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Liquid Chromatography Tandem Mass Spectrometry Analysis of Synthetic Coccidiostats in Eggs

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Abstract: Coccidiostats are synthetic drugs administered to animals, especially to poultry, to cure coccidiosis. In this paper, we present a selective liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to analyze residues of five synthetic coccidiostats in eggs: clazuril, diclazuril, robenidine, nicarbazin, toltrazuril and its two metabolites. The extraction efficiency was evaluated by testing several solvents, pH, different volumes and time of extraction. The clean-up procedures were optimized using different solid phase extraction cartridges and different eluants. The chromatographic separation was achieved in reversed phase using a gradient of 0.1% formic acid in water and acetonitrile, whereas the MS detection was performed in negative electrospray ionization (ESI) for all the analytes, except for the robenidine. The developed method has been validated according to Commission Decision 2002/657/CE. The validation parameters, as linearity, precision, recovery, specificity, decision limit (CCα), detection capability (CCβ), and robustness have been determined. The proposed method resulted simple, fast, and suitable for screening and confirmation purposes.

Keywords: synthetic coccidiostats; eggs; mass spectrometry

1. Introduction

Coccidiosis is a parasitic disease caused by the development and multiplication of coccidia in the intestine cells. It is caused by the development and multiplication of coccidian protozoa belonging to the *Eimeria* (the most predominant) or *Cryptosporidium* species. This infection is not lethal in healthy animals, but it can cause weight loss, low growth, and intestinal lesions, and every year, it leads to severe losses in meat and egg production [1].

Coccidiostats are drugs that are administered against the coccidiosis both for prophylactic chemotherapy and for health care [2]. To this aim, two different kinds of drugs are employed: ionophores and synthetic drugs. They are administrated in feed and/or in zootechnical supplements and some of them are included in the European Union register of Feed Additives [3]. In this paper, we investigated the simultaneous detection and quantification of clazuril, diclazuril, robenidine, nicarbazine and toltrazuril (including its main metabolites) in eggs (Figure 1).

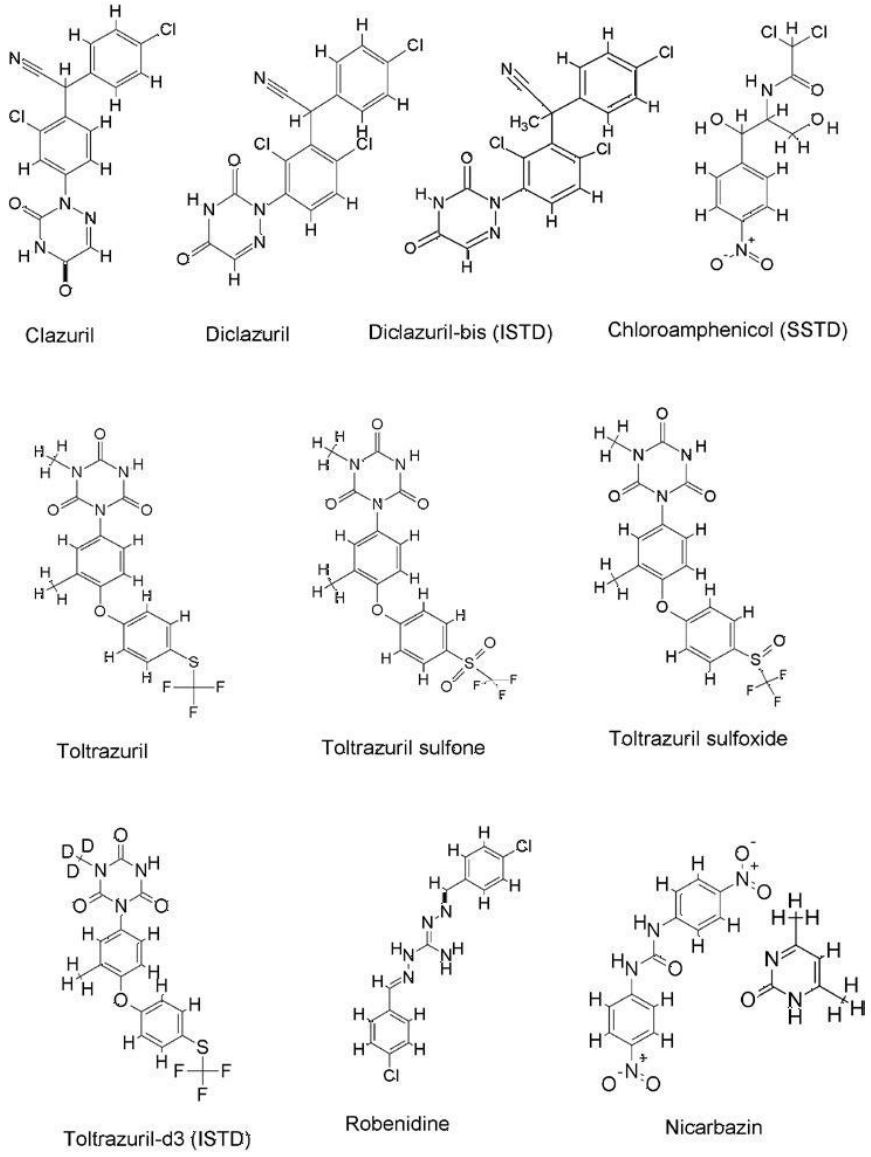

Figure 1. Molecular structures of the investigated coccidiostats and of the internal (ISTD) and external standards (SSTD).

Toltrazuril after administration to chicken by drinking water, is absorbed, metabolized and excreted as toltrazuril sulfone (main metabolite) and toltrazuril sulfoxide. Toxicology, metabolism and its characteristics are reported in the Summary Report on toltrazuril of the European agency for the evaluation of medicinal products [4,5]. Its use is regulated by Commission Regulation N 37/2010 [6].

Diclazuril is administered orally, and it is excreted by feces [7]. Diclazuril and toltrazuril act effectively against a large spectrum of coccidia. The first one is usually added to feed (about 1 ppm) for prevention purposes, whereas the other is added to water for disease care. Clazuril shows the same chemical-physical properties and acting way of diclazuril [8].

Nicarbazin is the equi-molar complex of 4,4-dinitrocarbanilide (DNC) and 2-hydroxy-4,6 dimetylpyrimidine (HDP) and it is administered by feed.

The DNC moiety is metabolized and excreted more slowly respect to HDP; consequently most of residue analyses for nicarbazin are based on determination methods for DNC moiety. Nicarbazin, being strongly electrostatic, can lead to cross contamination of feed production lines after milling of medicated feed [9].

Robenidine is administered by feed, like nicarbazin, and may give rise to phenomena of cross contamination [10]. In the past, it was discarded because of phenomenon of drug resistance, but it was then reintroduced for coccidian resistance to ionophores.

As a result of cross-contamination in feed intended for different species, the Commission decided to publish a Regulation 610/2012 [11], reviewing the allowable value of certain coccidiostats, previously set by the Regulation 124/2009 [12].

Several analytical methods have been developed for the analysis of one or more coccidiostats in different biological matrixes and with different techniques, as reported by Mortier et al. [13]. Most of the works use a chromatographic technique, especially high-performance liquid chromatography coupled with ultraviolet detector or mass spectrometric detector [14–28].

High resolution liquid chromatography-UV technique was used for the determination of nicarbazin and robenidine in eggs and in feeds [14–17]. Several authors used an HPLC-MS technique for single and multiresidual determination of these compounds in eggs, in muscle and in feedstuffs [18–25] reaching a lower Limit of Detection (LOD) than HPLV-UV. Sample preparation is a critical step in the analysis of coccidiostats [26], and sometimes highly specific and selective methods like immunoaffinity chromatography (IAC) can be used, [27], but this procedure, however, is very long, complex and expensive for routine analysis.

In 2012, a multiresidue method, including 20 coccidiostats, in eggs [28] was published. Five grams of egg were extracted by 20 mL of CH_3CN and after evaporation of the volume to dryness, the residue was reconstituted and injected to HPLC-MS/MS. Clazuril was not included in this study. Recently, an exhaustive review [29] summarized the analyses of coccidiostats in meat and other food.

At the moment, there are few papers about toltrazuril and its metabolites in eggs or in feedstuffs [30–32] and, to our knowledge, there are no scientific works about these five synthetic coccidiostats and the toltrazuril metabolites all together.

Due to the massive use of these drugs in poultry, in this study, we proposed a method for a simultaneous determination of the mentioned five synthetic coccidiostats in eggs together with toltrazuril metabolites, in order to be applied in research and routine control laboratories.

Starting from the literature about the topic and according to other authors [33], considering the ion suppression in the electrosray ionization to be one of the main problems (when analyzing drugs in complex matrices like eggs), we optimized a liquid–liquid extraction and solid phase extraction (SPE) purification of analytes before the analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in multiple reaction monitoring MRM mode. To this aim, several extraction solvents, extraction time, solvent volume, pH of extraction, clean-up cartridges and solvent for the elution were investigated for the highest recovery of the analytes from the eggs, and the results were evaluated to study the efficacy of the method. At the end, after the optimization of the mass spectrometric parameters and chromatographic conditions, the proposed method was validated according to Commission Decision 2002/657/CE [33,34].

2. Materials and Methods

2.1. Chemicals, Reagents and Solutions

Toltrazuril (TOL), Robenidine (ROB) hydrochloride, Chloroamphenicol (CAP) (External Standard ES), Diclazuril (DCLAZ) were from VETRANAL®, Fluka, Sigma-Aldrich (Milwaukee, WI, USA); Clazuril (CLAZ), Diclazuril (DCLAZbis) bis (Internal Standard ISTD) were from Janssen Animal Health (Beerse, Belgium); Nicarbazin (NIC) was from Sigma-Aldrich (Milan, Italy); Toltrazuril-d_3 (TOL-d_3) (ISTD), Toltrazuril sulfone, Toltrazuril sulfoxide were from Witega Laboratorien Berlin-Adlershof GmbH, Magnustrasse, (Berlin, Germany). Acetonitrile (AcN), methanol (MeOH), Ethyl-acetate (HPLC grade), acetone, ammonium acetate, ammonium formate (RPE), ammonium hydroxide, ammonium sulphate (RPE) were from Carlo Erba reagents (Milan, Italy). Dimethylformamide (DMF) was from R.P. Normapur AR, VWR (West Chester, PA, USA). Formic acid (98%–100%) and acetic acid were

from Merck (Darmstadt, Germany). Water was HPLC grade (generated by Milli-Q biocel, Millipore, purification system (Merck S.p.a. Vimodrone (MI) Italy).

Individual stock standard solutions were prepared at a concentration of 1 mg/mL.

Solvents used were: DMF for clazuril, diclazuril e nicarbazin and methanol for robenidine, toltrazuril, toltrazuril sulfone and toltrazuril sulfoxide. The stock solutions were stored at −20°C.

Working solutions were prepared by diluting stock solutions in acetonitrile to obtain solutions from 100 to 10 μg/mL. Standard mixtures were daily prepared by mixing adequate volume of each working solution and diluting in water-acetonitrile (30/70 v/v) up to a final concentration between 0.001 and 1.0 μg/mL. To enhance the quantification and the robustness of the method two internal standards were used: toltrazuril-d_3 for toltrazuril, toltrazuril sulfone and toltrazuri lsulfoxide; and diclazuril-bis for diclazuril, clazuril and robenidine. Stock solution were prepared in DMF for diclazuril bis and in methanol for toltrazuril-d_3 and stored at −20 °C.

2.2. HPLC-MS/MS Apparatus and Conditions

The LC system was a PE LC-200 Micro Pump (binary pump, vacuum degasser, autosampler AS 200) (Perkin-Elmer Sciex Instruments, Milan, Italy) coupled to a Triple Quadrupole LC-MS/MS Mass Spectrometer API2000 (Applied Biosystem, Foster, CA, USA) The MS system was controlled by Analyst software (version, Applied Biosystem, Foster, CA, USA). A chromatographic separation was achieved on reversed phase system using a C_{18} column (5 μm, 150 mm × 2.1 mm), Gemini, Phenomenex (Torrance, CA, USA) protected by a guard column containing the same packing material. Gradient elution was performed, starting from 70% of 0.1% aqueous formic acid (A) and acetonitrile (B) up to 20% A and 80% B in 15 min. Flow rate was 0.2 mL/min. A syringe pump (Harvard apparatus, Holliston, MA, USA) was connected to the interface for tuning purposes and to add ammonium hydroxide (0.08%) post column, before the mass spectrometer. All the experiments were performed in ESI (electrospray ionization) in negative mode, except for the robenidine, acquired in positive ionization. For each compound, the protonated or deprotonated molecular ion, $[M + H]^+$ or $[M - H]^-$, was chosen as a precursor and was subsequently fragmented by nitrogen, also used as drying and nebulising gas. The source block and desolvatation temperature were set at 120 °C and 380 °C, respectively. Dwell time was around 100 ms for each analyte.

2.3. Sample Preparation

One gram of homogenized eggs, purchased from the local market, was weighed in a falcon tube. Furthermore, 50 μL of the internal standard solution of TOL-d_3 and DCLAZbis were added to all samples. At this step, if necessary, samples were spiked by a specific amount of analyte standard solution mixture. The samples were then vortexed and allowed to stand for 15 min. Samples were extracted by 5 mL of AcN, placed in a ultrasonic bath for 5 min, then placed in a horizontal shaker for 20 min and finally centrifuged for 5 min at 4000 rpm. The supernatant (5 mL) was transferred into a graduated tube, diluted up to 25 mL withMilli-Q water and passed through a polymeric cartridge SPE Varian (60 mg) (Varian, Palo Alto, CA, USA), pre-activated with 3 mL of methanol and washed with 5 mL water. Two washing steps, using 5 mL of water and subsequently 5% aqueous MeOH, were tested before the elution with 5 mL of MeOH. The eluate was evaporated to dryness, using nitrogen at 40 °C. In addition, 50 μL of CAP solution (100 ng/mL) in aqueous AcN (70%) was used to reconstitute the dried residue and aliquots of 5 μL were injected into the LC–ESI-MS/MS system on a C_{18} column.

3. Result and Discussion

3.1. Optimization of the Chromatographic and Mass Spectrometric Conditions

Firstly, all the chromatographic conditions were optimized. Since all of the investigated compounds have an absorption in the UV region (between 240 and 350 nm), preliminary experiments were performed by HPLC with a diode array detector to select the column and the most suitable

chromatographic conditions for the purpose (data not shown). Three C_{18} columns were tested: a Discovery, Supelco, (Sigma Aldrich, Milan, Italy), a Zorbax (Agilent, 20063 Cernusco sul Naviglio, MI, Italy), anda Gemini (Phenomenex srl 40013—Castel Maggiore, Bo Italy) 4.6 × 150 mm, 5 µm at a flow rate of 1 mL·min^{-1}. The best selectivity was obtained by Gemini C_{18} column. Since the mobile phase composition has a significant effect on the peak shape and on the retention behavior of the analyte in the LC column, as well as on the MS response, modifications of the mobile phase (water-MeOH and water-AcN) in isocratic and gradient conditions with different additives were also tested.

The best performance, in terms of the mobile phase, was obtained by 0.1% formic acid-AcN in gradient elution with the addition of ammonium hydroxide (0.08%) at the exit of the column, prior to the mass spectrometer, in order to increase the ionization response.

As expected, the limits of quantifications (LOQs) were too high for the legal limits imposed for these substances; therefore, the method was further readapted to LC-MS/MS analysis and then validated according to the Commission Decision 2002/657/CE. The development of an MRM LC/MS/MS method requires experiments carried out by infusion at 10 µL·min^{-1} of standard solutions (2.5–10 ng·µL^{-1}), in order to determine suitable source parameters for the best sensitivity and signal to noise S/N ratio, as well as the molecule-related ions.

First of all, we selected the more effective ionization mode (ESI vs. Atmospheric pressure chemical ionization—APCI), enhancing the formation of protonated/deprotonated molecular ions of the target analytes. Experiments were carried out in negative and positive polarity using different mobile phase mixtures (MeOH and AcN) and additives such as: formic and acetic acid, ammonium formate and acetate, NH_3 and water. All the instrumental parameters, and potentials, such as ion source voltage (IS), cone potential (CP), and collision energy (CE), were optimized in order to maximize the quasi-molecular ion intensities on Q_1 and the MS/MS transitions in Q_3.The best results were obtained operating with ammonium formate 20 mM (Figure 2) in negative ion mode for all compounds, except for robenedine, more efficiently ionized in positive ion mode.

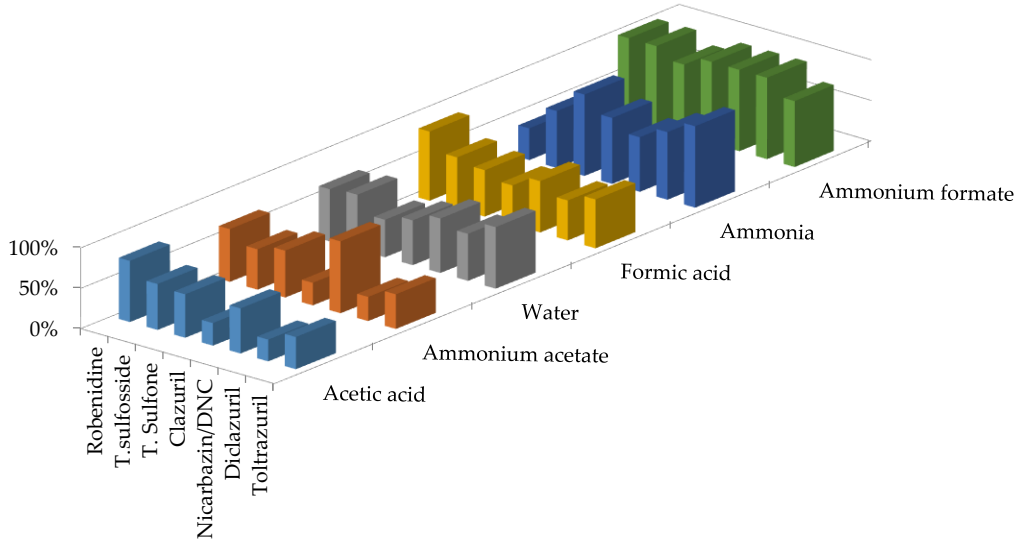

Figure 2. Effect of different additives in the mass spectrometric response of the investigated analytes.

For qualitative purposes, a minimum of 3.5 identification points are necessary. In LC-MS/MS, the transition of one precursor ion into two product ions corresponds to four identification points, and this criterion is accomplished except for diclazuril, toltrazuril and toltrazuril sulfone because they have only one fragment as a product ion. In this case, we used two precursor ions and one fragment from each precursor.

All the transitions and the optimized electrical parameters are reported in Table 1. For the development of the method to detect nicarbazin, we focused on the DNC moiety.

Finally, all of the analyses were performed in HPLC-MS/MS in MRM mode, according to Section 2.2 and acquired the most intense transitions from Table 1. To gain detection sensitivity, NH_3 was added post column to the eluate prior to the mass spectrometer. The analytes were eluted in less than 12 min.

Table 1. Summary of electric parameters, precursor and fragment ions chosen for the multiple reaction monitoring (MRM) analysis and chromatographic retention times.

Compound	Ionization	*m*/*z* Precursor Ion (Intensity %)	Capillary (kV)	Cone Voltage (V)	*m*/*z* Product Ion (Intensity %)	Collision Energy (eV)	Retention Time R_t (min)
Chloroamphenicol CAP (SSTD)	ES−	325.0 (10)	−4200	−35	194.0 (100) 152.0 (45)	−18 −20	5.7
Robenidine	ES+	334.1 (58)	+5300	50	138.0 (90) 155.0 (100)	35 28	6.7
Toltrazuril Sulfoxide	ES−	440.3 (60)	−4200	−40	42.1 (80) 371.0(100)	−25 −22	8.6
Toltrazuril Sulfone	ES−	456.2 (10) 456.2 (10)	−4200	−40	42.1 (100) 456.0 (100)	−30 −10	9.8
Clazuril	ES−	371.2 (9)	−4200	−40	299.9 (100) 265.0 (39)	−22 −22	9.8
Nicarbazin/DNC	ES−	301.0 (37)	−4200	−40	107.0(5) 137.0 (100)	−43 −20	10.1
Diclazuril	ES−	405.0 (6) 406.9 (6)	−4200	−40	333.8 (100) 335.8 (100)	−25 −25	10.5
Diclazuril-bis (ISTD)	ES−	419.0 (20)	−4200	−40	321.0 (80) 348.0(100)	−22 −20	10.8
Toltrazuril	ES−	424.0 (37) 424.0 (37)	−4200	−40	42.1 (100) 424.0 (100)	−38 −9	10.9
Toltrazuril-d3 (ISTD)	ES−	427.0 (30)	−4200	−40	42.1 (100)	−38	10.9

3.2. Optimization of the Clean-Up Procedure

The clean-up procedure was structured in the following steps:

- optimization of the liquid–liquid extraction (nature and volume of solvent, time and pH of extraction)
- optimization of the purification of the extracts (choice of the SPE cartridge, washing and elution conditions)

Preliminary experiments were done directly on the standard solution of analytes for the choice of the conditions to meet the goals of the best extraction and purification steps.

Successively, in order to optimize the clean-up procedure, 1 g of egg, spiked with each analyte at different level, was used. In order to find the best extraction conditions, we tested different kinds of solvents in different volumes: AcN, ethyl acetate, and a mixture of ethyl acetate:acetone (50:50, v/v), different pH, extraction times, and extraction in ultrasonic bath and in the horizontal shaker. In Table 2, the extraction efficiency for the tested organic solvents is shown. The best recovery was obtained with AcN, on average, 85% for all the compounds. Good results were obtained with ethyl acetate too, except for robenedine, where the recovery was only 10%. The mixture of ethyl acetate/acetone 50:50 v/v improved the extraction of toltrazuril and its metabolites (80%), but decreased the recovery of the other compounds to 30%. The main concern using the ethyl acetate/acetone mixture is due to a higher extraction of the lipid components as well, which can cause interferences and higher background.

Table 2. Extraction efficiency comparing different extraction solvents: ethyl acetate, acetonitrile, and ethyl acetate-acetone = 50:50.

Compound	Ethyl Acetate ($x_m \pm \sigma$)%	Acetonitrile ($x_m \pm \sigma$)%	Ethyl Acetate–Acetone = 50:50 ($x_m \pm \sigma$)%
Robenidine	10 ± 3.2	82 ± 1.0	33 ± 2.0
ToltrazurilSulfoxide	75 ± 2.2	72 ± 0.9	81 + 2.1
ToltrazurilSulfone	73 ± 1.5	70 ± 1.3	85 ± 1.5
Clazuril	95 ± 3.1	95 ± 0.8	45 ± 1.0
Nicarbazin/DNC	90 ± 2.8	98 ± 2.1	25 ± 4.2
Diclazuril	88 ± 1.0	95 ± 1.1	31 ± 0.5
Toltrazuril	75 ± 0.5	70 ± 1.5	82 ± 3.0

x_m average of three different tests. σ standard deviation.

The optimum conditions in terms of time/volume of extraction were, respectively, 5 min in ultrasonic bath with 5 mL of solvent. An additional 20 min in the horizontal shaker improved the extraction efficiency. The pH was another parameter under study. Acidifying at pH 5 or alkalizing at pH 9.5, the egg, before the extraction, did not improve the extraction efficiency (data not shown). In order to improve the sample clean-up, reducing the lipid components, a SPE procedure was mandatory. Different brands of polymeric SPE cartridges were tested: OASIS (Waters spa Milan, Italy), Strata X (Phenomenex srl 40013—Castel Maggiore, Bo, Italy) and Varian. The extracts were diluted five times with Milli-Q water before being loaded in the cartridge. Two washing steps (5 mL each), the first one with water and the second one with aqueous methanol (5%, 10%, and 15%) were done before the elution step. The higher concentration of MeOH should facilitate the clean-up. It was observed that 5% aqueous MeOH was the best compromise to avoid the loss of analytes (data not shown). Both MeOH and AcN were tested for the elution step, but MeOH gave the best results. Strata X and Varian (60 mg) cartridges were comparable, but the Varian was more reproducible and gave slightly higher recovery for the target analytes, on average 80% with a coefficient of variation CV, calculated over three replicates, below 15%. The eluate was dried under nitrogen, reconstituted and analyzed in HPLC/ESI-MS/MS in MRM mode, as described above. We experimentally calculated the loss due to the evaporation step between 1% and 5%.

The acquisition has been divided into three periods: The first one between 0 and 6.2 min in negative ionization where the CAP is acquired, the second one between 6.2 and 7.5 min in positive mode where the robenidine is acquired and finally the third one between 7.5 and 12.0 min, where clazuril, diclazuril, toltrazuril sulfone, toltrazuril sulfoxide and nicarbazin are acquired.

4. Validation Study

The validation method was carried out according to the Revision of Commission Decision93/256/EC [32]; therefore, instrumental linearity, specificity, recovery, precision, CCα and CCβ and robustness were studied.

4.1. Linearity and Matrix Effect

The matrix effect can greatly affect the reproducibility and accuracy of the method. The linearity was checked for three days by calibration curve both in solution and in matrix (adding analytes and ISTDs at the end of the clean-up) in the following range of concentrations: 1–10 $\mu g \cdot kg^{-1}$ for CLAZ and DCLAZ, (corresponding to 20–200 ng/mL in solution), and 5–20 $\mu g \cdot kg^{-1}$ for TOL and its metabolites (corresponding to 100–400 ng/mL in solution), 5–40 $\mu g \cdot kg^{-1}$ for ROB (corresponding to 100–800 ng/mL in solution) and 10–45 $\mu g \cdot kg^{-1}$ for NIC (corresponding to 200–900 ng/mL in solution). The linearity in the investigated ranges was very good, as demonstrated by the correlation factors $R^2 \geq 0.999$ (data not shown). To evaluate matrix effects (ME) the slope of matrix matched calibration curves and the slope of standard calibration curves were calculated. The slope ratio (R)

×100 is defined as the matrix effect (ME %). A value of 100% indicates that there is no matrix effect. There is signal enhancement if the value is >100% and signal suppression if the value is <100% [35]. Thanks to the proposed clean-up procedure, reducing the interfering compounds in the matrix that alter the ionization in the source of the mass spectrometer, the solution and matrix-matched calibration curve equations had an ME % within 10%. Therefore, for quantitative purposes, we used the solvent calibration curves.

4.2. Specificity

Specificity was tested on 20 representative blank samples, compared to spiked ones with analytes and ISTD. The acquisition, as discussed previously, was divided into three periods according to the retention time of the analytes. In the target regions, no interfering peaks were observed.

4.3.Recovery

Once the best extraction and clean-up conditions were chosen, the recovery of the whole procedure was determined forsix spiked blank samples for each concentration level for three days. The levels were: 1, 2, 3, 4, 6 $\mu g \cdot kg^{-1}$ for diclazuril and clazuril and 5, 7.5, 10 and 15 $\mu g \cdot kg^{-1}$ for TOL and its metabolites. For ROB, the levels were 5, 12.5, 25, 37.5 $\mu g \cdot kg^{-1}$, whereas, for NIC, the levels were 100, 150, 300, 450 $\mu g \cdot kg^{-1}$ (applying the dilution factor of 10 to be inside the calibration curve range). The recovery was calculated by differences among nominal values of spiked blank samples and experimental values. The results were obtained by a solvent curve, since the matrix effect was not considerable, and the average recovery was about 80%, considering 62% as a minimum for the robenidine and 95% as a maximum for the toltrazuril, as shown in Table 3.

Table 3. Results of recovery and precision of the method.

Analyte	Validation Level	Experimental Concentration	Recovery	Repeatability			Reproducibility
	($\mu g \cdot kg^{-1}$)	($\mu g \cdot kg^{-1}$) ± σ (n = 18)	(%)	(CV%) (n = 6) 1	2	3	(CV%) (n = 18)
Robenedine	5.0	3.1 ± 0.4	62	13.7	14.7	11.9	13.9
	12.5	8.5 ± 0.8	68	11.5	10.1	9.0	11
	25.0	18.5 ± 1.2	74	7.1	11	7.0	9
	37.5	28.5 ± 2.5	76	8.0	10	13	12.0
Toltrazuril Sulfoxide	5.0	4.0 ± 0.5	80	13.0	7.5	7.5	13.6
	7.5	6.4 ± 0.6	85	5.7	4.6	10.5	10.2
	10.0	7.5 ± 0.6	75	9.4	8.8	8.4	9.2
	15.0	11.4 ± 1.3	76	8.2	13.2	12.5	11.5
Toltrazuril Sulfone	5.0	4.4 ± 0.5	88	9.6	4.2	11.5	11.6
	7.5	6.6 ± 0.6	88	9.1	5.4	11.4	9.2
	10.0	8.1 ± 0.9	81	12.2	7.8	8.4	10.5
	15.0	12.7 ± 1.1	85	5.0	10.1	11.4	8.6
Clazuril	1.0	0.8 ± 0.1	80	12.5	11.9	12.1	12.0
	2.0	1.7 ± 0.2	85	12.4	7.9	11.0	11.0
	3.0	2.4 ± 0.2	80	6.1	8.4	8.5	8.9
	4.0	3.2 ± 0.3	80	3.2	10.3	10.3	10.2
	6.0	5.4 ± 0.3	90	2.9	4.8	8.0	8.7
Nicarbazin	100.0	75.0 + 9.0	75	11.0	9.0	13.0	12.0
	150.0	110.0 + 11.0	73	9.0	10.0	12.0	11.0
	300.0	234.0 + 10.0	78	5.0	8.0	7.0	8.0
	450.0	315.0 + 28	70	9.0	8.0	12.0	9.0
	100.0	75.0 + 9.0	75	11.0	9.0	13.0	12.0

Table 3. *Cont.*

Analyte	Validation Level	Experimental Concentration	Recovery	Repeatability			Reproducibility
	($\mu g \cdot kg^{-1}$)	($\mu g \cdot kg^{-1}$) $\pm$ σ (n = 18)	(%)	(CV%) (n = 6) 1	2	3	(CV%) (n = 18)
Diclazuril	1.0	0.8 $\pm$ 0.1	80	11.0	13.0	12.1	12.1
	2.00	1.8 $\pm$ 0.3	90	7.0	5.6	8.8	13.7
	3.0	2.6$\pm$ 0.2	87	7.2	5.5	7.0	10.2
	4.0	3.2 $\pm$ 0.3	80	9.2	9.1	8.9	9.5
	6.0	5.1 $\pm$ 0.5	85	9.9	12.2	8.4	11.5
Toltrazuril	5.0	4.4 $\pm$ 0.3	88	9.3	7.7	8.8	14.1
	7.5	7.1 $\pm$ 0.5	95	5.5	4.1	9.5	6.4
	10.0	8.7 $\pm$ 0.9	87	11.7	12.4	10.1	10.9
	15.0	12.7 $\pm$ 1.2	85	8.1	9.2	11.3	9.8

4.3. Precision: Repeatability and Reproducibility Intra-Laboratory

Precision has been evaluated as repeatability and reproducibility intra-laboratory. Repeatability is measured as relative standard deviation (CV%) of repeated measures of six aliquots for each concentration level for three consecutive days, and the reproducibility as CV% of repeated measures of all three days (18 samples for each concentration level).The results are shown in Table 3; they are good for all analytes with CV% below 20% and they respect the recommendations of the 657/2002 Directive.

4.4. CCα and CCβ and Robustness (Minor Changes)

The decision limit (CCα) and the detection capability (CCβ) has been estimated according to Lynn Vanhaecke et al. [36] and Commission Decision 2002/657/EC [34].

The CCα is between 2.2 $\mu g \cdot kg^{-1}$ for diclazuril and 320 $\mu g \cdot kg^{-1}$ for nicarbazine, whereas the CCβ is between 2.2 $\mu g \cdot kg^{-1}$ for diclazuril and 350 $\mu g \cdot kg^{-1}$ for nicarbazine. The results are reported in Table 4. Robustness has been evaluated by Youden test on eight spiked blank samples at the CCβ concentration level. The parameters shown in Table 5 were chosen to evaluate how small changes can affect the proposed method, so one variable was chosen in the extractive step (extraction volume) and the other five variables in the purification step, including the stability of analytes at the boiling point of the solvent. The effect of each factor was calculated by determining the difference between the value of the variable at the highest and at the lowest level. Differences were not significant; in other words, the investigated parameters have no effect on the characteristics of the method, and, therefore, the method can be defined robust.

Table 4. CCα and CCβ values for the investigated analytes.

Analyte	CCα ($\mu g \cdot kg^{-1}$)	CCβ ($\mu g \cdot kg^{-1}$)
Robenidine	28.0	30.0
Toltrazuril sulfoxide	5.1	6.1
Toltrazuril sulfone	5.8	6.7
Clazuril	2.2	2.6
Nicarbazin/DNC	320.0	350.0
Diclazuril	2..2	2.6
Toltrazuril	6.0	6.9

CCα Decision limit.

Table 5. Youden experiment table for the robustness of the method.

Variable Number	Experiment Number							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Lot of SPE	lotA	lotA	lotA	lotA	lotA	lotA	lotA	lotA
Extraction Volume Acetonitrile (mL)	5.5	5.5	4.5	4.5	5.5	5.5	4.5	4.5
Dilution Volume with Water (mL)	30	20	30	20	30	20	30	20
Washing Volume for SPE with Water (mL)	5.5	5.5	4.5	4.5	4.5	4.5	5.5	5.5
Washing Volume for SPE with MeOH 5% (mL)	5.5	4.5	5.5	4.5	4.5	5.5	4.5	5.5
Elution Volume for SPE (mL)	5.5	4.5	4.5	5.5	5.5	4.5	4.5	5.5
Drying temperature (°C)	44	36	36	44	36	44	44	36

SPE solid phase extraction cartridge.

5. Conclusions

Due to themassive use of coccidiostats in poultry farming, for the prevention and treatment of coccidiosis, we developed a sensitive, simple, rapid and robust LC-MS/MS method, to detect, in eggs, simultaneously different synthetic coccidiostats for a total of seven analytes (five compounds and two metabolites), usually not analysed all together. Possible critical factors were examined and several clean-up strategies were tested to find the parameters and the conditions to meet the goals of the best extraction and purification step for screening and confirmation purposes. Experimental data showed that, thanks to a proper sample preparation, the matrix effect was drastically decreased, reducing the endogenous substances liable to interfere with the assay. This circumstance allowed us to use the solvent calibration curve for quantitative purposes.

The method has been validated in conformity with the main lines of the UE requirements for detecting residues of veterinary drugs in animal products and can be used to detect residues, in eggs, of the five coccidiostats at the level of μg/kg.

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