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Efficient and sensitive detection of residues of nine coccidiostats in egg and muscle by liquid chromatography–electrospray tandem mass spectrometry

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Abstract

We present a method based on electrospray liquid chromatography tandem mass spectrometry (LC–MS/MS) for determining in muscle and eggs the following nine coccidiostats: halofuginone, diclazuril, dinitrocarbanilide (the main metabolite of nicarbazin), robenidine, monensin, lasalocid, narasin, salinomycin, and maduramicin. Dinitrocarbanilide-d8, nigericin, and diclazuril-bis were used as internal standards. The method uses extraction in acetonitrile followed by a clean-up on an SiOH solid-phase extraction column. High-performance liquid chromatography (HPLC) separation was performed on a Purospher® C_{18} column (125 mm × 3 mm i.d.) protected by a guard column, the mobile phase being a water–acetonitrile gradient (each gradient component containing 0.1% formic acid) at a flow rate of 1 ml min^{-1}. For unequivocal identification of each analyte, two ions were detected and chosen for multiple reaction monitoring (MRM). Validation was carried out on spiked muscle and egg samples. The method described meets all the criteria of Decision 2002/657/EC and is easy to use in routine analysis. Validation results are presented with the measured CCα and CCβ values. This whole method allows extraction and analysis of up to 24 samples per day.

Keywords: Coccidiostats; Polyether ionophores; Residues; Meat; Eggs; Mass spectrometry

1. Introduction

Coccidiosis leads to severe health problems in several classes of livestock. It has a significant economic impact on farmers if no control is in place for its prevention. Historically, poultry has shown the greatest susceptibility to coccidiosis because it is most often produced intensively. In poultry, coccidiosis is caused by protozoa of the genus *Eimeria*, which spend most of their life in the intestinal tract of the host animal. Coccidiosis is extremely important in the poultry industry, but economic losses also occur in the production of cattle, sheep, goats, rabbits, and swine.

A wide range of drugs is available for the prevention and treatment of coccidiosis. Nicarbazin, halofuginone (HFG), robenidine, diclazuril, monensin, salinomycin, narasin, lasalocid, and maduramicin (see Fig. 1) are the most common anticoccidial drugs used.

Coccidiostats are considered feed additives for poultry and must meet a number of legislative criteria of Regulation 1831/2003/EC [1]. For these substances, withdrawal times have been established, but residues are frequently found in products derived from poultry eggs and meat.

Nicarbazin, a mixture of 4,6-dimethyl-2-hydroxypyrimidine (DHP) and 4,4′-dinitrocarbanilide (DNC) in a 1:1 molar ratio, is a drug used to prevent coccidiosis in broiler chickens. The DHP moiety is excreted rapidly following withdrawal of the drug, but DNC is less rapidly eliminated and is thus considered the only residue of concern left from nicarbazin in meat. Nicarbazin is not licensed for use in egg-laying chickens.

Diclazuril is a benzene acetonitrile derivative with antiprotozoal activity. For example, it exerts potent action against *Eimeria* species. Diclazuril is still allowed for laying hens.

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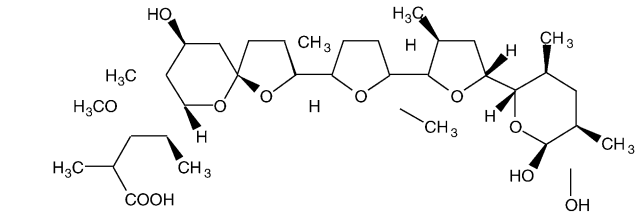

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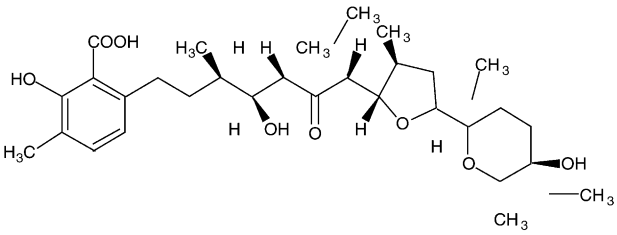

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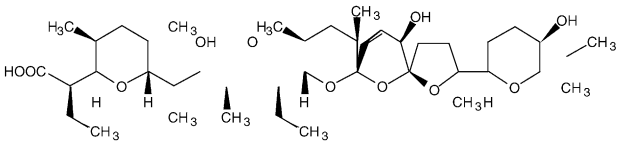

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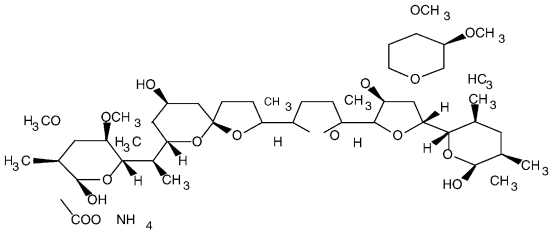
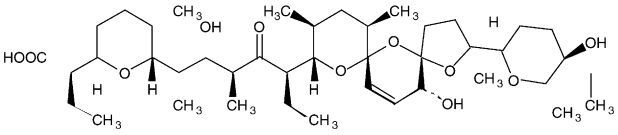
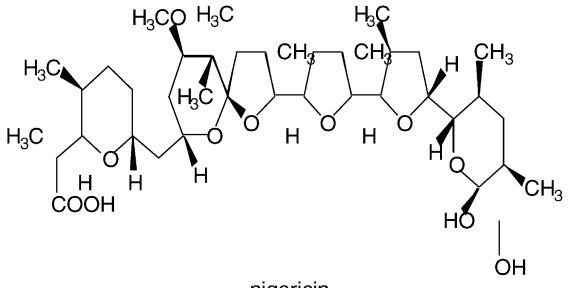


maduramicin

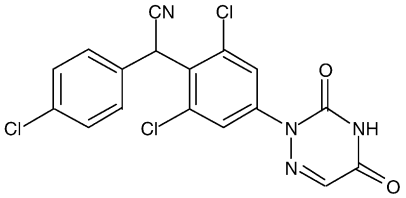


salinomycin

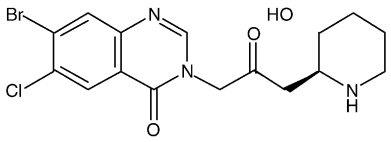
(a)



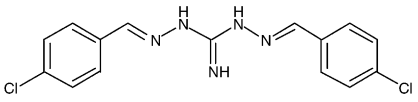
nigericin



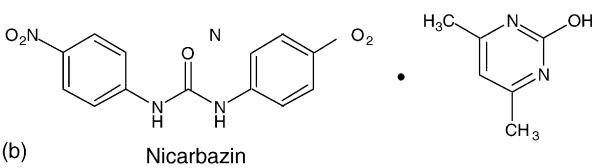
Diclazuril



halofuginone



Robenidine



(b) Nicarbazin

Fig. 1. Molecular structures of coccidiostats: (a) polyether ionophores (monensin, lasalocid, salinomycin, narasin, maduramicin, nigericin (IS)); (b) other coccidiostats (diclazuril, halofuginone, dinitrocarbanilide, robenidine).

Halofuginone is used worldwide in commercial poultry production to prevent coccidiosis. The European Agency for the Evaluation of Medicinal Products (EMEA) has established HFG as the marker residue and has set maximum residue limits (MRLs) of 10 and 30 $\mu g\,kg^{-1}$ for bovine muscle and liver, respectively, but HFG is not licensed for use in commercial egg production. Robenidine hydrochloride, i.e. 1,3-bis(*p*-chlorobenzylidene amino) guanidine hydrochloride, has a structure unique among anticoccidials. It is the only anticoccidial of the guanidine group and is unrelated chemically to any coccidiostat in use. This minimises the risk of cross-resistance.

Monensin, salinomycin, narasin, lasalocid, and maduramicin belong to a group of polyether antibiotics naturally produced by certain strains of *Streptomyces*. They are relatively large molecules with molecular masses of 613–940. The usual structural features include a carboxylic acid at one terminus and a hydroxyl group at the other. These compounds readily form cyclic complexes with cations, particularly the sodium ion, and have the ability to cross biological membranes.

Owing to the widespread use of these drugs on farms, there is a risk that residues will be present in animal products intended for human consumption. Suitable methods must therefore be available for detecting in animal tissues the presence of such residues, arising from improper use of anticoccidials or from cross-contamination.

Several analytical approaches have been used to determine one or more anticoccidial drugs in different biological matrixes: colorimetry [2,3], high-performance liquid chromatography (HPLC) with UV [4,5], fluorescence detection [6,7], and immunoassays [8]. In addition, a number of authors have described the use of LC coupled to mass spectrometry to determine nicarbazin [10,12–14], diclazuril [12,19], halofuginone [12], robenidine [12], and polyether ionophores [9,11,13,15–18]. The methods described herewith detect and/or quantify a limited number of compounds.

The aim of the present study was to develop a selective and sensitive multiresidues method. It has been developed and validated for the detection of nine coccidiostats among which five polyether molecules which present physico-chemical features different from the other coccidiostats. The validation has been realised on muscle and egg samples. The method can also be used for liver samples.

2. Experimental

2.1. Reagents, chemicals, and biological specimens

Reference products were dinitrocarbanilide (39,015-1), monensin (M-5273), lasalocid (46372), narasin (N-1271), salinomycin (S-4526), nigericin (N-7143) from Sigma (St. Louis, MO, USA), diclazuril (R064433), diclazuril-bis (R062646) from Janssen (Beerse, Belgium), robenidine (TC4123), maduramicin (TC4146) from Alpharma (Technical Center, Willow Island, USA), halofuginone from Intervet Belgium and dinitrocarbanilide-d8 (DNC-d8) from Witega (Laboratoire Berlin-Adlershof GmbH, Germany). Nigericin, diclazuril-bis, and dinitrocarbanilide-d8 were used as internal standards (IS).

Individual standard solutions were prepared by placing approximately 10 mg standard in a glass tube and adding an appropriate amount of solvent to reach a concentration of 1 $mg\,ml^{-1}$. The solvents used for preparing stock solutions were ethanol for lasalocid, narasin, salinomycin, monensin, robenidine, diclazuril-bis, and nigericin, dimethylformamide for diclazuril, water for halofuginone and maduramicin, and *N*,*N*-dimethylacetamide for dinitrocarbanilide. Secondary standard solutions (10 $\mu g\,ml^{-1}$) were obtained by diluting 0.1 ml stock solution in 9.9 ml solvent. Dilutions were then prepared from this solution to obtain a suitable range of working solutions. These solutions were stored in the dark at 4 °C.

All chemicals and reagents, including formic acid (98–100%), anhydrous sodium sulfate, dimethylformamide, *N*,*N*-dimethylacetamide, and ethanol (Merck, Darmstadt, Germany) were pro analysi. Acetonitrile and water were HPLC grade (Acros Organics, Geel, Belgium). Filters for filtration of the HPLC solvent were from Millipore (Millex GV, 0.22 μm).

Biological materials were subjected to solid-phase extraction (SPE) on a silica SPE column (500 mg, volume 3 ml) (Baker, Deventer, Holland).

Muscle samples from untreated chickens and egg samples from untreated hens were used as blanks and, after spiking with the different coccidiostats, as quality control specimens.

2.2. Apparatus

The following devices were used for extraction and clean-up: a balance, a mechanical shaker (Ika Labortechnick, Staufen, Germany), a centrifuge (RC-3B Refrigerated Centrifuge, Sorvall Instruments, Wilmington, USA), and a nitrogen evaporator (Turbo vap LV, Zymark Corporation, Hopkinton, USA). The HPLC equipment consisted of a Waters Alliance 2690 system (Waters Ltd., Watford, Herts, UK). For mass spectrometry, a Micromass Quattro Ultima tandem mass spectrometer (Micromass UK, Altrincham, UK) was used.

Glassware and other recipients were chosen as was suitable for each step of the procedure.

2.3. Chromatographic conditions

Chromatography was performed on a 125 mm × 3 mm column (Purospher, C_{18}, 5 μm) protected by a guard column (Purospher, C_{18}, 5 μm) (Merck). A gradient of water (A) and acetonitrile (B) was applied, each containing 0.1% formic acid. The flow rate was 1 $ml\,min^{-1}$. We used a two linear-step elution gradient. The initial conditions, maintained for 0.5 min, were A–B (100/0, v/v). During the first gradient step, the percentage of eluent B was increased to 40% over 4 min

while eluent A decreased to 60% (v/v). These conditions remained for 1 min. During the second gradient step, eluent B was brought to 100% over 3 min while eluent A gradually reached 0% (v/v). These conditions remained for 2 min. A 4-min post-run was used to return to the initial conditions.

2.4. MS–MS parameters

A solution of coccidiostats (0.1 μg ml^{-1} in the mobile phase) was infused into the detector at a rate of 10 μl min^{-1} and the capillary cone voltage was adjusted to yield the $[M + Na]^+$ ion for the ionophore compounds, the $[M - H]^+$ ion for robenidine and halofuginone, and the $[M - H]^-$ ion for dinitrocarbanilide, diclazuril, diclazuril-bis, and DNC-d8.

The analysis was performed in the positive- and negative-ion electrospray modes (ESP). The first 7.4 min of the run were in the positive ESP mode for robenidine and halofuginone. Then, we switched to the negative ESP mode until 9 min for dinitrocarbanilide, DNC-d8, diclazuril, and diclazuril-bis. Finally, we switched to the positive ESP mode for the polyethers.

Nitrogen was used as a desolvation gas at a flow rate of 600 l h^{-1} and also as a cone gas at a flow rate of 100 l h^{-1}. This HPLC flux was split 1/2, resulting in an approximate spray flux of 500 μl min^{-1}. The cone voltage generally varied from 30 to 35 V depending on the best signal of ionisation products. The other MS–MS parameters were: capillary voltage, 2.0 kV; source temperature, 130 °C; desolvation temperature, 450 °C; collision gas pressure, 2×10^{-3} mbar.

Product ion scans were studied so as to choose the best ions for monitoring each molecule in multiple reaction monitoring (MRM). The dwell times were 0.08 ms.

2.5. Sample preparation

A 5-g portion of blank or spiked sample (minced muscle, whole egg) was weighed into a 50-ml Falcon® tube and mixed with a small volume (20 μl) of internal standards (dinitrocarbanilide-d8, 10 ng; diclazuril-bis and nigericin, 5 ng). After a 10-min equilibration period, the muscle or egg was mixed vigorously with 10 g anhydrous sodium sulfate until a powdery or granular mixture was obtained. The granular mixture was not allowed to set at the bottom of the tube. If this occurred, the tube was inverted and with a tapping action the solid was dislocated to allow maximum contact with the solvent. Acetonitrile (15 ml) was added to the mixture, which was then vortexed for 1 min. The tube was placed on a horizontal shaker for 60 min at 300 motions min^{-1}. The mixture was then centrifuged at 4000 g for 20 min at 4 °C. All supernatants were further purified by solid-phase extraction. A silica cartridge was conditioned with 5 ml acetonitrile. Then an aliquot of extract (≈12 ml) was passed through the cartridge under gravity and the eluate was collected in a 15-ml conical plastic tube. The silica cartridge was washed with a further 2 ml of acetonitrile and this was collected in the glass tube. The combined eluate was evaporated dry under a nitrogen flow at 40 °C. The extracts were dissolved in 300 μl acetonitrile. Aliquots of supernatant were transferred to conical autosample vials and a 50-μl loop was used to inject the extract into the LC–MS/MS system.

3. Results and discussion

The aim of this work was to develop a multiresidue method, applicable to egg and muscle matrixes, for the simultaneous extraction, detection, and confirmation of nine coccidiostats among which polyethers.

The first step was to develop a simple work-up procedure for the simultaneous extraction of the nine coccidiostats. Best results were obtained by a method described by Matabudul et al. [13] for the determination of five anticoccidial drugs in animal livers and eggs by LC/MS–MS. The described method proved suitable for liver, muscle, and egg samples. By combining the simple and rapid extraction procedure with the elaborate monitoring of nine coccidiostats by LC/MS–MS, an analyst can assay up to 24 samples in a day's work with an overnight run.

Percentage recoveries after extraction were determined on muscle samples spiked with the various coccidiostats at 2 μg kg^{-1}. The formula used was: MS response of a compound obtained for samples spiked before clean-up, taking into account the response of the internal standard, divided by the MS response obtained with a blank matrix spiked after clean-up, and multiplied by 100. These results obtained for muscle were acceptable for all nine coccidiostats and are summarised in Table 1. Values ranged from 40% for halofuginone to 60% for dinitrocarbanilide, diclazuril, and lasalocid.

The second step of this work was to develop a quick, selective, and sensitive LC/MS–MS procedure. Identification of precursor and product ions in the positive and negative electrospray modes and preliminary optimisation of instrument settings were performed by continuous infusion of the individual compounds at 0.1 μg ml^{-1} in a mixture of acetronitrile/water (50/50, v/v) containing 0.1% formic acid. Fig. 2 shows the mass spectra (precursor and product ions) obtained for dinitrocarbanilide and diclazuril when the apparatus was tuned to the negative electrospray mode and for

Table 1
Extraction recoveries of the nine coccidiostats determined at 2 μg kg^{-1} level by means of spiked muscle samples

Compound	Extraction recovery (%)
Halofuginone	40
Robenidine	56
Dinitrocarbanilide	60
Diclazuril	60
Maduramicin	52
Monensin	56
Salinomycin	44
Narasin	53
Lasalocid	60

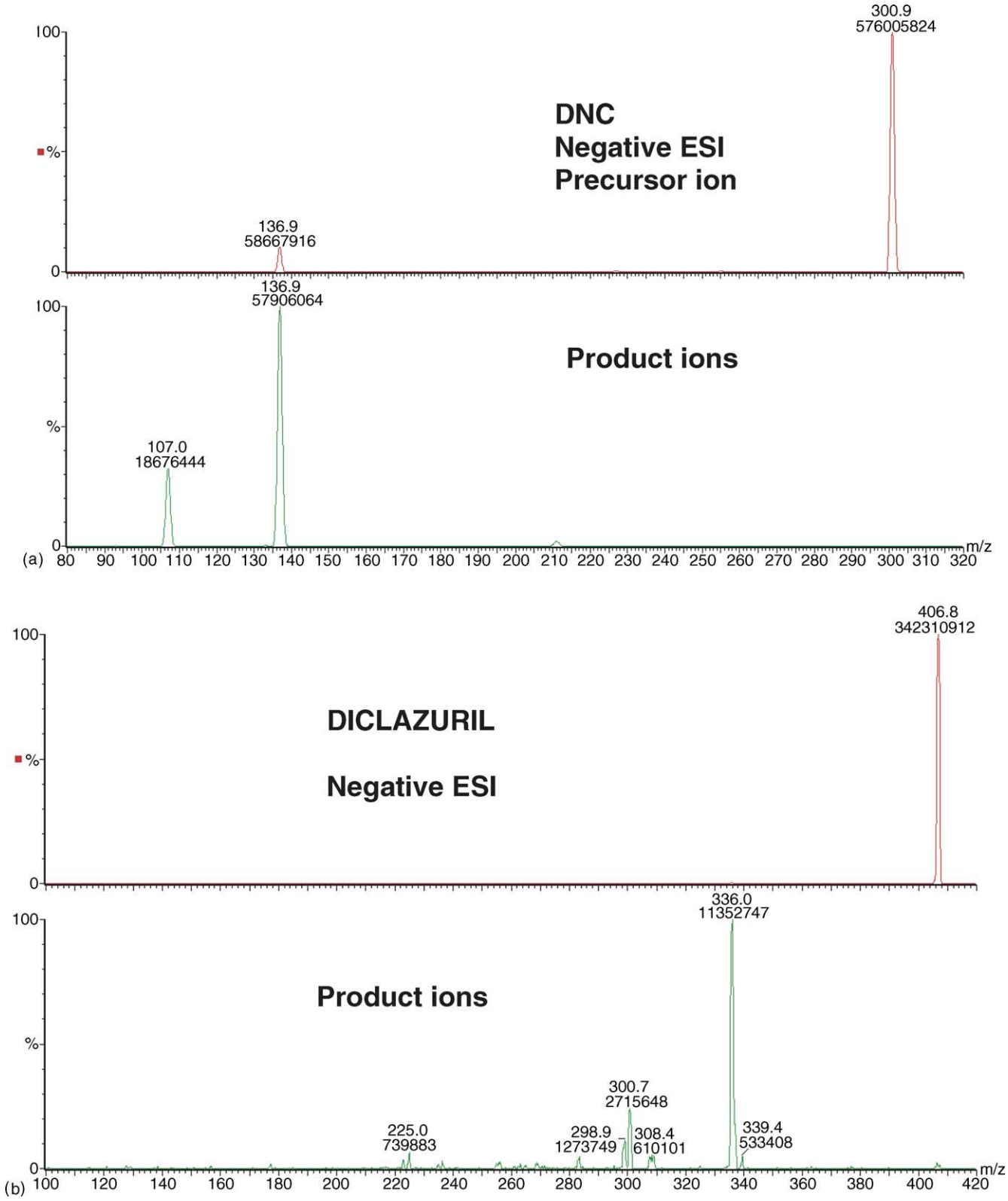
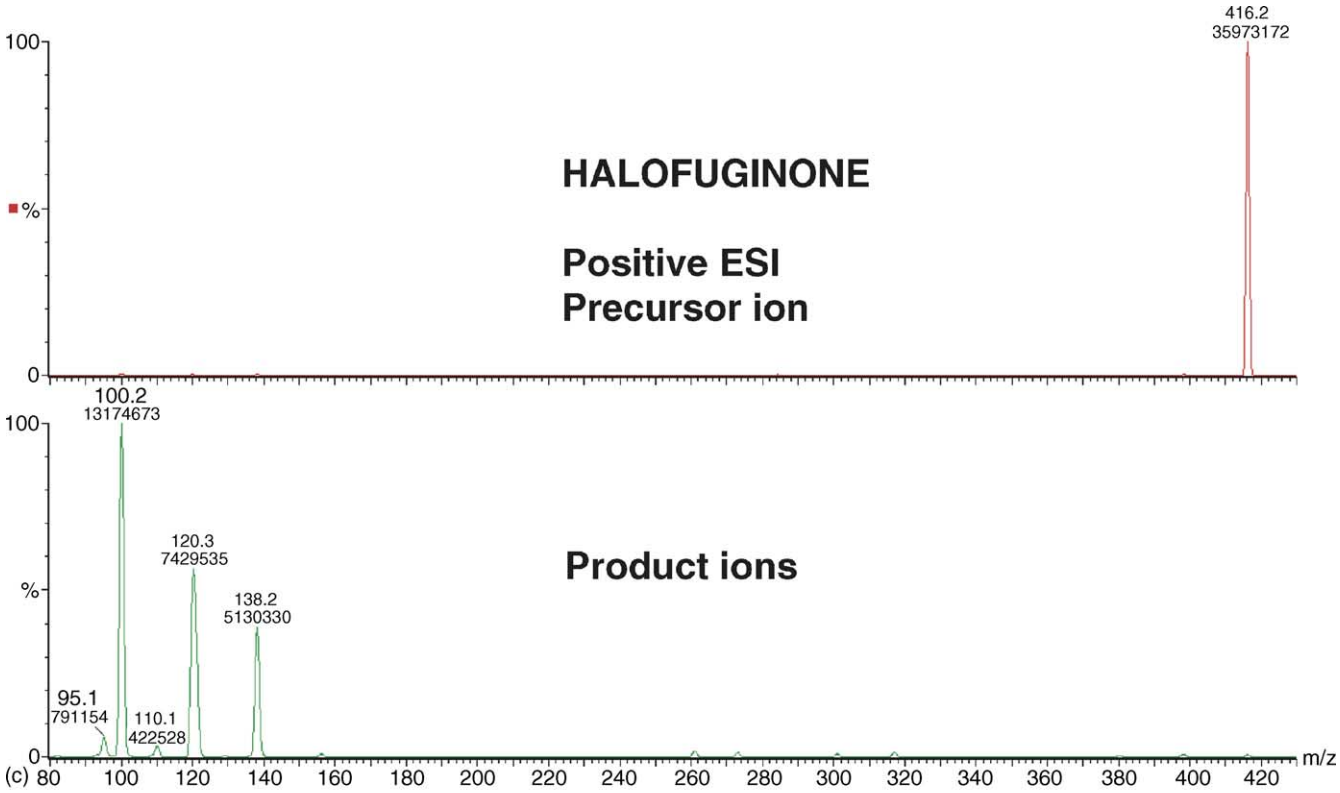


Fig. 2. ESI–MS and ESI–MS/MS spectra of anticoccidial drugs: (a) dinitrocarbanilide, (b) diclazuril, (c) halofuginone, (d) salinomycin; the infusion was performed in the negative mode for dinitrocarbanilide, diclazuril and diclazuril-bis (IS), and in ESI+ for other coccidiostats.



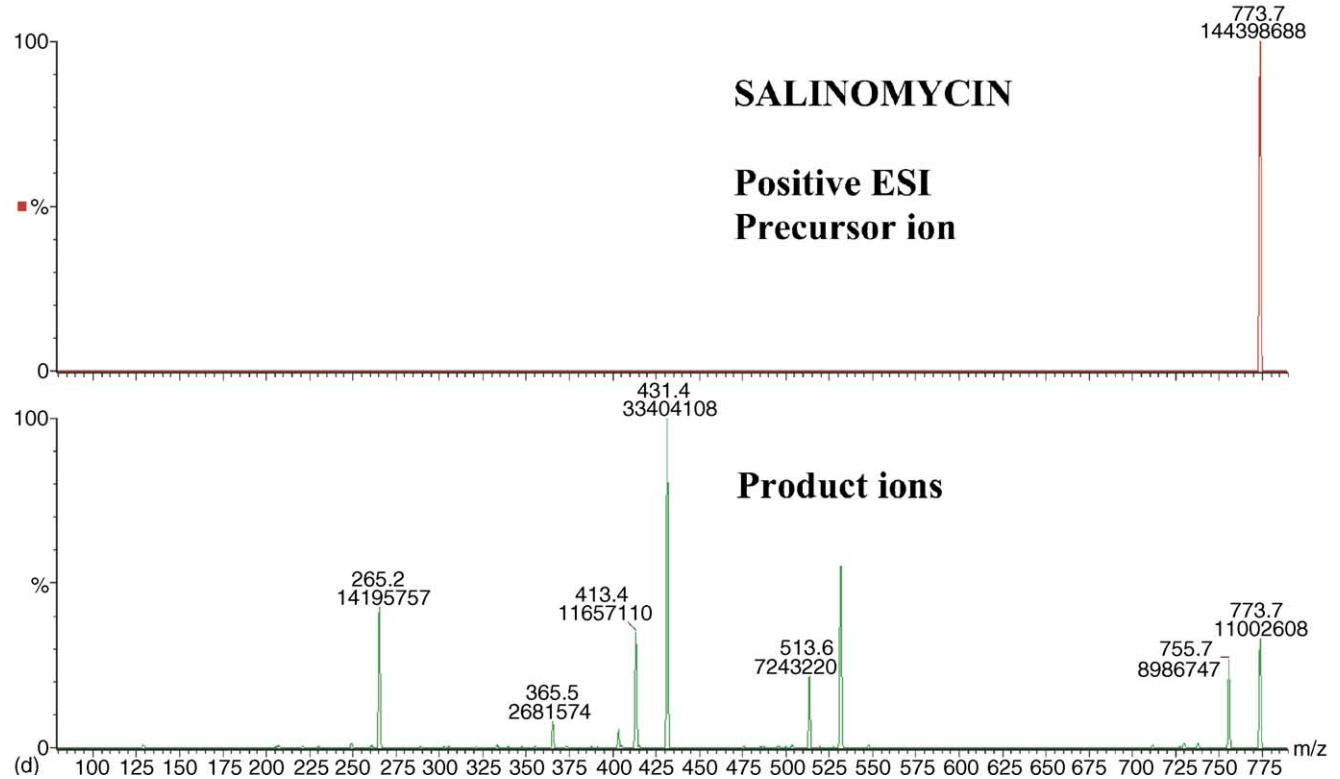


Fig. 2. (*Continued*).

Table 2
Precursor ions, product ions, collision energy used for detection and confirmation of the coccidiostats with electrospray in the negative mode for dinitrocarbanilide, diclazuril, diclazuril-bis (IS) and in the positive ion mode for the other coccidiostats

Compound	Precursor ion (*m*/*z*)	Product ions (*m*/*z*)	Collision energy (e/V)
Lasalocid	613.4	377.3	35
		359.3	35
Narasin	787.5	431.2	50
		531.2	55
Salinomycin	773.5	431.2	50
		265.2	55
Monensin	693.4	461.2	50
		479.2	55
Maduramicin	939.8	877.5	50
		719.5	70
Halofuginone	416.0	100.3	21
		120.0	20
Robenidine	334.1	138.2	23
		111.2	41
Dinitrocarbanilide	301.0	137.0	10
		107.0	40
Diclazuril	407.0	336.0	20
		301.0	30
Dinitrocarbanilide-d_8	309.0	141.0	15
Nigericin	748.5	730.5	40
Diclazuril-bis	421.0	323.0	25

halofuginone and salinomycin when the instrument was tuned to the positive electrospray mode. Sodium adducts gave the best responses for the ionophores. The positive-ion fullscan electrospray spectrum for salinomycin is shown in Fig. 2d. The most prominent ion is the sodium complex ($M+23$) at *m*/*z* 774. To enhance the analysis specificity, MS/MS was applied for confirmation of residues in muscle and eggs samples. Mass spectrometry was optimised to obtain an MRM transition for each precursor ion (see Table 2).

The chromatography conditions were adjusted with the aim to develop a quick method. This aim was reached by using a gradient. Different combinations of acetonitrile, methanol, formic acid, and ammonium acetate solutions with different pH values were used to find the optimal LC/MS–MS conditions: retention times ranging in minutes, signal-to-noise ratios as high as possible, stable responses, and peak shapes. Compound separation was less important, thanks to the mass selectivity afforded by the detection method.

The best chromatographic conditions were: a gradient system consisting of acetonitrile and water, each containing 0.1% formic acid, at a flow rate of 1 ml min^{-1}. The presence of formic acid in the mobile phase improved peak shapes in the chromatogram.

Table 3
Decision limits (CCα) and detection capabilities (CCβ) in muscle

Compound	CCα (μg kg^{-1})	CCβ (μg kg^{-1})
Lasalocid	0.1	0.2
Narasin	0.3	0.4
Salinomycin	0.07	0.1
Monensin	0.2	0.3
Maduramicin	0.4	0.5
Halofuginone	0.6	1.00
Robenidine	0.2	0.5
Dinitrocarbanilide	0.4	0.5
Diclazuril	0.3	0.4

The third step was validation of the qualitative method just described. This was done according to Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results [20].

First, 20 representative blank samples were analysed to check for interfering peaks in the regions where target analytes were expected to elute. No interference was observed. The method's power of discrimination was further checked by comparing the results obtained for a standard solution of each individual compound (1 μg ml^{-1}) in the presence and absence of the other compounds. No interference was detected between the 12 compounds (9 coccidiostats, internal standards).

In practice, we estimated the CCβ by trial and error, as the lowest tested fortification level giving no false compliant result in an analysis of 20 spiked blank samples (hence, less than 95% false compliant results). Typically, this corresponded with a signal-to-noise ratio of about 6. The CCβ values obtained in this way are listed in Table 3. Analysis of the 20 spiked samples also enabled us to estimate the repeatability by determining the standard deviation of the signal amplitude. The decision limit (CCα, Table 3) was then calculated by subtracting 1.64 times this standard deviation from the CCβ value.

The detection limit of the assay was found to depend mainly on the sensitivity of coccidiostat detection by the LC/MS system. It was influenced by such factors as the cleanliness of the source, the position of the electrospray probe, the composition of the mobile phase, and the flow rate of the mobile phase into the source. The sensitivity remained good provided the source was cleaned after every 100 samples analyses or so.

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 products ions is followed, four identification points are earned. Here, this criterion was met for all nine coccidiostats studied. A chromatogram of a muscle sample spiked at 2 μg kg^{-1} is shown in Fig. 3.

To demonstrate the method's practicability and applicability, a total of 100 incurred samples were analysed on the same HPLC column, the guard column being changed once during the study.

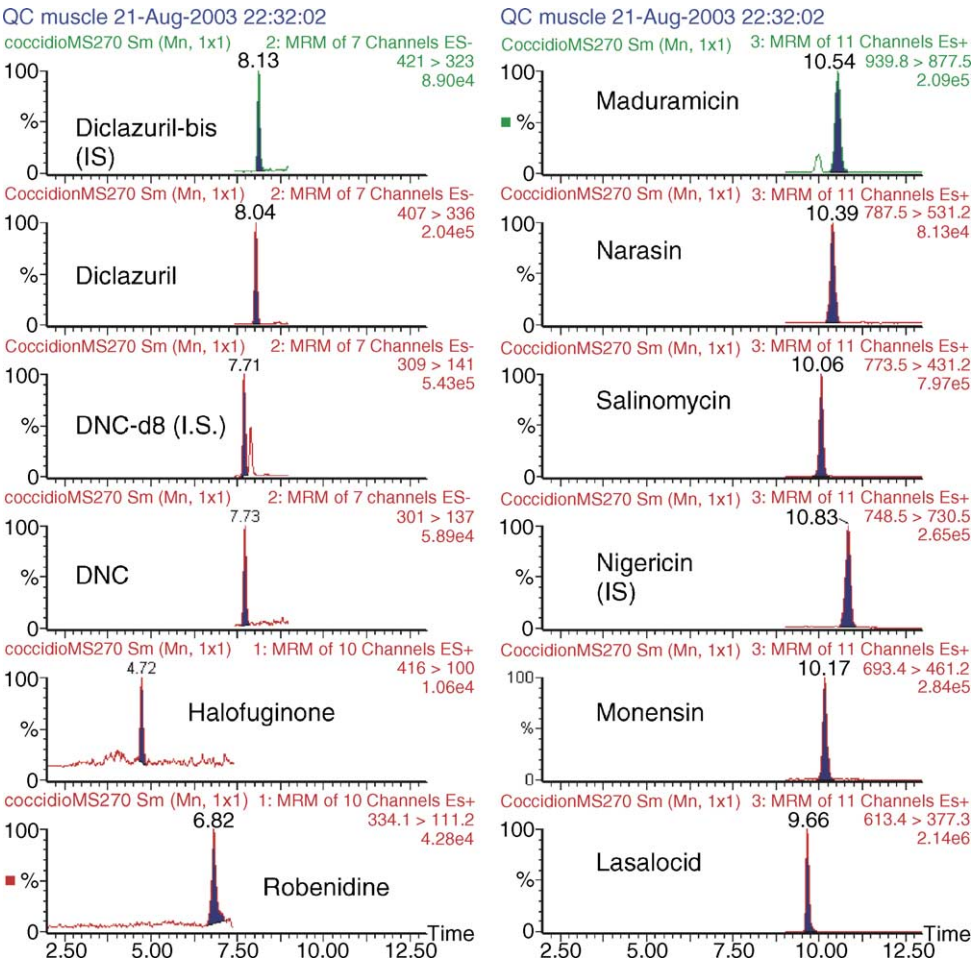


Fig. 3. MRM chromatograms of muscle samples spiked at 2 $\mu g\,kg^{-1}$ with the different coccidiostats.

4. Conclusions

We have developed a sensitive, simple, and very selective liquid chromatography–tandem mass spectrometry method that can detect simultaneously and at very low levels ($\leq 1\,\mu g\,kg^{-1}$) a range of nine coccidiostats. We observed no endogenous substances liable to interfere with the assay. The method was validated in conformity with the main lines of the revised EU requirements for detecting residues of veterinary drug substances in animal products.

Furthermore, since the method requires only a simple extraction with a short run time, large sample batches (more than 20 samples) can be processed daily.

We, thus, recommend the present method for routine analysis of residues of the most widely used coccidiostats.

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