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Liquid chromatographic tandem mass spectrometric determination of five coccidiostats in poultry eggs and feed

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Abstract

A method is described which permits the quantitative detection of the chemical coccidiostats halofuginone, robenidine, diclazuril, nicarbazin and dimetridazole and its main metabolite 2-hydroxydimetridazole in poultry eggs and feed. Sample preparations were kept very simple and are based upon extraction with an organic solvent. Sample extracts were injected into the liquid chromatography tandem mass spectrometry (LC–MS/MS) system on a C_{18} column and a gradient elution was performed. Dimetridazole-D_3 and diclazuril-bis, a structural analogue of diclazuril, were used as internal standards. Detection was performed on a triple quadrupole mass spectrometer in the selected reaction monitoring mode after ionisation in the positive or negative electrospray ionisation mode. Argon was applied as collision gas for collision induced dissociation. Validation of the methods was performed based on Commission Decision 2002/657/EC [Official Journal of the European Communities L221 (2002) 8].

Keywords: Coccidiostats; Eggs; Feed; Residues; LC–MS/MS

1. Introduction

Coccidiosis is a contagious condition affecting livestock, especially poultry, throughout the world. Particular in warm, humid environments it causes intestinal lesions, which result in diarrhoea and related health problems in the animal. The disease is carried by unicellular organisms belonging to the genus *Eimeria* in the class Sporozoa. In its acute form, coccidiosis causes high mortalities, in its sub-acute form, small numbers of oocysts can cause poor weight gain, poor feed conversion and poor egg production in poultry. Of all domestic animals, industrially bred poultry and rabbits are particularly prone to this disease. The economic damage caused by coccidiosis in modern poultry production is so serious that practically all poultry farms have resorted to feeding anticoccidial drugs as a feed additive to pullets and broiler breeders for 12–16 weeks and to broiler chickens for almost their entire life. Despite the use of anticoccidial drugs, coccidiosis remains one of the biggest causes of loss in poultry production.

A wide range of anticoccidial drugs are available to treat and prevent coccidiosis. Besides the ionophoric coccidiostats, such as narasin, monensin, lasalocid and salinomycin, there is also a class of chemical coccidiostats. The most common chemical coccidiostats are nicarbazin, halofuginone, diclazuril and robenidine. Dimetridazole, which belongs to the group of nitroimidazoles, formerly was used as anticoccidial but is now listed in Annex 4 of Council Directive 2377/90 [2] and is, as a consequence, a forbidden compound.

According to Regulation 1831/2003/EC, coccidiostats are at the moment licensed as feed additives [3]. The coccidiostats can be used at a prescribed concentration and during a certain time interval for broilers and young chickens but not for laying hens. It was shown in the past that accidental cross contamination of feed could lead to residues of the

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compounds in eggs. Especially carry-over from medicated feed into non-medicated feed at the feeding mill is found to be a main reason for the presence of residues in eggs.

With a view to a decision on the phasing out of the use of coccidiostats as feed additives by 31 December 2012, the European Commission shall submit to the European Parliament and the Council a report on the use of these substances as feed additives and available alternatives before 1 January 2008. In the mean time, there are no maximum residue levels (MRLs) set for eggs, and thus the compounds cannot be present in eggs. Hence the zero tolerance principle has to be applied. In practice, however, different EU member states apply another approach. In Belgium, an action limit of 10 $\mu g\,kg^{-1}$ has been proposed by the scientific committee of the Belgian Food Agency for monensin, salinomycin, diclazuril, lasalocid, maduramycin, narasin, nicarbazin, robenidine and the group of sulphonamides. In the United Kingdom on the other hand, an action limit of 100 $\mu g\,kg^{-1}$ for nicarbazin in eggs has been set.

Nicarbazin is the generic name of the equimolar complex of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP). When chickens are given nicarbazin in the feed, the HDP fraction is absorbed and excreted more rapidly than the DNC fraction and consequently most residue analyses for nicarbazin are based on methods for the DNC molecule. Thus, for the development of a method to detect residues of nicarbazin in eggs, we focused only on the DNC compound.

As mentioned above, dimetridazole or 1,2-dimethyl-5-nitroimidazole belongs to a group of compounds called the nitroimidazoles. The major pathway of elimination of dimetridazole is hydroxylation of the 2-methyl group to 2-hydroxymethyl-1-methyl-5-nitroimidazole. The fact that dimetridazole is metabolized rapidly and that the main metabolite, 2-hydroxydimetridazole, is present in higher concentrations in tissues and eggs emphasizes the need to monitor for both of these compounds when one is performing residue analysis.

Several analytical methods have been described to determine one or more of the coccidiostats in different matrices based on different techniques. For the determination of diclazuril, only two methods could be found for the egg matrix [4,5]. High performance liquid chromatography (HPLC) with UV detection [6] and gas chromatographic (GC)–mass spectrometric (MS) [7] methods were described for the detection of diclazuril in feed. Dirikolu et al. [8] published a liquid chromatographic method with a diode array detector for the detection in horse plasma and Croubels et al. [9] described a LC–MS/MS method for the detection of diclazuril in animal plasma. Besides the method described by our own lab [5], only one MS method for the detection of robenidine was reported [4]. For the detection of robenidine in the feed matrix, a thin layer chromatographic [10], a photometric [11] and a polarographic method [12] were described. Quite a few papers have been published describing the determination of dimetridazole and its main metabolite 2-hydroxydimetridazole in feed, tissues and eggs. In these papers, methods based on techniques such as differential pulse polarography [13], LC [14–22], GC [23–29] and LC–MS [4,5,30–36] were described. Out of all the papers which have been published on the determination of halofuginone in different matrices, only few of them used LC–MS [4,5,37–39]. Other techniques used were LC with UV detection [40–47], gas–liquid chromatography [48] and capillary isotachophoresis combined with capillary zone electrophoresis [49]. Most papers describing a method for the detection of nicarbazin in different matrices, use LC with UV detection [50–64]. Also papers describing LC–MS [4,5,59,65–69], electrochemical methods [70,71] and pulse polarographic methods [70,72,73] are available.

The aim of this work was to develop a sensitive and specific method for the quantitative detection of the chemical coccidiostats halofuginone, robenidine, diclazuril, nicarbazin and dimetridazole and its metabolite 2-hydroxydimetridazole in poultry egg and feed. The molecular structures of these compounds are presented in Fig. 1. The method described for the egg matrix, is based on a previously published method [5], which has been adapted to improve sample throughput and robustness. Both methods were validated according to the most recent European legislation concerning residue analysis [1]. The methods described are suitable according to the EU criteria for the confirmation of five different coccidiostats and one metabolite.

2. Experimental

2.1. Chemicals and reagents

Dimetridazole (>99%), nicarbazin (99.5%) and dinitrocarbanilide (97%) were from Sigma (Bornem, Belgium). Diclazuril and internal standard R062646, a structural analogue of diclazuril and further referred to as diclazuril-bis, 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 diclazuril-bis, respectively. 2-Hydroxydimetridazole was purchased at Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (BgVV) in Berlin, Germany. Halofuginone and robenidine (99.8%) were gifts from Intervet (Mechelen, Belgium) and Alpharma Animal Health (Antwerpen, Belgium), respectively. Dimetridazole-D_3 was bought at RIVM (Bilthoven, The Netherlands).

Acetonitrile, methanol and hexane (MS quality) were from Biosolve (Valkenswaard, The Netherlands) and ethanol (96%) was from BDH Laboratory Supplies (Poole, England). Dimethylformamide (pro analysi) and dimethylsulfoxide (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).

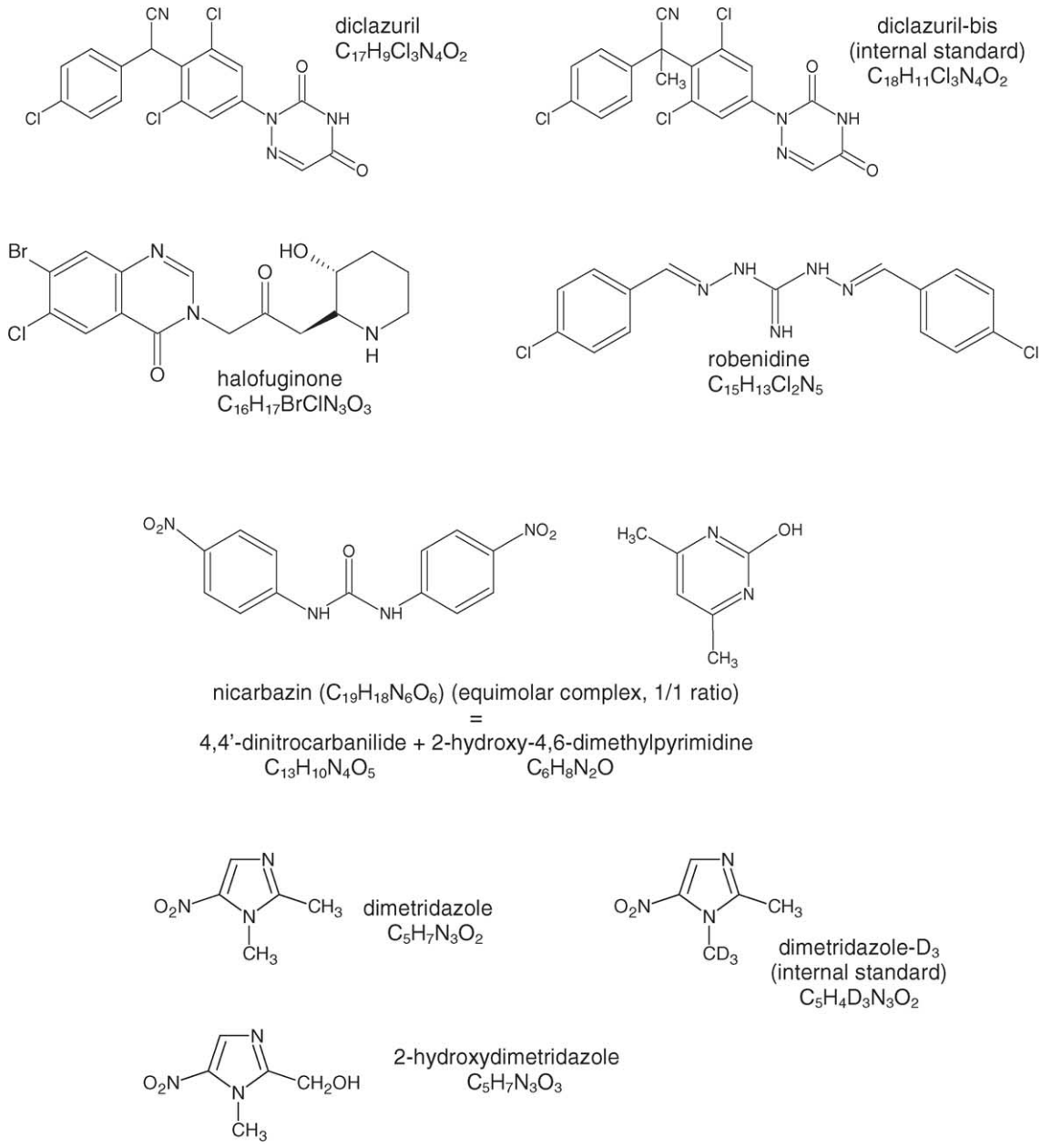

Fig. 1. Molecular structures of the coccidiostats studied and both internal standards.

Standard stock solutions of 1 mg ml^{-1} of the different compounds were prepared and stored at −18 °C. The solvents used for preparing the stock solutions were dimethylformamide for diclazuril and diclazuril-bis, a mixture of acetonitrile–water (50/50, v/v) for dimetridazole, 2-hydroxydimetridazole and halofuginone, dimethylsulfoxide for dinitrocarbanilide and nicarbazin and ethanol for robenidine. A stock solution of internal standard dimetridazole-D_3 of 100 ng μl^{-1} was prepared according to the procedure of RIVM. The standard stock solutions were stable for at least 8 months when stored at −18 °C. Working standard solutions of 100 ng μl^{-1}, 10 ng μl^{-1}, 1 ng μl^{-1} and 0.1 ng μl^{-1} were prepared daily by diluting the stock solutions in a mixture of acetonitrile–water (50/50, v/v). Tuning solutions of 0.1 ng μl^{-1} were made by diluting the working solution of 10 ng μl^{-1} in water–acetonitrile (50/50, v/v) containing 0.1% formic acid.

2.2. Sample preparation

2.2.1. Egg

The sample preparation procedure described in Mortier et al. was used as a basis [5]. In short, the eggs were broken and after mixing its content with an ultra-turrax, 10 g homogenized egg was weighed in a disposable 50 ml centrifuge tube and, depending on whether measurements were

carried out in the electrospray positive or negative ionisation mode, 5 μg kg^{-1} diclazuril-bis or 10 μg kg^{-1} dimetridazole-D_3 was added as internal standard, respectively. After vortex mixing, samples were allowed to stand for 10 min. Ten millilitres acetonitrile was added and the sample was vortex mixed for 1 min and placed in an ultrasonic bath for 5 min. The sample then was centrifuged (IEC, Centra MP4, VWR, Leuven, Belgium) during 10 min at 1550 × *g*. The supernatant was then transferred into a graduated tube and concentrated to a volume of 4 ml under nitrogen in a waterbath at 60 °C. Also a second procedure was used which included an additional step with hexane. In this second procedure, after centrifugation, 10 ml of the acetonitrile layer was transferred into another tube to which 10 ml hexane was added. After gently mixing, this mixture was centrifuged (IEC, Centra MP4, VWR, Leuven, Belgium) at 600 × *g* during 5 min. The hexane layer was carefully removed and the remaining acetonitrile layer was transferred into a graduated tube and concentrated to a volume of 1 ml under nitrogen in a waterbath at 60 °C. Finally, the remaining extracts of both methods were filtered through a 0.22 μm filter. The first procedure was used for the detection of dinitrocarbanilide, robenidine and diclazuril. The second procedure, including the hexane wash step, was used for the determination of dimetridazole, 2-hydroxydimetridazole and halofuginone

2.2.2. 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 [66]. Five grams of feed was weighed in a 50 ml centrifuge tube. Internal standard was added at a concentration of 1000 μg kg^{-1}. The sample was vortex mixed and after 10 min, 25 ml 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*. Five millilitres of supernatant was transferred in a tube and was evaporated to dryness under nitrogen in a waterbath 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.

2.3. LC–MS/MS

2.3.1. Liquid chromatography

A model 2695 Alliance LC system (Waters, Milford, Massachusetts, USA) 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, Illiniois, USA). In comparison with the method published earlier [5], another LC–MS system was used. The new LC system contained a column oven and hence now the optimal column temperature was determined and found to be 35 °C. The chromatographic conditions were basically the same as those published earlier. Nevertheless, HPLC eluent A was replaced by water/acetonitrile (95/5, v/v), containing 0.1% formic acid in order to prevent a hydrophobic collapse. 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 re-equilibrated for 10.2 min before the next injection. The flow rate was 0.25 ml min^{-1} and the injection volume was 10 μl. No split was necessary to introduce the LC eluent in the mass spectrometer.

2.3.2. Mass spectrometry

The MS equipment consisted of a Waters Micromass Quattro Ultima Pt (Altrincham, Cheshire, UK) equipped with a Z-spray system. The MS system was controlled by version 4.0 of the MassLynx software. Tuning was performed by infusion of solutions of the coccidiostats of 0.1 ng μl^{-1} in acetonitrile–water (50/50, v/v) containing 0.1% formic acid at a flow rate of 10 μl min^{-1} in an LC flow of 200 μl min^{-1} using a T-piece. For all compounds, ionisation was performed in the electrospray mode. Cone voltage, capillary voltage and RF1 voltage were tuned to optimise the presence of the $[M+H]^+$ ion for dimetridazole, 2-hydroxydimetridazole, dimetridazole-D_3, halofuginone and robenidine and the $[M-H]^-$ ion for diclazuril, diclazuril-bis and dinitrocarbanilide. These ions were then used for collision induced dissociation with argon. Collision energy was tuned to optimise the fragmentation of the precursor ion into the most abundant product ions. Nitrogen was used as cone gas and desolvation gas at flow rates of 60 l h^{-1} and 700 l h^{-1}, respectively. The source block and desolvation temperature were set at 120 °C and 300 °C, respectively. Collision gas pressure was 2.5×10^{-3} mbar.

As stated in Commission Decision 2002/657/EC [1] 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 earned. For diclazuril, only one major product ion could be generated, i.e. *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 selected reaction monitoring (SRM). 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, i.e. $^{37}Cl_3$, $^{35}Cl^{37}Cl_2$, $^{35}Cl_2{}^{37}Cl$ and $^{35}Cl_3$ versions of diclazuril as a deprotonated molecule, respectively. According to the natural occurrence of the different isotopes, 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 (Fig. 2). Product ions in the MS/MS mode of *m*/*z* 407 and 405 are 336 and 334, respectively. 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 earned for diclazuril.

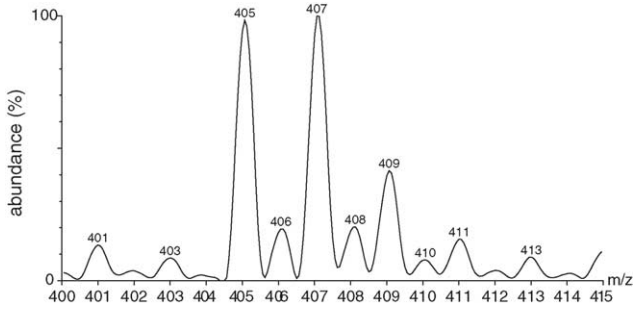

Fig. 2. MS spectrum of diclazuril: the ions at *m*/*z* 411, 409, 407 and 405 in the negative MS mode for diclazuril represent the chlorine isotopes, i.e. $^{37}Cl_3$, $^{35}Cl^{37}Cl_2$, $^{35}Cl_2{}^{37}Cl$ and $^{35}Cl_3$ versions of diclazuril as a deprotonated molecule, respectively.

The optimized MS parameters are presented in Table 1. The LC effluent was connected to the interface via a divert valve to avoid clogging of the cone of the mass spectrometer. The instrument was operated in the SRM mode with a dwell time of 0.35 s, an interchannel delay of 0.01 s and an interscan delay of 0.1 s.

3. Results and discussion

In 2003, our lab already reported a method for the simultaneous detection of five coccidiostats in eggs. Then, our main objective was to develop a multiresidue method which allowed a rapid screening of egg samples. Since LC–MS/MS, and more specific SRM, guarantees a very specific detection, clean up was kept as simple as possible. Although this method was perfectly suitable for this rapid screening, a major drawback was the clogging of the cone and consequently a lower sample throughput. Therefore, we decided to try to improve this method. Last year, we organized an animal experiment in which laying hens were fed feed containing several coccidiostats. In the samples originating from this experiment, only one compound would be present per egg sample. Therefore, it was not longer necessary that our method was a multiresidue method. In order to reduce the source contamination the mass spectrometer, our main concern was to remove the fat. Therefore, the additional step with hexane was applied. Visual inspection of the cone after 15 injections, evidenced that the removal of the fat with hexane was really beneficial. This method was found to be suitable for the detection of dimetridazole, its major metabolite 2-hydroxydimetridazole and halofuginone. For dinitrocarbanilide, robenidine and diclazuril on the other hand, this method could not be used since these compounds are lipid soluble and as a result would be removed together with the hexane layer. So for these three compounds, an alternative way for reducing source contamination was used. We connected the LC effluent to the interface via a divert valve. In this way it is possible to reduce the amount of effluent that comes into the mass spectrometer and consequently also the contamination is really reduced. This effect could be used optimally since only one compound and his internal standard needed to be monitored, so the timeframe in which the SRM transitions have to be recorded, was very short.

To enhance ruggedness and quantification of the method, an internal standard was added. For the measurements performed in the electrospray positive mode, dimetridazole-D_3 was found to be suitable. No deuterated compound could be obtained for use as internal standard in the electrospray negative mode, but diclazuril-bis, which only differs in the presence of one methylgroup with diclazuril, works very good as internal standard.

The most recent EU legislation concerning residue analysis was used as a guideline for the validation of the presented method. Linearity of the LC–MS/MS response was first tested by making three individual standard calibration graphs in the concentration range 0–100 ng ml^{-1} for dimetridazole, 2-hydroxydimetridazole, halofuginone, robenidine and diclazuril and in the concentration range 0–20 ng ml^{-1} for dinitrocarbanilide on three different days. A smaller concentration range was use for dinitrocarbanilide due to the smaller linear range for this compound. Dimetridazole-D_3 and diclazuril-bis were added at a concentration of 10 ng ml^{-1} and 5 ng ml^{-1}, respectively. The determination coefficients (R^2) were at least 0.99 for all compounds for the three graphs. Detailed results are presented in Table 2. For each compound, three calibration graphs of spiked egg samples were made. This was done on three different days in the concentration range 0–200 μg kg^{-1} for dimetridazole, 2-hydroxydimetridazole, halofuginone, robenidine and diclazuril and in the concentration range 0–75 μg kg^{-1} for dini-

Table 1
Mass spectrometric conditions

Compound	Ionisation mode	*m*/*z*, precursor ion	Cone voltage (V)	RF lens 1 (V)	Capillary (kV)	*m*/*z*, product ion	Collision energy (eV)
Diclazuril	ES−	404.9, 406.9	50	50	3.0	334.1, 336.1	16, 16
Dimetridazole	ES+	142.1	50	35	1.0	96.1, 81.2	12, 20
2-Hydroxy dimetridazole	ES+	158.1	40	30	1.0	140.1, 55.2	9, 14
Dinitrocarbanilide	ES−	301.1	35	35	2.5	137.1, 107.1	8, 30
Halofuginone	ES+	416.0	50	40	3.2	100.2, 120.1	20, 18
Robenidine	ES+	334.1	50	40	3.2	155.1, 138.1	18, 24
Dimetridazole-D_3	ES+	145.0	50	30	1.0	99.2	12
Diclazuril-bis	ES−	419.0	50	50	3.2	321.0	25

Table 2
Detailed results of the regression analysis of the standard calibration curves, calibration curves in the egg and feed matrix

	Matrix	Mean slope ± standard deviation	Mean R^2	Number of data points	Concentration range ($\mu g\,kg^{-1}$)
Diclazuril	Standard	1.15 ± 0.02	0.998	8	0–100
	Egg	0.71 ± 0.07	0.998	10	0–200
	Feed	3.29 ± 0.15	0.995	11	0–2000
Dimetridazole	Standard	9.35 ± 0.41	0.999	8	0–100
	Egg	1.02 ± 0.05	0.999	10	0–200
	Feed	0.88 ± 0.05	0.995	11	0–2000
2-Hydroxy dimetridazole	Standard	0.52 ± 0.03	0.998	8	0–100
	Egg	0.60 ± 0.10	0.997	10	0–200
Dinitrocarbanilide/nicarbazin	Standard	16.40 ± 0.40	0.998	6	0–20
	Egg	27.06 ± 5.57	0.993	8	0–75
	Feed	14.21 ± 1.41	0.987	11	0–2000
Halofuginone	Standard	0.75 ± 0.01	0.999	8	0–100
	Egg	0.60 ± 0.09	0.993	10	0–200
	Feed	0.24 ± 0.01	0.992	11	0–2000
Robenidine	Standard	1.52 ± 0.06	0.999	8	0–100
	Egg	1.29 ± 0.03	0.994	10	0–200
	Feed	0.12 ± 0.03	0.978	11	0–2000

trocarbanilide. The determination coefficients were at least 0.985. Linearity was also tested with spiked feed samples. Individual calibration curves in the range 0–2000 $\mu g\,kg^{-1}$ were made and good linearity results were obtained. It has to be noted that egg samples were spiked with dinitrocarbanilide while feed samples were spiked with nicarbazin. Detailed results of the regression analysis are presented in Table 2.

Specificity means the ability of a method to distinguish between the analyte being measured and other substances. This was tested in three different ways. First, individual standard stock solutions of 10 $ng\,ml^{-1}$ of the different coccidiostats studied were analysed on the presence of the other coccidiostats. No interferences could be observed. A second way of determining specificity is spiking blank matrix with a range of compounds that can be encountered with the compound of interest and analysing this spiked sample on the presences of the target analyte. This was done by spiking a blank egg sample with 50 μg/kg of avermectins (doramectin, abamectin, ivermectin, moxidectin and eprinomectin), ionophoric coccidiostats (narasin, salinomycin, lasalocid and monensin), β-lactam antibiotics (benzylpenicillin, cloxacillin, dicloxacillin, oxacillin, ceftiofur, cefapyrin, cefalexin, nafcillin, cefazolin, ampicillin and amoxicillin) and other nitroimidazoles (ronidazole and metronidazole). No interferences were detected. Also the analyses of 20 blank egg samples of different origin, was carried out in order to test specificity. All three approaches proved the specificity of the method for the compounds studied.

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 $1-\alpha$ whether the identified analyte is present. According to Commission Decision 2002/657/EC, CCα can be determined in different ways. For the egg matrix, CCα was determined by analysing 20 samples spiked at a concentration for which a signal to noise ratio of at least 3 for two transitions was obtained. For the feed matrix, CCα was determined by fortification of blank material. The acquired signal was plotted against the added concentration. The corresponding concentration at the *y*-intercept plus 2.33 times the standard deviation of the intercept equals the decision limit. 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 $1-\beta$. To determine CCβ for the egg matrix, 20 blank samples were spiked at the decision limit and analysed and quantified using a calibration curve in the matrix. The value of the decision limit plus 1.64 times the standard deviation of the measured content equals the detection capability. In the calibration curve approach, CCβ was calculated by adding 1.64 times the standard deviation of the intercept to the value of CCα. For substances for which no permitted limit has been established, α and β equal 1% and 5%, respectively. In the egg matrix, CCα varied from 0.5 $\mu g\,kg^{-1}$ for diclazuril to 2 $\mu g\,kg^{-1}$ for 2-hydroxydimetridazole. The CCα and CCβ values for both matrices are summarised in Table 3. Chromatograms of eggs spiked at CCα levels are presented in Figs. 3–5.

Table 3
Summary of CCα and CCβ values

Compound	Egg ($\mu g\,kg^{-1}$)		Feed ($\mu g\,kg^{-1}$)	
	CCα	CCβ	CCα	CCβ
Diclazuril	0.5	0.6	2.1	2.2
Dimetridazole	1	1.1	1.6	2.4
2-Hydroxydimetridazole	2	2.2	–	–
Dinitrocarbanilide/nicarbazin	1	1.2	5.8	8.6
Halofuginone	1	1.1	10.7	14.5
Robenidine	1	1.2	8.8	12.5

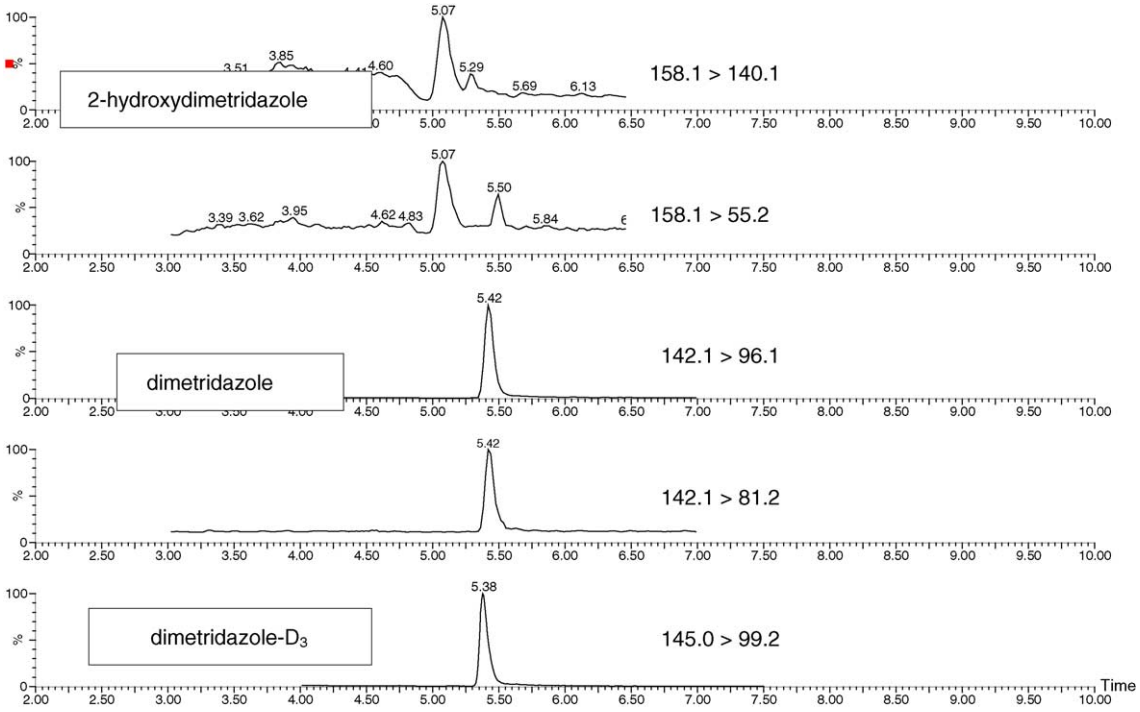

Fig. 3. Chromatogram of an egg spiked at 2 μg kg^{-1} 2-hydroxydimetridazole, 1 μg kg^{-1} dimetridazole and 10 μg kg^{-1} dimetridazole-D_3 (internal standard).

Since no certified reference material was available, trueness was proved with recovery. Repeatability and within-laboratory repeatability were determined to prove precision. Spiked samples were analyzed on three concentration levels at three different occasions. Six replicates of known negative egg or feed samples were spiked at three concentrations, extracted and analysed. Quantification was performed with a calibration curve in the matrix. Detailed results of the trueness and precision experiments are presented in Table 4. For the egg matrix, mean recoveries between 87.7% and 108.2% were obtained, while for the feed matrix, mean recoveries varied between 95.1% and 105%. Commis-

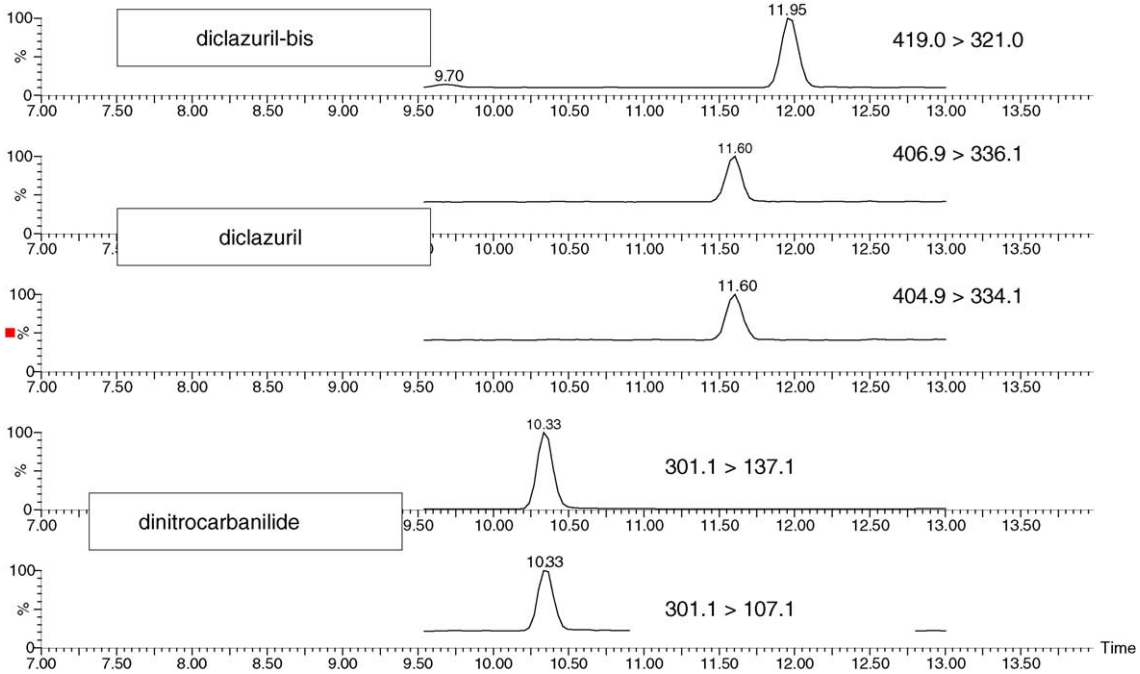

Fig. 4. Chromatogram of an egg spiked at 0.5 μg kg^{-1} diclazuril, 1 μg kg^{-1} dinitrocarbanilide and 5 μg kg^{-1} diclazuril-bis (internal standard).

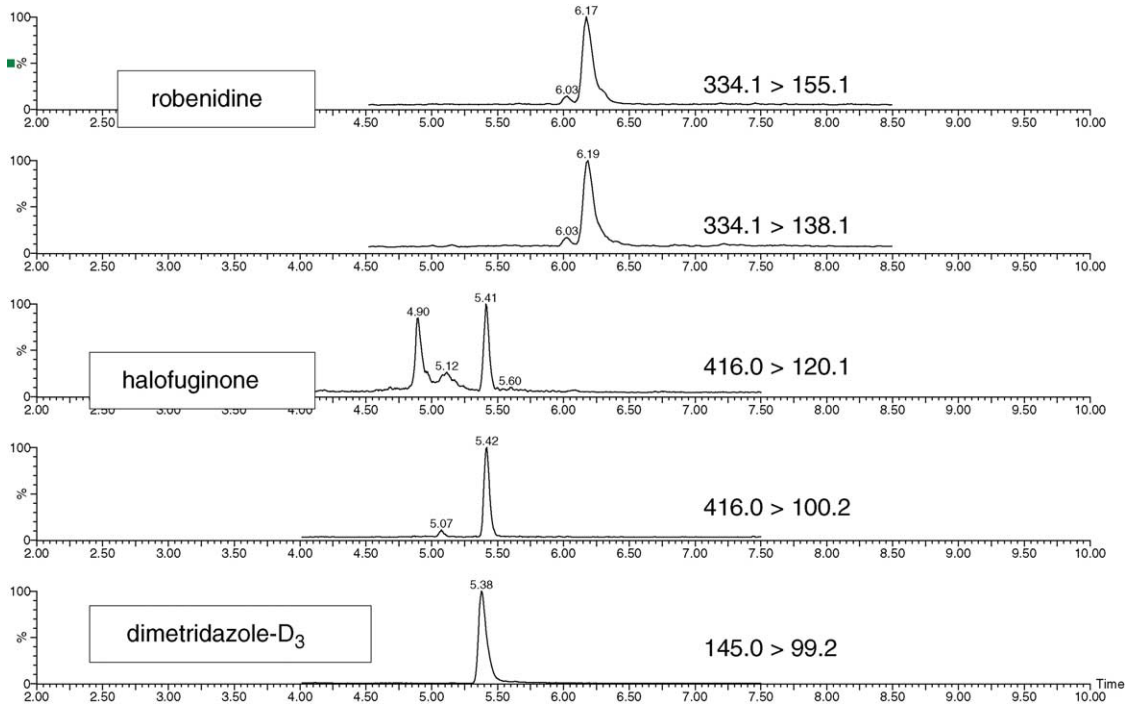

Fig. 5. Chromatogram of an egg spiked at 1 μg kg^{-1} robenidine and halofuginone and 10 μg kg^{-1} dimetridazole-D_3 (internal standard).

sion Decision 2002/657/EC states that for concentrations between 1 μg kg^{-1} and 10 μg kg^{-1}, a recovery between 70% and 110% should be obtained. For concentrations above 10 μg kg^{-1}, recovery should fall between 80% and 110%. No values outside these limits were found.

For analyses carried out under repeatability conditions, the intra-laboratory coefficient of variation (CV) should not exceed two-thirds of the values calculated by the Horwitz equation. This means that the CV should not exceed 23.7%, 21.3%, 16.7%, 15.1%, 11.8% and 10.6% for the concentration levels of 5 μg kg^{-1}, 10 μg kg^{-1}, 50 μg kg^{-1}, 100 μg kg^{-1}, 500 μg kg^{-1} and 1000 μg kg^{-1}, respectively. This criterion is fulfilled for all compounds in both matrices. However, Commission Decision 2002/657/EC states that for mass fractions lower than 100 μg kg^{-1} 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 4
Detailed results of the inter- and intra-day precision and recovery experiments in the egg and feed matrix

		Concentration A[a]		Concentration B[a]		Concentration C[a]	
		Mean CV (%)	Mean rec (%) ± standard deviation	Mean CV (%)	Mean rec (%) ± standard deviation	Mean CV (%)	Mean rec (%) ± standard deviation
Diclazuril	Egg	4.9	95.5 ± 4.4	3.8	108.2 ± 0.6	3.8	108.1 ± 1.4
	Feed	4.1	102.8 ± 3.2	3.9	105.0 ± 3.6	4.4	101.5 ± 4.9
Dimetridazole	Egg	2.4	101.4 ± 1.2	2.1	101.8 ± 2.3	1.5	99.5 ± 2.2
	Feed	2.5	99.6 ± 2.41	2.6	101.7 ± 1.9	1.4	102.7 ± 1.4
2-Hydroxy dimetridazole	Egg	5.1	87.7 ± 10.2	3.7	93.5 ± 15.6	3.4	93.7 ± 7.5
Halofuginone	Egg	6.5	96.8 ± 2.4	3.8	105.0 ± 2.6	2.0	100.4 ± 1.0
	Feed	4.6	95.1 ± 1.6	4.5	95.8 ± 1.8	6.2	99.8 ± 2.5
Robenidine	Egg	7.4	104.9 ± 2.3	4.9	100.6 ± 3.4	3.6	101.1 ± 1.0
	Feed	6.2	102.0 ± 1.9	5.9	96.3 ± 1.7	4.9	99.9 ± 0.8
Dinitrocarbanilide/nicarbazin	Egg	6.2	101.2 ± 6.7	6.3	99.9 ± 4.8	4.7	97.1 ± 5.4
	Feed	7.5	97.0 ± 11.6	4.7	99.1 ± 5.2	3.6	96.3 ± 2.8

[a] For the egg matrix: for diclazuril, dimetridazole, 2-hydroxydimetridazole, halofuginone and robenidine: conc. A = 5 μg kg^{-1}; conc. B = 50 μg kg^{-1}; conc. C = 100 μg kg^{-1}and for dinitrocarbanalide: conc. A = 5 μg kg^{-1}; conc. B = 10 μg kg^{-1}; conc. C = 50 μg kg^{-1}. For the feed matrix: conc. A = 50 μg kg^{-1}; conc. B = 500 μg kg^{-1}; conc. C = 1000 μg kg^{-1}.

For analyses carried out under within-laboratory reproducibility conditions, the within-laboratory CV should not be greater than the reproducibility CV. This reproducibility CV, calculated according to the Horwitz equation, equals 35.5%, 32.0%, 25.1%, 22.6%, 17.8% and 16.0% for the concentration levels of 5 $\mu g\,kg^{-1}$, 10 $\mu g\,kg^{-1}$, 50 $\mu g\,kg^{-1}$, 100 $\mu g\,kg^{-1}$, 500 $\mu g\,kg^{-1}$ and 1000 $\mu g\,kg^{-1}$, respectively. No values above these were obtained for neither the egg or feed matrix. Also here applies the remark that CVs should be as low as possible when the concentration levels are lower then 100 $\mu g\,kg^{-1}$.

4. Conclusions

A sensitive and selective LC–MS/MS method for the detection of five coccidiostats and one metabolite was described. The addition of a wash step with hexane and the use of a rheodyne divert valve improved the ruggedness of the method for the egg matrix described earlier significantly. The methods were validated based on Commission Decision 2002/657/EC.

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