	0.4	0.4
	rsd (%)	7.6	8.9	8.6	8.4
	mean recovery (%)	94.9	88.5	90.6	91.3
50 μg/kg	no. of replicates	6	6	6	18
	mean (μg/kg)	51.2	49.7	48.4	49.8
	sd (μg/kg)	2.5	1.3	1.0	2.0
	rsd (%)	4.8	2.6	2.0	4.0
	mean recovery (%)	102.4	99.3	96.8	99.5
500 μg/kg	no. of replicates	6	6	6	18
	mean (μg/kg)	486.3	478.6	482.7	482.5
	sd (μg/kg)	6.8	7.1	8.9	7.2
	rsd (%)	1.4	1.5	1.8	1.5
	mean recovery (%)	97.3	95.7	96.5	96.5

The decision limit or CCα is in the case of banned substances the lowest concentration level at which the method can discriminate with a statistical certainty of 1 − α whether the identified analyte is present. CCα was determined by analyzing 20 samples spiked at a concentration for which a signal-to-noise ratio of at least 3 for two transitions was obtained. The detection capability or CCβ is the lowest concentration at which the method is able to detect truly contaminated samples with a statistical certainty of 1 − β. Twenty blank samples were spiked at the decision limit and analyzed and quantified using a matrix containing calibration curve. The value of the decision limit plus 1.64 times the standard deviation of the measured content equals the detection capability. For substances for which no permitted limit has been established, α and β equal 1% and

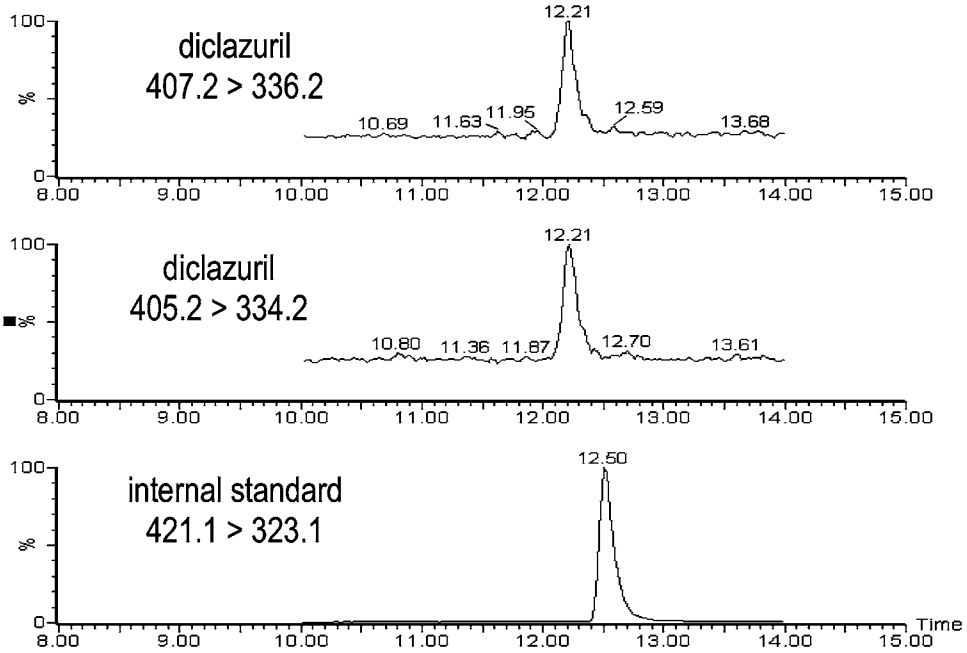

Figure 4. Chromatogram of meat spiked at CCα level (0.5 μg/kg).

5%, respectively. For CCα and CCβ, values of 0.5 and 0.6 μg/kg were obtained, respectively. Analyzing 20 samples spiked at 0.5 μg/kg resulted in a mean recovery of 111% and a relative standard deviation of 9.9%. A chromatogram of a sample spiked at CCα level is shown in **Figure 4**.

Validation of the Method for Poultry Liver. Linearity was determined by spiking negative liver samples with 25−1200 μg/kg of diclazuril and analyzing them. The assay proved to be linear up to the highest concentration tested. Detailed results are presented in **Table 2**. Recovery and repeatability were determined by spiking six negative samples at 5 and 50 μg/kg and quantifying them with a matrix containing calibration curve. A mean recovery of 104% and a relative standard deviation of 4.7% for the 5 μg/kg level and a mean recovery of 103% and a relative standard deviation of 1.9% for the 50 μg/kg level were obtained. These values fall within the criteria set by Commission Decision 2002/657/EC (*1*). The detection limit was not determined. However, no sensitivity problems were encountered: the lowest concentration measured in the liver samples was 23 μg/kg, and for that concentration a signal-to-noise ratio of 800 was obtained for the second transition.

Validation of the Method for Poultry Feed. Three calibration graphs of spiked feed samples were made in the concentration range 0−2000 μg/kg. The determination coefficients were at least 0.9934. Detailed results of the regression analysis are presented in **Table 2**. Inter- and intraday precision and recovery were assessed by extracting and analyzing six replicates of known diclazuril negative feed spiked at 50, 500, and 1000 μg/kg with diclazuril on three separate days. Spiking of the feed was performed by adding an appropriate amount of one of the working standard solutions (as described in the Reagents and Standards section) to 5 g of feed, that is 250 μL of a standard solution of 1 ng/μL, 250 μL of a standard solution of 10 ng/μL, and 50 μL of a standard solution of 100 ng/μL for spiking at the 50, 500, and 1000 μg/kg level, respectively. Quantification was performed with a matrix containing calibration curve. Detailed results are presented in **Table 4**. An overall recovery of 102.9%, 105.0%, and 101.5% for the concentration levels 50, 500, and 1000 μg/kg were obtained, respectively. As mentioned above, Commission Decision 2002/657/EC states that for concentrations above 10 μg/kg, recovery should fall within the range −20% to +10%. This criterion was fulfilled. Overall relative standard deviations ranged from 5.6% to 6.6%. These values fall within the limits set by Commission Decision 2002/657/EC, namely, a maximum intralaboratory CV of 16.7%, 11.8%, and 10.7% for a concentration of 50, 500, and 1000 μg/kg, respectively.

Table 4. Detailed Results of the Inter- and Intraday Precision and Recovery Experiments for Feed

concn level		day 1	day 2	day 3	overall
50 μg/kg	no. of replicates	6	6	6	18
	mean (μg/kg)	53.1	51.3	49.9	51.4
	sd (μg/kg)	4.5	0.7	1.2	2.9
	rsd (%)	8.5	1.4	2.4	5.6
	mean recovery (%)	106.1	102.6	99.8	102.9
500 μg/kg	no. of replicates	6	6	6	18
	mean (μg/kg)	543.9	508.4	523.1	525.1
	sd (μg/kg)	45.9	6.1	11.1	29.8
	rsd (%)	8.4	1.2	2.1	5.7
	mean recovery (%)	108.8	101.7	104.6	105.0
1000 μg/kg	no. of replicates	6	6	6	18
	mean (μg/kg)	988.7	985.5	1070.6	1014.9
	sd (μg/kg)	94.2	18.9	19.4	66.9
	rsd (%)	9.5	1.9	1.8	6.6
	mean recovery (%)	98.9	98.6	107.1	101.5

For each series of samples analyzed, two spiked unknown samples were analyzed. Recoveries between 91% and 108% for the breast samples, between 81% and 108% for the thigh samples, and between 91% and 118% for the liver samples were obtained. Also for each set of samples, an evaluation of the MS identification criteria according to Commission Decision 2002/657/EC (*1*) was performed. Each diagnostic ion had a signal-to-noise ratio of at least 3. Also the ion ratio was checked: the relative intensity of the second ion (i.e., 407.2 > 336.2), expressed as a percentage of the intensity of the most intense ion, corresponds to that of a spiked sample at a comparable concentration within the tolerances set by Commission Decision 2002/657/EC (*1*). The chromatographic criterion set by the just-mentioned Commission Decision was verified as well. The ratio of the chromatographic retention time

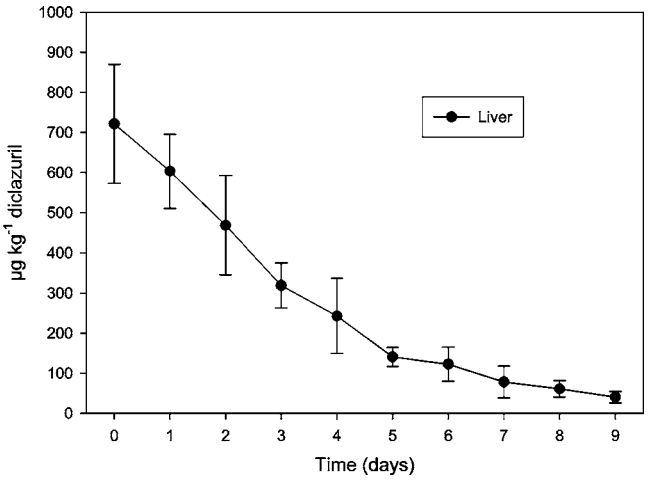

Figure 5. Depletion of residues of diclazuril in poultry liver after withdrawal of medicated feed containing 730 μg/kg diclazuril.

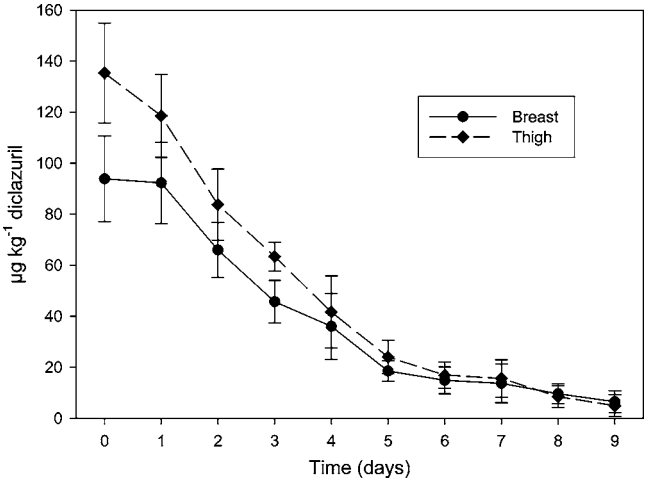

Figure 6. Depletion of residues of diclazuril in poultry breast and thigh muscle after withdrawal of medicated feed containing 730 μg/kg diclazuril.

of the analyte to that of the internal standard, that is, the relative retention time of the analyte, must correspond to that of a spiked sample at a tolerance of ±2.5%. All criteria were fulfilled for all sets of samples.

The commercial feed theoretically containing 1 mg/kg diclazuril was analyzed with the method described above. Analysis was performed in triplicate: a mean value of 730 μg/kg with a relative standard deviation of 4.8% was obtained.

Liver and muscle samples of the three negative control birds were analyzed to be sure that no contamination had happened during the animal treatment. All samples were found to be residue free.

The results of the analysis of the liver and breast and thigh samples are presented in **Figures 5** and **6**. The first six chickens were slaughtered on day 33 of the experiment. As the withdrawal of the medicated feed took place after the removal of these chickens, diclazuril concentrations found in their tissues can be considered as steady-state concentrations. These data are labeled as "day 0" in **Figures 5** and **6**.

Five days after withdrawal of the medicated feed, that is, the withdrawal time, diclazuril residues were still present as well in liver as in muscle tissues. A mean concentration of 140.7, 18.5, and 24.0 μg/kg was found in liver, breast, and thigh, respectively. Although 5 days are clearly not enough to obtain residue-free samples, MRLs set by Codex Alimentarius are not exceeded. Moreover, Codex Alimentarius MRLs were never exceeded, not even at steady-state conditions. The steady-state concentration and the initial residue concentrations seem to be higher in the thigh than in the breast muscle tissue. To verify whether the differences in residue values between thigh and breast muscle were statistically significant an analysis of variance (ANOVA) was carried out on the individual measurements for each sampling time. The data were log-transformed prior to analysis as a variance-stabilizing measure and to avoid the negative effect of correlations between mean values and variances, which were observed in the nontransformed data. Homogeneity of variances was verified with a Bartlett χ^2 test. Differences between the individual matrices were calculated using a post-hoc Fisher test. This analysis was done in Statistica 6.1 (Statsoft, Tulsa, OK).

Table 5. Parameter Estimations and Confidence Intervals for the *b* Parameter of Eq 1

	breast	thigh	liver
b	0.000 16	0.000 14	0.000 023
$b_{-95\%}$	0.000 13	0.000 12	0.000 021
$b_{+95\%}$	0.000 19	0.000 15	0.000 026

Significant differences ($P < 0.05$) could be observed between breast and thigh for the first three data points. A potential explanation for this phenomenon can be a higher blood flow in the thigh. After 2 days withdrawal, no significant differences between breast and thigh could be observed. The liver values were significantly different from the values for both muscle types at each sampling time during the whole measuring period.

To compare the time evolution of the depletion curves a similar procedure as outlined in De Ruyck et al. was followed (*16*). A suitable function was fitted to the three data series. Using TableCurve2D (Systat Software, Point Richmond, CA), a function was found that could adequately be fitted to the data (eq 1):

$$\frac{1}{y} = a + bt^3 \qquad (1)$$

with y the value of diclazuril in the sample, t the time (d), and a and b the parameters to be fitted. Most important parameter is b, because this is directly related to the shape of the curve. Parameter a is the value of the intercept at time $t = 0$. Therefore, only b is considered further.

Table 5 shows the parameter estimations and the 95% confidence intervals for the three cases. When the confidence intervals overlap, the parameters and consequently the depletion kinetics are not significantly different. The depletion in breast and thigh is not significantly different; in the liver it is considerably faster.

Although Codex Alimentarius has also set MRLs for kidney and skin/fat, we preferred to analyze only liver and muscle since those are the tissues to which the consumer is exposed. The consumption of kidney and fat is, at least in Belgium, negligible. Pharmacokinetic data show that for turkeys as well as broilers, the liver is the target tissue, that is, the highest residue concentrations are found in the liver (*17*). Lower concentrations are found in kidneys, skin/fat, and muscle in a decreasing order (*17*, *18*). So by analyzing liver and muscle, we analyzed the tissue with the highest and lowest residue concentrations.

Reanalyzing five breast samples and five thigh samples 7 months after the first analysis showed no degradation when the samples are stored at −18 °C. For the liver samples, a degradation of 25% was found when five samples were reanalyzed 5 months after the first analysis. Because the liver

samples were originally analyzed only a couple of weeks after the chickens were slaughtered, this degradation should not be considered as a problem for this study.

The methods developed for the determination of diclazuril in poultry feed, meat, and liver proved to be very specific and sensitive and to allow a high sample throughput. The methods were applied to the samples obtained from an animal experiment. This experiment revealed that a withdrawal time of 5 days, as prescribed, is not sufficient to produce residue-free tissues.

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