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PII: S0021-9673(20)30009-1
DOI: https://doi.org/10.1016/j.chroma.2020.460857
Reference: CHROMA 460857

To appear in: *Journal of Chromatography A*

Received date: 15 October 2019
Revised date: 2 January 2020
Accepted date: 6 January 2020

Please cite this article as: D. Mooney , C. Coxon , K.G. Richards , L. Gill , P.-E. Mellander , M. Danaher , A new sensitive method for the simultaneous chromatographic separation and tandem mass spectrometry detection of anticoccidials, including highly polar compounds, in environmental waters, *Journal of Chromatography A* (2020), doi: https://doi.org/10.1016/j.chroma.2020.460857

Highlights

- Highly polar and other anticoccidials can be separated on one analytical column.
- SPE allows for the efficient isolation of 26 anticoccidials from water.
- Validation has proven the method's applicability to surface and groundwaters.
- The method's detection capability ranges from ppq (pg L^{-1}) to ppt (ng L^{-1}) levels.

A new sensitive method for the simultaneous chromatographic separation and tandem mass spectrometry detection of anticoccidials, including highly polar compounds, in environmental waters

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Abstract

A sensitive and selective method was developed and validated for the determination of 26 anticoccidial compounds (six ionophores and twenty chemical coccidiostats) in surface and groundwater samples at parts-per-quadrillion (pg L^{-1}) to parts-per-trillion (ng L^{-1}) levels by ultra-high performance liquid chromatography with tandem mass spectrometry detection (UHPLC-MS/MS). A range of different analytical columns and mobile phase compositions were evaluated to enhance selectivity and retention of a number of highly polar and basic anticoccidials along with other non-polar coccidiostats. A combined separation, including these problematic polar compounds, was achieved on a phenyl-hexyl column, by binary gradient elution with water/acetonitrile using ammonium formate and formic acid as additives. The anticoccidial residues were extracted from raw, unfiltered, water samples (250 mL) using polymeric divinylbenzene solid phase extraction (SPE) cartridges, with subsequent elution (methanol:acetonitrile:ethyl acetate, 40:40:20, v/v) and concentration prior to determination. The method recovery (at a concentration representative of realistic expected environmental water concentrations based on literature review) ranged from 81–105%. The method was successfully validated for 26 anticoccidials, at four concentration levels, in accordance to Commission Decision 2002/657/EC and SANTE/11813/2017 guidelines. Trueness and precision, under within-laboratory reproducibility conditions, ranged from 88–111% and 0.9–10.3% respectively.

Keywords: Chemical coccidiostats; Ionophores; Environmental water; SPE; UHPLC-MS/MS.

1. Introduction

Anticoccidials, interchangeably referred to as coccidiostats, are used to control coccidiosis and other protozoan infections in food producing animals [1, 2]. Coccidiosis is a parasitic intestinal disease caused by protozoa of the genus *Eimeria*. Anticoccidials can be classified into two main groups: the ionophores which are naturally occurring polyether antibiotic type compounds, and the synthetic/chemical anticoccidials [3]. Chemical anticoccidials are generally used at much lower concentrations compared to the ionophores, given that they have higher efficacy toward the parasites [4].

Poultry have a high susceptibility to coccidiosis, which causes intestinal lesions and diarrhoea in the animal, resulting in poor weight gain and poor feed conversion. Due to the high number of birds housed at any one time, outbreak of infection poses huge economical loss. Very often, the damage to the bird occurs before it becomes symptomatic and hence, if infected, it is often difficult for the bird to recover, given their very short life cycle (approx. 42 weeks) [5]. As a result, it is more financially viable to administer anticoccidials prophylactically as opposed to therapeutically, with broilers treated for a large portion of their life-cycle.

In the European Union (EU), there are 11 anticoccidials licensed as feed additives under Regulations 1831/2003/EC [6], for use on intensively reared species, primarily poultry (broilers, turkeys, and layers), where the substance is administered in feed. These include the ionophores salinomycin, narasin, monensin, lasalocid, maduramicin and semduramicin, and the chemical anticoccidials robenidine, decoquinate, halofuginone, nicarbazin and diclazuril. In addition, some anticoccidials are authorised in the EU as veterinary medicines as listed under Commission Regulation No 37/2010 [7], which are used to a lesser extent in poultry, cattle, swine, sheep and rabbits. There are also a number of anticoccidials authorised for use outside of the EU, which include aklomide, arprinocid clopidol, diaveridine, ethopabate, nequinate and roxarsone [1, 4].

Of the information available, it has been reported that up to 95% of some anticoccidials can be excreted as the unmetabolised active parent drug e.g. diclazuril (85–95%) [9] and lasalocid (74–77%) [10]. This, combined with the prophylactic use, provides for a potentially persistent source of anticoccidials that can enter the environment, primarily via the spreading

of poultry manure and slurry [11]. Once in the environment, these compounds have the potential to: sorb and concentrate in soil, be washed to surface waters by overland flow, or be leached to groundwaters, depending on their mobility and fate, on which information is generally lacking. The main concern with anticoccidials in the environment relates to resistance issues caused by long term exposure to low levels, and potential eco-toxicological effects on aquatic and terrestrial organisms, given the antimicrobial potency of anticoccidials [4, 12, 13]. In a prioritisation exercise in the UK, Boxall et al. [14] classified 56 different veterinary drugs to be of "high priority" in terms of risk to the environment, based on (a) their potential to reach the environment in large amounts and (b) their hazard to aquatic and terrestrial organisms (based on available eco-toxicity data). Twelve different anticoccidial compounds were included in this high priority group.

There has been a significant amount of work carried out on instrumental detection methods for anticoccidials, with the majority, and most extensive, of these methods relating to matrices of food of animal origin (e.g. poultry eggs, muscle, milk and liver) [1, 15, 16]. Clarke et al. [3] carried out a comprehensive overview of anticoccidial analysis in meat and other food products, providing a good overview of their history and advancements in their analysis and detection techniques. Based on this review, and published methods, liquid chromatography tandem mass spectrometry (LC-MS/MS) is currently considered the most powerful technique for determining anticoccidial residues in complex matrices. Instrumental detection is usually carried out using a reversed phase separation, with detection by tandem mass spectrometry using rapid polar switching electrospray ionisation (ESI). Notably, the Clarke et al. review highlights the complexity of analysis due to the broad range of physicochemical properties of anticoccidial compounds (e.g. highly polar amprolium and clopidol in contrast with some non-polar ionophores), with the authors emphasising the need to improve anticoccidial analysis to include these polar compounds. Since this review, some attempts have been made to incorporate highly polar compounds such as amprolium; however retention and peak shape still remained an issue based on the chromatograms presented [17].

In regard to environmental matrices, none of these comprehensive detection methods developed for food applications have been adapted and applied for environmental samples, with most methods for environmental samples incorporating no more than 12 anticoccidial

compounds, and very few methods incorporating both groups of anticoccidials [18, 19]. Amongst the methods available for environmental water samples, extraction and clean-up is generally performed by solid phase extraction (SPE), typically using reversed phase polymeric sorbents [20-26], eluted with methanol for subsequent evaporation and detection. The best overall method is considered to be that proposed by Herrero et al. [27] for the determination of five ionophores from river water and sewage treatment plant influent/effluent using Oasis HLB SPE cartridges with good recoveries and sensitivity achieved for river water.

In a comprehensive review assessing analytical strategies for analysis in the environment, Hansen et al. [4] decided to report solely on ionophore compounds due to the scarcity of methods for the analysis of chemical anticoccidials in environmental samples. In concluding, the authors expressed an urgent need for development of robust, sensitive methods capable of monitoring both classes of anticoccidials in environmental matrices. Taking all of the above into consideration, the overall aim of this study was to firstly develop a more comprehensive chromatographic separation and detection method for the quantitative confirmatory determination of a larger suite of both ionophore and synthetic/chemical anticoccidials, particularly the highly polar and/or basic compounds, which to date have required separation on alternative column chemistries. This detection method would also include anticoccidials licensed outside the EU, to allow for a broader application in different geographical regions. The second focus of this study was to develop and optimise a sample clean-up procedure based on SPE, capable of extracting these anticoccidials from unfiltered raw samples, for particular application to surface and groundwaters. This extraction procedure would be more advantageous compared to previously reported methods as the analysis of unfiltered samples would avoid the loss of contaminants on filtering, which most methods to date have failed to consider, as was also highlighted by the Hansen et al. review.

2. Experimental

2.1 Chemicals, standards and consumables

Ultra-pure water (UPW) (18.2 MΩcm) was generated in house using a Millipore water purification system (Cork, Ireland). The following super purity grade solvents ("SpS") were purchased from Romil Ltd. (Cambridge, UK): acetonitrile (MeCN), ethyl acetate (EtOAc), methanol (MeOH) and propan-2-ol (IPA). Dimethyl sulfoxide (DMSO), ethylene glycol

(EG), 99.5% deuterated MeOH (MeOH-$_d$), ammonium formate puriss p.a. (puriss pro analysis) and formic acid (HCOOH) (98-100%) were sourced from Sigma-Aldrich (Dublin, Ireland). Acetone puriss and ammonium acetate puriss p.a. (Fluka) (>98%) were purchased from Honeywell Research Chemicals (Honeywell Riedel-de Haen; Seelze, Germany). Acetic acid (CH_3COOH) (100%) and ammonia solution (25% w/v) were obtained from Merck (Darmstadt, Germany). The ammonia solution was used to prepare 0.1 and 0.5M ammonium hydroxide (NH_4OH) solutions for sample pH adjustment. Concentrated hydrochloric acid (HCl) (36%) was sourced from BDH Chemicals Ltd. (Poole, UK) and used to prepare a 0.1M HCl solution for pH adjustment.

Neat analytical standards of aklomide (AKLO), amprolium hydrochloride (AMP), clopidol (CLOP), cyromazine (CYROM), decoquinate (DECO), diaveridine (DIAV), diclazuril (Diclaz), diminazene aceturate (DIMIN), dinitolmide (DINITOL), 4',4''-dinitrocarbanilide (DNC), ethopabate (ETHO), imidocarb dipropionate (IMIDO), maduramicin ammonium (MAD), monensin sodium salt hydrate (MON), nafamostat mesylate (NAFAM), narasin (NAR), nitromide (NITRO), pentamidine (PENT), piperazine (PIP) robenidine hydrochloride (ROB), roxarsone (ROX), salinomycin monosodium salt hydrate (SAL) and toltrazuril (TOL) were purchased from Sigma-Aldrich Ireland (Dublin, Ireland). Arprinocid (ARPRIN), 3-Amino-2-methyl-5-nitrobenzamide (3-ANOT), buquinolate (BUQUIN), halofuginone hydrobromide (HALO-HBr), isometamidium chloride hydrochloride (ISOMET), nequinate (NEQUIN), toltrazuril sulphoxide (TOL-SO) and toltrazuril sulphone (TOL-SO_2) were purchased from Witega (Berlin, Germany), as were the isotopically labelled internal standards: decoquinate-d_5 (DECO-d_5), dinitrocarbanilide-d_8 (DNC-d_8), ethopabate-d_5 (ETHO-d_5), halofuginone hydrobromide-$^{13}C_6$ (HALO-HBr-$^{13}C_6$), imidocarb-d_8 2HCl hydrate (IMIDO-d_8) and robenidine hydrochloride-d_8 (ROB-d_8). The deuterated Cyromazine internal standard cyromazine-d_4 (CYROM-d_4) was purchased from C/D/N Isotopes Inc. (Quebec, Canada). Semduramicin sodium (SEMD) was obtained from the Community Reference Laboratory (CRL) (Berlin, Germany), while lasalocid A sodium (LAS) (Dr Ehrenstorfer GmbH, Augsburg, Germany) was sourced through LGC Standards (Middlesex, UK).

Glass amber bottles, 1000 mL and 500 mL, were purchased from Sci Chem Scientific and Chemical Supplies Ltd. (Cork, Ireland). Glass wool (both silanised and unsilanised) was purchased Lennox Laboratory Supplies (Dublin, Ireland). Polypropylene tubes (15 mL, conical) were obtained from Sarstedt Ltd (Wexford, Ireland). Large volume SPE reservoirs

(150 mL) were purchased from Biotage (Uppsala, Sweden) and connected on top of the SPE cartridge using 1–6 mL adapters purchased from UCT Ireland Ltd, (Wexford, Ireland). Final extracts were filtered through Captiva Econo PTFE 0.2 µm filters from Agilent Technologies Ltd. (Cork, Ireland).

A number of different SPE cartridges were assessed as part of the initial method development steps for sample preparation and clean-up including: Isolute ENV+ (200 mg, 6 mL) and Isolute ENV+/C_{18} dual layered (400 mg, 6 mL) purchased from Biotage (Upsala, Sweden), STRATA-X (200mg, 6mL) (Phenomenex, Cheshire, UK), UCT Enviro-Clean HL-DVB (200 mg, 6 mL) from United Chemical Technologies Ireland Ltd. (Wexford, Ireland), and Oasis HLB (200 mg, 6 mL) and Oasis MCX (500mg, 6mL) from Waters (Dublin, Ireland). The analytical UHPLC column chemistries assessed for the chromatographic separation included: Luna Omega Polar C_{18} (50 × 2.1 mm, 1.6 µm) (Phenomenex, Cheshire, UK), Selectra PFPP (100 × 2.1 mm, 3.0 µm) (UCT, Wexford, Ireland), Triart C_{18} (100 × 2.0 mm, 1.9 µm) (YMC, Kyoto, Japan) and Zorbax Eclipse Plus Phenyl-Hexyl Rapid Resolution HD (100 × 3.0 mm, 1.8 µm) (Agilent, Cork, Ireland).

2.2 Preparation of standard solutions

Individual primary stock solutions were prepared by dissolving the appropriate weight of certified standard material in suitable solvents, selected based on solubility. CLOP (0.5 mg mL^{-1}), DIAV, HALO, NICARB, NITRO (all 2 mg mL^{-1}), DICLAZ and ROB (both 4 mg mL^{-1}) were prepared in DMSO. NEQUIN, BUQUIN and DECO (0.1, 1 and 2 mg mL^{-1} respectively) were prepared in 10% (v/v) formic acid in MeCN (quinolone solvent). ETHO (2 mg mL^{-1}) was prepared in MeCN, while all remaining analytical standards were prepared in MeOH at a concentration of 2 mg mL^{-1}, except CYROM, MAD, NAR and ROX which were prepared at 4 mg mL^{-1}. All deuterated or labelled internal standards were prepared at a concentration of 1 mg mL^{-1}, in the same solvent as their corresponding analyte, from which a mixed intermediate solution was prepared for all internal standards, except DECO-$_{d5}$ which remained separate. Internal standards requiring MeOH, were prepared in MeOH-d.

Six mixed intermediate solutions were prepared at a concentration of 25 µg L^{-1}, each containing different analytes as specified in Table 1 (Std. Group A–F). In addition, 1 µg mL^{-1} intermediates were prepared for groups A-D. All intermediates were prepared in MeCN, except group C intermediates, which were prepared in quinolone solvent. This solvent was incorporated based on the work carried out by Moloney et al.[1], who reported the necessity

of the added formic acid to keep the group C analytes in solution. A set of eight mixed working calibration solutions (Calibrants 1–8) were prepared in MeCN by dilution of the respective intermediate mixed working solution (A, B and D-F), as described in Supplementary Table S1. A second series of calibrants for group C compounds were prepared in quinolone solvent. All working solutions were stored at -18 °C or below in glass amber vials with equilibration to room temperature before use.

2.3 UHPLC-MS/MS determination

Instrumental determination was performed using an Agilent 1290 Infinity™ II UHPLC system (equipped with an 8 tray multi-sampler and dual needle injector), coupled to an AB Sciex 6500+ quadrupole linear ion trap (QTRAP) mass spectrometer with IonDrive™ technology including a Turbo V source, an IonDrive QJet Guide and an IonDrive High Energy Detector+ (HED). The mass spectrometer was controlled using Analyst® software provided by Sciex (Version 1.7.0.). An Analyst® Device Driver (ADD) application (Version 1.3) provided by AB Sciex, was necessary to interface and control the Agilent LC. Data was processed and reviewed using MultiQuant™ (version 3.0.3) provided by AB Sciex.

2.3.1 UHPLC conditions

All analytes were chromatographically separated on an Agilent Zorbax Eclipse Plus Phenyl-Hexyl Rapid Resolution HD threaded analytical column (100 × 3.0 mm, 1.8 µm particle size) fitted with an in-line filter (0.2 µm pore size). A binary gradient elution was performed using 2mM ammonium formate + 0.01% formic acid in water (mobile phase A) and 0.1% formic acid in MeCN (mobile phase B), at a flow rate of 0.6 mL min^{-1}. The gradient starting condition was 99.9% mobile phase A, with the profile as follows: 0.0–2.0 min (99.9% A), 2.0–4.0 min (70% A), 4.0–8.0 min (30% A), 8.0–11.0 min (30% A), 11.0–13.0 min (0.1% A), 13.0–14.5 min (0.1% A), 14.50–14.6 min (99.9% A) and 14.6–16.5 min (99.9% A). An integrated divert valve was incorporated to divert the LC flow to waste for the first and last 2 min of the gradient. Extracts were injected in pure DMSO, using a 2.5 µL injection volume. The autosampler needle was rinsed after each injection with a H_2O:MeOH:IPA (40:40:20, v/v) solution, while a H_2O:IPA (90:10, v/v) solution was used for seal wash. The column temperature was maintained at 40 ± 1 °C while the auto-sampler was maintained at 20 °C to prevent solidification of the DMSO extracts.

2.3.2 MS/MS conditions

Anticoccidial residue detection was performed using an electrospray ionisation interface with rapid polar switching i.e. in both ESI positive (+) and negative (-) mode. Data was gathered using multiple reactions monitoring (MRM) mode with the acquisition segmented to produce MRM windows around each analyte retention time with a span of 60 or 90 s, dependent on the peak width. Transitions were selected and adapted from the in-house methods described by Moloney et al. [1], with some additional compounds included. Compound specific parameters were tuned using a teed infusion of individual compounds (100 or 500 ng mL^{-1}), using a Hamilton syringe (10 µL min^{-1}), into the MS source with mobile phase (A:B, 50:50 v/v, 0.6 mL min^{-1}). Generic source conditions were used to allow sufficient desolvation and ionisation in the source (± 4500V, 450°C, curtain gas pressure 20 psi and GS1 and GS2 both at 40 psi). The transitions followed for each analyte are as summarised in Table 1. The MS/MS source conditions were then optimised for the least sensitive analytes using flow injection analysis (FIA) and the final optimised conditions are summarised as follows: ion spray voltage (+)4500V/(-)4500V; source temperature 550°C; collision gas nitrogen (N_2); CAD gas High; entrance potential (EP) 10 volts; curtain gas pressure 40 psi; ion source gas 1 (GS1) pressure 60 psi; ion source gas 2 (GS2) pressure 60 psi and Q1/Q3 unit resolution. Collision energies (CE) and de-clustering potentials (DP) were optimised for each fragment, and are also summarised in Table 1.

2.4 Sample Collection, Control Samples and Quality Control

Samples were collected in the same manner as previously described by the authors in discussing the analysis of anthelmintic drug residues [28]. Samples were stored in the dark at 4 °C until analysis, which was always carried out within 10 days of collection, as determined by matrix stability studies. Control samples were also produced as described in the previous paper [28], with the exception of the negative control and QC samples, which in this instance consisted of a 250 mL negative control aliquot contained in a 500 mL glass amber bottle. Similarly, internal QC checks consisted of system suitability checks, negative control samples, solvent blank injections and retention checks.

2.5 Procedural Matrix Calibration

Matrix calibration curves were prepared by fortification of negative control water samples (250 mL) with 100 µL of both sets of calibrant standards (Calibrant 1–8) as described in Appendix A Table A1. An additional lower calibration point was produced for some analytes

by fortification with 100 µL of a solution consisting of Calibrant 1 diluted 1 in 5, while an additional higher calibration point was produced by fortification with 200 µL of calibrant 8 (i.e. 2 × Cal 8). For each analyte, a minimum of 8 points were used to construct a calibration curve, with the individual calibration range for each analyte shown in Table 2 above. All calibrants, quality control samples and samples were fortified with the working mixed internal standard solution (100 µL) and DECO-d_5 (100 µL), corresponding to sample concentration of 100 ng L^{-1} for CYROM-d_4, DECO-d_5, ETHO-d_5, HALO-HBr $^{13}C_6$ and ROB-d_8 and 500 ng L^{-1} for DNC-d_8 and IMIDO-d_8.

2.6 Sample preparation - Final SPE method

Water samples were weighed (250 ± 0.1 g corresponding to 250 ± 0.1 mL) directly into glass amber bottles (500 mL) and equilibrated to room temperature. Extracted matrix calibrants were fortified with the working calibrant solutions, with internal standard added to all calibrants, controls and test samples, as described above (Section 2.5). All samples were shaken (60 s), modified with MeOH (7.5 mL), and shaken again (60 s). Samples were subsequently adjusted to pH 8.5 ± 0.05 with NH_4OH (0.5M and/or 0.1M). The sample-modifier mixtures (257.5mL) were extracted using UCT Enviro Clean HL DVB (200 mg, 6 mL) SPE cartridges packed with glass wool (2.5 ± 0.2 g). Prior to loading, SPE cartridges were conditioned with MeOH:MeCN (50:50, v/v) (5 mL), MeOH (5 mL) and equilibrated with UPW, pH 8.5 (5 mL). Samples were loaded under vacuum at a rate of 5–6 mL min^{-1}. Once loaded, samples bottles were rinsed with H_2O: MeOH (95:5, v/v) (10 mL) and added to the SPE cartridge. The SPE cartridges were then washed with a further aliquot of H_2O: MeOH (95:5, v/v) (5 mL). Cartridges were dried under vacuum (30 mins) and eluted with MeOH:MeCN:EtOAc (40:40:20, v/v) (3 × 4 mL) into 15 mL polypropylene tubes. DMSO (500 µL) was added to each sample as a keeper solvent and then vortexed (30 s). Samples were concentrated under nitrogen using a TurboVap LV (50°C, 15–20 psi, 60–90 min). Extracts (in 500 µL DMSO) were sonicated (5 min) and vortexed (60 s) prior to filtration through 0.22 µm syringe filters into glass HPLC vials (Waters; Dublin, Ireland) containing 300 µL glass inserts, for instrumental determination.

2.7 Method Validation procedure

There are no definitive legislative validation guidelines available pertaining to the performance of analytical methods for the determination of veterinary pharmaceuticals in environmental water samples. As a result the developed method was validated using a similar

approach to that previously described by the authors of this work [28], using an amalgamation of validation criteria from SANTE/11813/2017 (guidelines for pesticides in food) [29] and European Legislation 2002/657/EC (guidelines for veterinary residues in food) [30]. Validation was performed at four concentration levels (Table 3) across the calibration curve, and around a target level (TL) of 100 ng L^{-1} (set based on pesticide legislation in drinking water [31] and groundwater [32]), to be consistent with the method sensitivities for the different analytes. Identification, specificity, selectivity, matrix effects, limits of detection and limits of quantification were all assessed as per the Mooney et al. approach [28]. Linearity was assessed by examining calibration curves produced with a minimum of 8 points using a $1/x^2$ fit, on five different occasions. Trueness and Precision as relative standard deviation (RSD) were both assessed under within lab repeatability (WL_r) and within lab reproducibility (WL_R) conditions, using fortified negative control samples. The WL_r study involved fortification at each of four validation levels in replicates of $n = 8$. For WL_R, a total of $n = 29$ replicates at each validated concentration level, were analysed over 5 different days ($n = 6$ on each day, except one day with $n = 5$). Matrix effects (ME) were assessed similarly using the post extraction spiking method as described by Matuszewski et al. [33], using 25 negative control samples from different sources. ME were assessed at two concentration levels, equivalent to calibrant L2 and L7 for each analyte. The criteria adhered to for each parameter are specified in Supplementary Information Table S2.

3. Results and Discussion

3.1. Method development:

3.1.1 UHPLC-MS/MS

Precursor and product ions were assessed by teed infusion of individual analytes along with mobile phase into the MS, with detection using generic source parameters that were further optimised by flow injection analysis (FIA) once transitions were selected (final conditions as in Section 2.3.2). This approach was used as mobile phase was necessary to assist with the formation of particular adducts.The product ion transitions obtained and selected (Table 1) were in agreement with the in-house method developed by Moloney et al. [1] and consistent with those reported amongst literature, as summarised by the Clarke et al. review [3]. NICARB was detected as its active component dinitrocarbanilide (DNC). Semduramicin-sodium was detected and fragmented using the 890 *m/z* precursor, which is produced by loss of the free sodium and subsequent formation of an ammonium adduct (895.1 - 23 + 18 *m/z*).

Fragments (833.2 *m/z* and 851.1 *m/z*) were also obtained for the protonated semduramicin sodium molecular ion (896 *m/z*), however intensities were not very reproducible. TOL, TOL-SO and TOL-SO_2 proved difficult to fragment in either ESI (+) or (-), as experienced and discussed by previous authors. No product ions were achieved for TOL-SO and TOL-SO_2, however some product ions were obtained for TOL, as follows: *m/z* 371, *m/z* 367, *m/z* 99 and *m/z* 42. The authors were unable to verify any of these transitions given that no other method has been published with similar product ions, except for *m/z* 42, which may be unspecific and prone to background interference for such a small fragment ion.

A number of additional compounds, not included in the Moloney paper [1], were incorporated as highlighted in Table 1. Tuning experiments, for the majority of these compounds, showed protonated $[M + H]^+$ molecular ions, with the exception of NITRO and DINITOL (dinitolmide also called zoalene) which formed deprotonated $[M - H]^-$ ions. The products formed from these additional compounds were in agreement with those included in the Clarke et al. review, or other literature [34-36], with the exception of AKLO and ANOT. Clarke et al. noted that AKLO does not easily fragment, thus is monitored using the deprotonated $[M - H]^-$ *m/z* 199 only, and therefore is unsuitable for confirmatory analysis. In this current work, AKLO was monitored using the protonated molecular ion *m/z* 201, with product ions observed at *m/z* 183, 155, 138 and 110, with the *m/z* 138 and 155 ions selected as quantifier and qualifier ions respectively. The quantifier ion monitored in this experiment for ANOT was consistent with other reported literature, however a *m/z* 133 qualifier fragment monitored by Wu et al. [37] was not observed in this current work. Instead, a *m/z* 153 ion was monitored as a qualifier, likely formed by cleavage of the amide group.

Four different UHPLC column chemistries were assessed, namely PFPP, Triart C_{18}, Omega polar C_{18} and phenyl-hexyl. Initial work indicated that both the PFPP and phenyl hexyl columns showed good retention of most compounds, including the problematic highly polar compounds, which are not well retained by reversed phase chromatography chemistries and are normally analysed by HILIC phases. Further assessment of the PFPP indicated problems with drifting and inconsistent retention times for a number of compounds including CLOP, IMIDO, ISOMET and NAFAM. It is proposed that this issue is likely due to the capability of the PFPP stationary phase to operate in both reversed phase and HILIC mode, where the very polar basic compounds are retained initially by reverse phase interactions; however as the percentage of organic phase increases, the retention mechanism switches to HILIC mode.

Efforts to address this issue resulted in a significantly increased run time, and as a result PFPP was omitted from further consideration, with phenyl-hexyl selected for final consideration. The particular phenyl-hexyl phase used contains a special high purity ZORBAX support that is designed to reduce or eliminate strong adsorption of basic and highly polar compounds.

A number of authors have reported improved retention and peak shape for a number of anticoccidials by incorporation of formic acid (HCOOH) into mobile phases [1, 34], therefore the effect of varying concentrations of HCOOH (0.01–1%, v/v), in both A and B mobile phases, was assessed using the phenyl hexyl column. Acetic acid was also assessed as a commonly used alternative additive. Chang et al [34] also reported the use of ammonium formate to further improve peak shape, and hence, varying concentrations (1–10 mM) of ammonium formate in mobile phase A were also assessed. Optimal results for 31 different anticoccidial compounds were achieved when using a binary gradient separation on the phenyl hexyl column using a 0.01% HCOOH and 2 mM ammonium formate aqueous phase (mobile phase A) and a 0.1% HCOOH in MeCN organic phase (mobile phase B). Higher concentrations (0.1%) of HCOOH in mobile phase A, had a negative effect on peak shape and intensity for some analytes, as did the use of the acetic acid additive. In addition, a number of different injection solvents, including DMSO, EG and H_2O:MeCN (80:20, v/v) were assessed, with DMSO achieving better sensitivity and peak shape for a number of compounds, including AMP and CYROM.

The gradient profile was optimised in order to reach optimal chromatographic separation, with the overall conditions as previously described in Section 2.3.1. All 31 anticoccidials and the six internal standards were successfully eluted within the first 12 minutes of the gradient, as demonstrated by the extracted ion chromatograms (EIC) in Figure 1. After elution of the compounds, the gradient was held at 99.9% B for 1.5 min to remove any less polar co-extractives from the column. During this period the LC continued to flow directed into the MS source, with the organic solvent anticipated to provide some cleaning of the ion source probe and spray plate. Subsequently, the gradient was returned to the starting point (99.9% A), with a minimum 2 min hold determined to be necessary for column re-equilibration. On injecting a solvent standard on a number of different occasions (5 different runs), the gradient was found to be robust and reproducible, with the variation in retention times for all analytes ≤0.02 min (Table 2), except for AMP (≤0.03 min) and ISOMET (≤0.08 min). All analytes

satisfied the SANTE criterion (± 0.1 min)[29]. Retention was also verified by injection on columns with different product batch numbers, with no adjustment necessary to retention windows.

3.1.2. Sample Preparation

The development and optimisation of a SPE procedure for anticoccidials was carried out using an approach similar to that described for anthelmintic residues [28], with the main steps depicted in Figure 2. Six different polymeric sorbents (described in Section 2.1) were assessed as part of this work for the extraction of anticoccidials from water, given that they are the most commonly used amongst literature [20-26]. These included five different reversed phase sorbents and one mixed mode phase used for the extraction of basic compounds with cationic functional groups. The HLB, ENV+ and HL-DVB cartridges all performed similarly, with satisfactory recovery (>70%) for the majority of analytes, with the exception of CLOP, DIAV, HALO, IMIDO, ISOMET, NAFAM and PENT, which gave lower recovery (<50%), while AMP and CYROM demonstrated recoveries of 50 and 60% respectively. The dual layered ENV+/C_{18} cartridge also showed similar results, although the loading rate was much slower and a higher vacuum required. Recoveries of a number of the poorly recovered basic compounds were improved using the MCX cartridge, however this was at the expense of less basic and neutral compounds such as the toltrazurils (TOL, TOL-SO and TOL-SO_2), NICARB, DICLAZ and the quinolones (BUQUIN, DECO AND NEQUIN) which were not retained on this sorbent phase. Overall the HL-DVB cartridge was selected for further assessment, given that better reproducibility (all <15% RSD) and more consistent flow rates were achieved compared to the other cartridges.

Further spiking experiments were carried out to assess the recovery of analytes on the HL-DVB cartridge. On spiking directly onto the cartridge (as opposed to loading in water), all analytes achieved satisfactory recoveries (69–116%) except HALO (22%), indicating that recovery losses occurred prior to, or during, the loading of samples onto the SPE cartridge. To further improve recovery, six different elution solvent compositions (described in Figure 2) were assessed, with the elution volume restricted to 12 mL due to tube size and evaporation time in the TurboVap LV evaporator. MeOH, MeCN and MeOH:MeCN (50:50, v/v) gave the best overall recovery results, but the MeOH:MeCN mixture provided better precision. Results indicated that EtOAc did not improve the recovery of analytes, however it provided enhanced sensitivity for analytes including the toltrazurils and two ionophores,

namely, NAR and SAL. This was attributed to the more hydrophobic EtOAc extracting fewer polar interferences. Additional elution compositions incorporating EtOAc were assessed, with the overall optimal elution solvent determined to be MeOH:MeCN:EtOAc (40:40:20, v/v). On assessing elution volumes, the 12 mL volume was maintained given there was no significant increase in recovery with the larger volumes. Following optimisation of the elution conditions, further experiments were carried out to identify the possible cause of lower recoveries for some analytes, namely AMP, CYROM, HALO, IMIDO, ISOMET, NAFAM and PENT. Breakthrough experiments (two stacked cartridges eluted and analysed separately) showed minimal breakthrough of analytes. Analysis of the sample bottle rinsate (rinsed with elution solvent) indicated that there was minimal adsorption of analyte to the bottle given that no more than 5% of any analyte was detected in rinsate.

The Water Framework Directive [38] and the Environmental Quality Standards Directive [39] require the measurement of "whole water" concentrations of pollutants (including both dissolved fractions and suspended solid fractions). Filtration of water samples prior to analysis may consequently remove any contaminants sorbed to suspended solids in the sample, therefore does not allow for the measurement of whole water concentrations. Glass wool was incorporated into the SPE procedure to allow for the analysis of the water without filtration, with the glass wool eluted simultaneously with the SPE cartridge. The glass wool was also necessary to prevent blocking of the SPE cartridge by the unfiltered samples. In order to investigate the effect of the glass wool on recovery, experiments were carried out using ultrapure water in which analytes were extracted with and without glass wool, assessing both silanised and unsilanised glass wool. Results indicated that IMIDO, ISOMET, NAFAM and PENT were strongly retained to active sites on the unsilanised glass wool, with subsequent elution failing to remove these analytes from the glass wool. However, use of the unsilanised glass wool proved beneficial for a number of analytes (AMP, ARPRIN, CLOP and CYROM) with up to a 70% improvement in recovery compared the use of silanised or no glass wool, indicating that the recovery of these compounds was primarily due to adsorption to the unsilanised glass wool, as opposed to retention on the sorbent. Overall, better recoveries were achieved for a greater number of analytes using the unsilanised glass wool.

Sample modification experiments assessed the use of organic modifier (MeOH, 0–30%) and pH adjustment (pH 2–10) to address the retention of analytes to the unsilanised glass wool.

Addition of >10% MeOH modifier demonstrated a notable decrease in recovery for a few compounds (e.g. AMP and CYROM), with recoveries dropping below 20%, while 15 other analytes showed a slight improvement in recovery with higher modifier, particularly the ionophores. Further experiments looked at refinement of the modifier, with the addition of 3% MeOH modifier selected as the optimum, despite no improvement in recovery of IMIDO, ISOMET, NAFAM and PENT. These findings are somewhat consistent with those reported by Song et al. [22] who reported the use of approx. 9% MeOH. A pH range between 8.5 and 10 produced the best overall results, with improved recovery demonstrated for a number of compounds, namely the ionophores, AMP, CYROM and HALO. A pH of 8.5 was selected for the final method, given that there was evidence of precipitation of some compounds when the pH was adjusted to 10. The improved recovery of AMP and CYROM is proposed to be due to reduced adsorption of analyte on the glass wool, and more retention on the reversed phase sorbent as the analytes are fully unionised at the higher pH. This selected pH is also consistent with the findings of the Hansen et al [4] review, which suggested a range of pH 7–9, as reported amongst literature, to be sufficient for extraction of the ionophore anticoccidials. At pH values greater than their pK_a (reported as 4–8 [4, 21, 40]), the ionophores remain un-protonated and form neutral highly lipophilic complexes with cations, allowing for better retention on the reverse phase SPE.

The overall optimised conditions for the final method (as described in Section 2.6) were assessed at two levels (one low and one high), with concentrations of each analyte equivalent to calibrant L2 and calibrant L7 respectively. The overall recovery results are as presented in Figure 3. The SPE procedure was unsuitable for the extraction of four compounds (IMIDO, ISOMET, NAFAM and PENT), due to what is proposed to be the lack of retention on the cartridge, or the retention and insufficient elution of analyte from the glass wool. For the other analytes, at the lower concentration, the recoveries ranged from 81–105%, with precision ranging from 0.9–8.8% RSD. At higher concentrations, recovery of some analytes was slightly lower, however the minimum criteria were satisfied (recovery of 70–120%) with recoveries ranging from 77–105% and precision between 0.8–5.8 %.

3.2. Method validation

3.2.1 Identification

For each compound, one precursor and two daughter ions were monitored giving a total of four identification points, satisfying the confirmation criteria set out in 2002/657/EC. In some cases (e.g. DICLAZ), five points were achieved by monitoring two different precursor ions. TOL, TOL-SO and TOL-SO_2 failed to meet confirmatory criteria due to insufficient identification points, as a result of the poor fragmentation, commonly reported amongst literature. However, these three analytes were still incorporated for screening purposes. The 2002/657 criterion for relative retention time (RRT, ≤2.5% deviation) was adhered to and satisfied for all analytes. For ion ratio (R, relative intensities), the SANTE criterion of 30% (ΔR) was applied, given the value specified in 2002/657/EC varied from 20–50% (ΔR) depending on the magnitude of the value. In this work, the ion ratio criteria of < 20% deviation were for the majority of analytes.

3.2.2 Specificity, Selectivity Linearity, Limits of Detection (LOD) and Limits of Quantification (LOQ)

No cross-talk or isobaric interferences were observed on injecting analytes and internal standards. The selectivity of the method was initially evaluated through application to 30 different groundwater and surface water samples collected from different sources. No major matrix interference peaks were observed at the same retention time of the analytes.

Linearity was assessed by visual inspection of calibration curves and by verification of residuals and coefficient of determination (R^2) values. Acceptable linearity was set as $R^2 \geq 0.98$ (2002/657) and residual deviations of no greater than ± 20% from the calibration plot. The majority of curves were produced with using a linear fit and $1/x^2$ weighting, however a number of analytes (ARPRIN, CLOP, DIAV, BUQUIN and NEQUIN) required a quadratic fit, attributed to the detector approaching saturation at the higher concentrations. For almost all analytes, mean R^2 values (n = 5 runs) were >0.99 (Table 2) meeting the validation criterion. The one exception was ROX, with insufficient linearity achieved through all validation runs, thus this analyte was omitted from the method.

The LOQ was determined as the lowest spiking level which satisfied the method performance criteria set out by SANTE for trueness and precision, in combination with the minimum signal to noise (S/N) (Supplementary Table S2). The LOQ for the majority of analytes corresponds to the lowest calibrant level of the calibration curve, ranging from 0.1–20 ng L^{-1} as summarised in Table 3. Adhering to minimum performance capabilities specified for

pesticides under Council Directive 98/83/EC [31] and assuming similar applicability to anticoccidials, the method LODs were required to be ≤ 25 ng L^{-1} (calculated as ≤ 25% of the specified parametric value for pesticides of 0.1 µg L^{-1}). LODs, as summarised in Table 3, ranged from 0.005 to 5 ng L^{-1} (ppt; parts-per-trillion), thus all analytes satisfied the minimum performance capability criterion. In terms of sensitivity, this developed method performs similar to, or better than other methods (see Section 4), with detection capabilities as low as part-per-quadrillion (ppq; pg L^{-1}) levels.

3.2.3 Matrix effects (ME)

Traditionally, ME are calculated using the formula first described by Buhrman et al.[41] :

$$ME\ (\%) = 100 - (B/A) \times 100 \quad \text{(Eq. 1)}$$

where A is the response of analyte in neat solvent and B is the response of analyte at the same concentration, in post spiked matrix extracted samples. However, this approach can be counter-intuitive, given that a resulting negative ME value represents ion enhancement (increase in response), while a positive value indicates ion suppression (decrease in response). In an attempt to avoid such confusion, Matuszewski et al. [33] used an adapted approach whereby they measured ME as "absolute ME" calculated as (B/A × 100), in which a resulting ME value >100% indicated ion enhancement, while values <100% indicated suppression.

In this paper, a similar approach to Matuszewski et al. was used, where matrix effects were assessed at two levels, one low (Cal L2) and one high (Cal L7), and calculated as follows:

$$ME\ (\%) = (B - A)/A \times 100 \quad \text{(Eq. 2) [42]}$$

Using this approach, negative (-) ME values indicated suppression (decrease in analyte response due to endogenous and/or exogenous matrix components), while positive (+) values indicated enhancement (increase in analyte response due to matrix components). The mean matrix effects (n = 25) of analytes at the higher concentrations (equivalent to Cal L7, validation L4) ranged from –12% for DINITOL (analyte suppression) up to +5% for MAD (enhancement), satisfying the SANTE criteria (ME ± 20%). The range of ME for each individual analyte across the entire 25 samples is shown in Table 2. The most suppression in any one sample was 22% (ME -22%) for ANOT (as demonstrated in Figure 4(b)), while the highest enhancement in any one sample was observed for MAD (+18%) (Figure 4(a)). Very

good precision was demonstrated between the 25 different samples, with RSD values for each analyte ranging between 1.3 and 7.2%. At lower concentrations (equivalent to Cal L2, validation L1) the effect of matrix was slightly more prominent, with the mean ME ranging from -22% (suppression) to +24% (enhancement). Precision at the lower concentration, however, was still satisfactory, with RSD for all analytes <9.9%. Isotopically labelled internal standards were incorporated into the method for six anticoccidials, with the precision further improved for these six analytes when the IS was employed for quantification. Suitable internal standards were not available for the majority of analytes and as a result matrix calibration was employed to address any potential matrix effects, further satisfying validation criteria.

3.2.4. Trueness and Precision

Trueness criteria were set as 70–120% based on SANTE guidelines, while precision (in terms of RSD) was set as ≤20% as the 2002/657/EC guidelines were not appropriate. Trueness and precision data for WL_r and WL_R conditions are summarised in Table 3. Under WL_r conditions, the trueness across the four validation levels ranged from 86–114%, with all analytes meeting the set criteria. The trueness for all analytes under WL_r conditions at the lowest validation level was >95%, demonstrating very high accuracy even at ppq (pg L^{-1}) to ppt (ng L^{-1}) levels. WL_r precision (RSD_r) for all analytes across the four validation levels was in the range of 0.5–8.2%. For a number of analytes such as NEQUIN and BUQUIN, the WL_r trueness decreased with increasing concentration, however it was were still acceptable. Under reproducibility conditions (WL_R), trueness ranged from 88–111%, with all analytes meeting the acceptance criteria. Precision for the majority of analytes under reproducibility conditions (RSD_{wR}) WAS <5%, with the overall range between 0.9–10.3%. Overall this method has been shown to be very accurate and precise for the 23 confirmatory analytes and three screening analytes (TOL, TOL-SO and TOL-SO_2).

3.3 Applicability

The method presented above has been applied for the determination of the 26 anticoccidial compounds as part of a spatial sampling programme, whereby >100 groundwater samples were collected from sites throughout the Republic of Ireland during November/December 2018. Seven different anticoccidial compounds, consisting of four ionophores (lasalocid, monensin, narasin and salinomycin) and three chemical coccidiostats (amprolium, diclazuril and nicarbazin), were detected during the sampling campaign. The concentration ranges of

each anticoccidial detected are as shown in Table 4. Further information and details of this spatial occurrence study are currently in preparation for publication.

4. Comparison with other existing methods for environmental water samples

As highlighted in the introduction, based on literature review, there are very few methods available for the determination of anticoccidial residues in water samples, with the majority of methods reported being for the separate analysis of ionophores [20, 21, 24, 25, 27] or a limited number of chemical anticoccidials [43]. The method proposed by Herrero *et al.* [27] extracted five ionophores (LAS, MAD, MON, NAR, SAL) from river water using HLB (150mg) SPE cartridges, with good recoveries reported, ranging from 89–97%. An LOQ of 1 ng L^{-1} was reported for all analytes except MAD (5 ng L^{-1}), with LODs ranging from 0.5 -1 ng L^{-1}. Martinez-Villalba et al. [19] proposed a method for the determination of eight anticoccidials (including the three chemical anticoccidials DICLAZ, NICARB and ROB) using C_{18} SPE. Recoveries of all analytes were in the range of 85–100% except for ROB (60%), while LODs were in the range of 11–71 ng L^{-1}. The method developed as part of this study is capable of determining 26 anticoccidial compounds, including six ionophores and 20 chemical anticoccidials. This new method performs better for all of the analytes reported by Herrera et al., with LOQs of 0.1 ng L^{-1} for LAS, MON, NAR and SAL and 1 ng L^{-1} for MAD. Similarly, detection capabilities of this developed method are much improved compared to the results reported by Martinez-Villalba et al.. In particular, this work reports higher recovery of ROB, with lower reported detection limits (at least 50 times lower) for the three chemical anticoccidials reported by Martinez-Villalba et al. This new method allows for detection limits down to the part-per-quadrillion (pg L^{-1}) level, depending on the analyte.

5. Conclusions

A comprehensive LC-MS/MS detection method has been developed which allows for the simultaneous separation and detection of 31 anticoccidial drugs in one single injection. A sample extraction procedure based on SPE has been developed and optimised for the extraction of these anticoccidial residues from raw, unfiltered, environmental water samples at ppq to ppt levels. This extraction procedure was suitable for extraction of 26 anticoccidials, with four compounds not retained by the SPE due to their high hydrophilicity. The method has been extensively validated for these 26 analytes, over a broad range of concentration levels, in-line with expected environmental levels, based on review of currently available

literature. The developed detection method is advantageous compared to other reported methods as it allows the simultaneous detection of highly polar, basic compounds such as amprolium and cyromazine, along with other analytes such as the ionophores, on the same analytical column. In addition, the combination of the developed SPE procedure with this detection method allows for the determination of a broader range of both ionophore and chemical anticoccidial residues (26), compared to currently available methods which incorporate <10 anticoccidials. Overall the method has been deemed fit for purpose for the confirmatory analysis of 23 anticoccidials, and screening of an additional three compounds (TOL, TOL-SO and TOL-SO_2), according to appropriate validation guidelines.

CRediT author statement

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

6. Acknowledgements

This work was carried out as part of a joint project between Trinity College Dublin and Teagasc through the Walsh Fellowship scheme, and it forms part of the Groundwater Spoke of the Irish Centre for Research in Applied Geosciences (iCRAG). This work has emanated from research supported in part by a research grant from Science Foundation Ireland (SFI) under Grant Number 13/RC/2092 and is co-funded under the European Regional

Development Fund and by iCRAG industry partners. The UHPLC-MS^3 system used as part of this work was funded by an SFI Research Infrastructure Grant (16/RI/3710).

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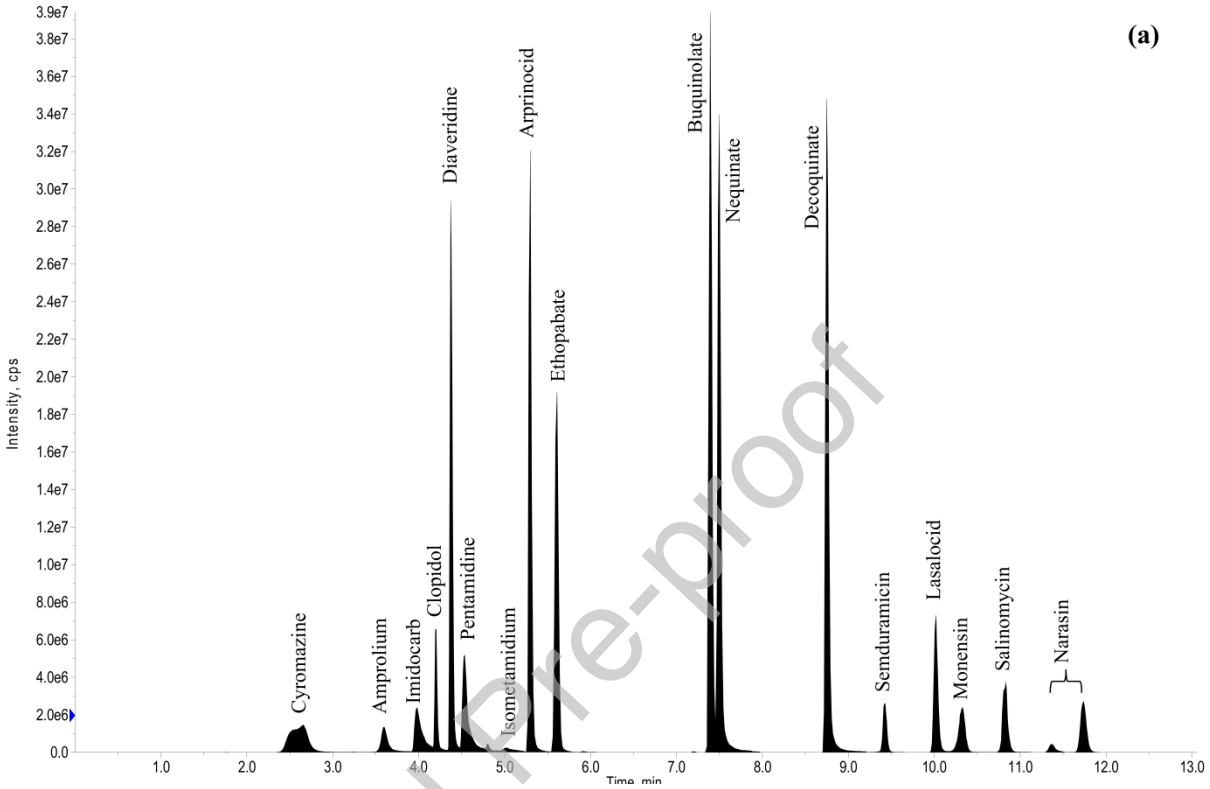
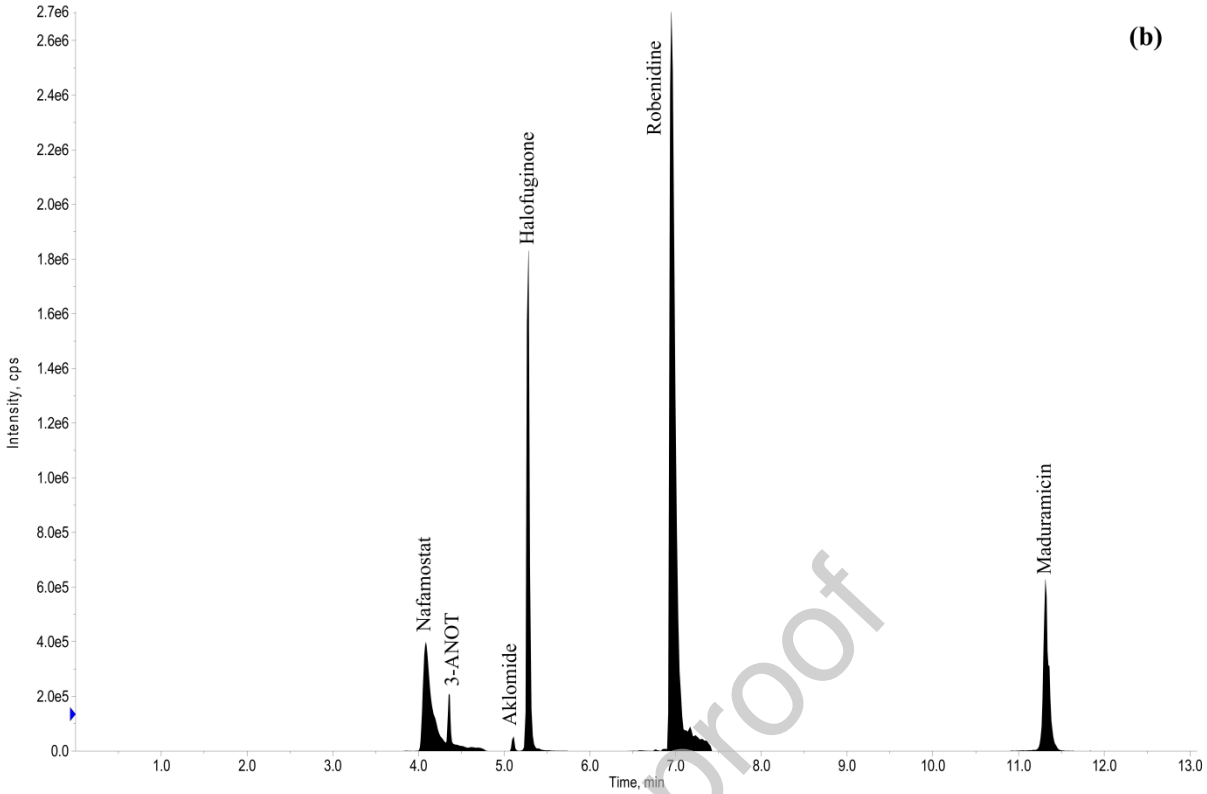
8. Appendices

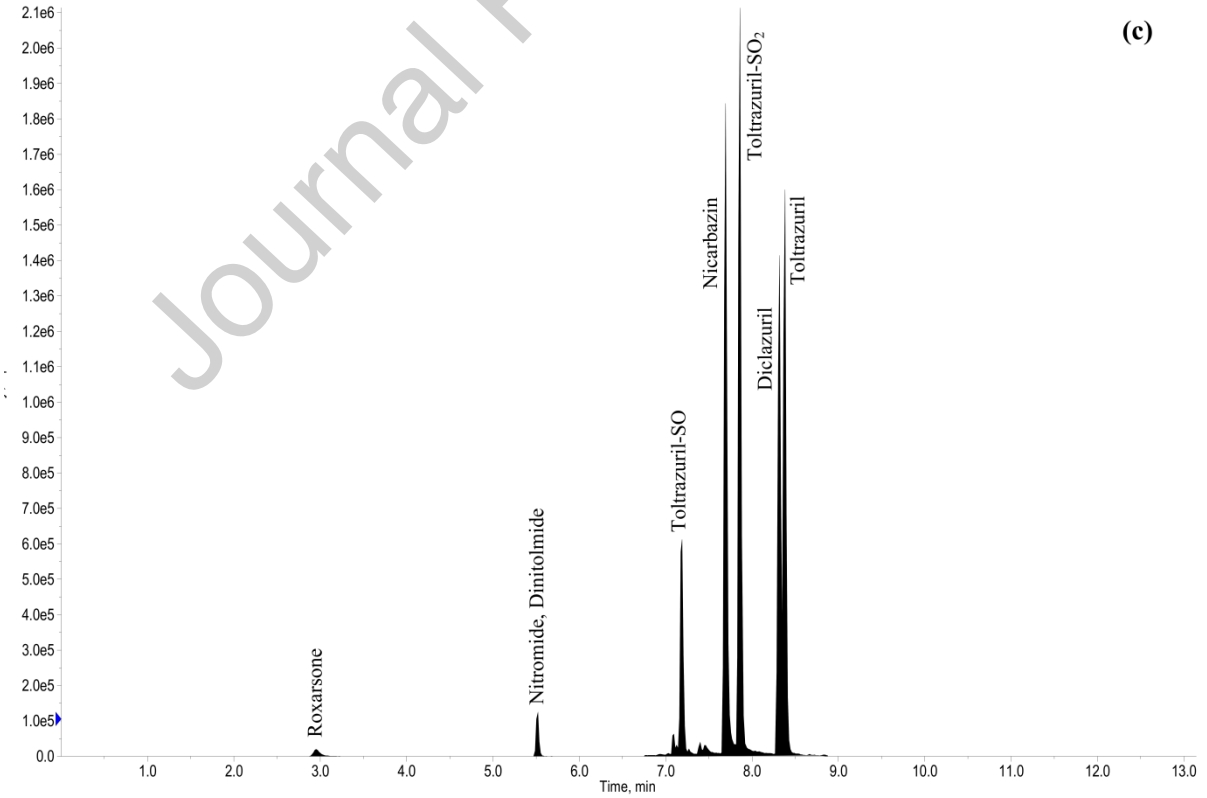
Appendix A

See Table A1

Figure Captions







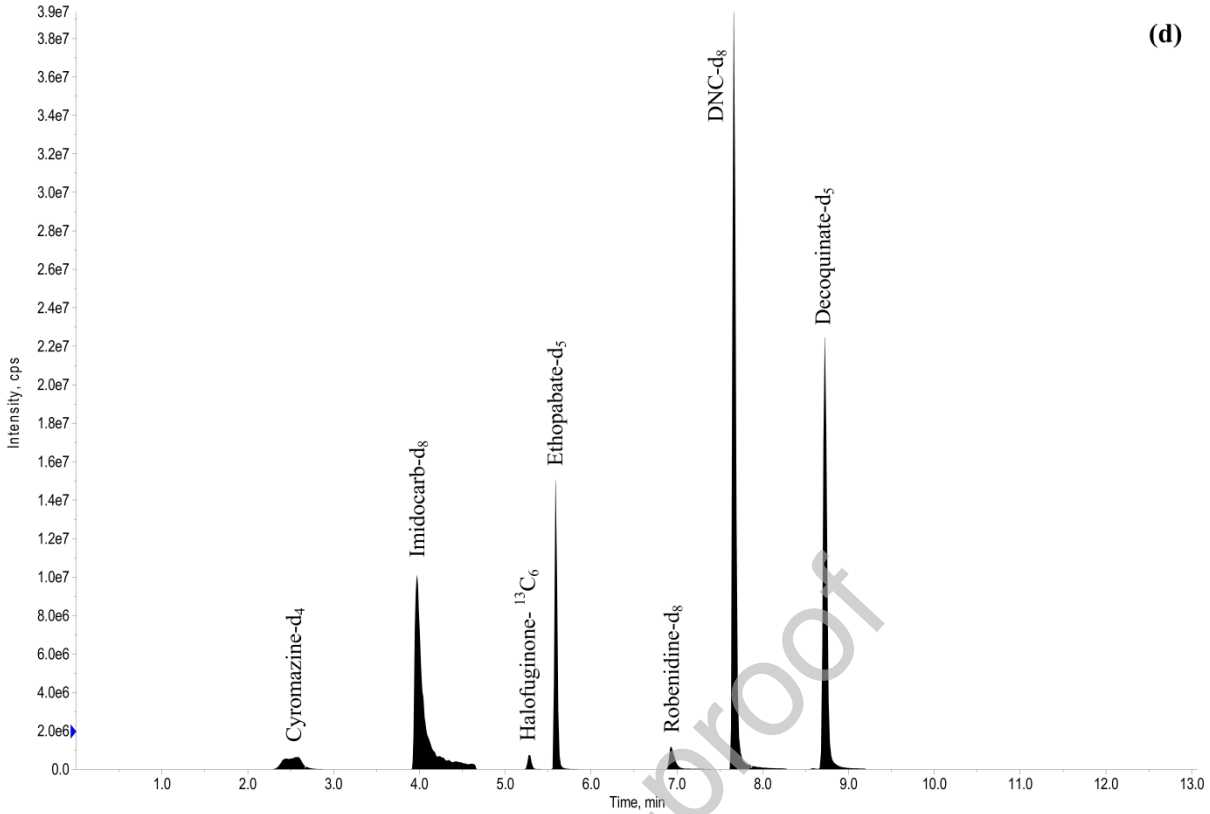


Figure 1. Overlay of LC-MS/MS extracted ion chromatograms (EIC) for all 31 anticoccidial analytes (positive mode (a-b) and negative mode (c)) at concentrations equivalent to calibrant level L2 (2.5/7.5/20/25 ng L^{-1}) (Table A1), and the seven internal standards (d), in a fortified blank water sample.

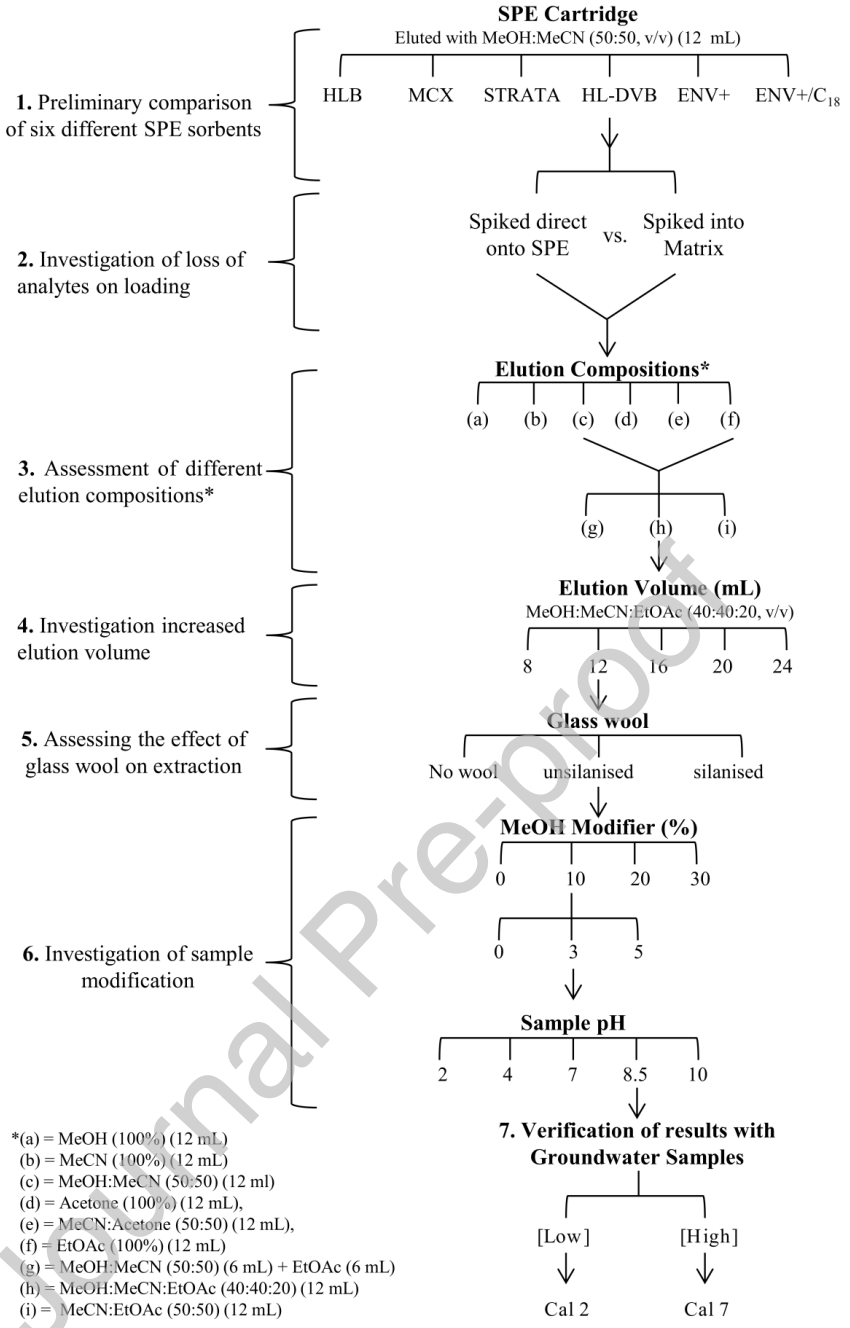


Figure 2. Summary of the main steps carried out as part of the development and optimisation of the SPE procedure for extraction of anticoccidial residues from water.

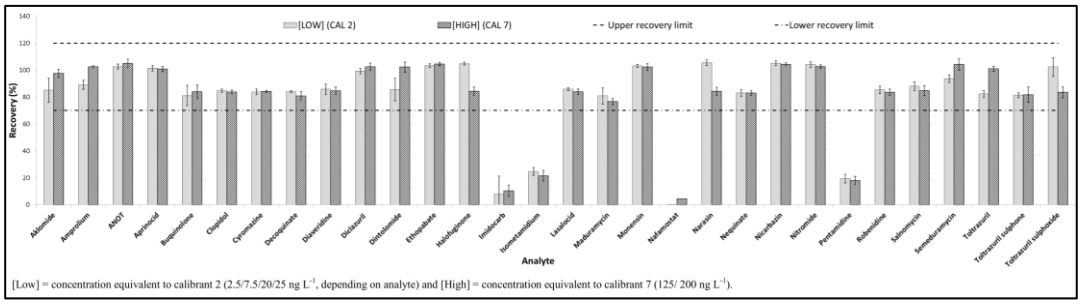


Figure 3. Overall mean recoveries and precision (% RSD shown by error bars) (n = 3) for all anticoccidial compounds, at two concentrations using the final optimised conditions: 250 mL environmental water samples, modified with MeOH (7.5 mL) and pH adjusted to pH 8.5, extracted using UCT-HL-DVB (200mg, 6 mL) SPE cartridges, washed with MeOH:H_2O (95:5, v/v) and eluted with MeOH:MeCN:EtOAc (40:40:20, v/v) (3 x 4 mL).

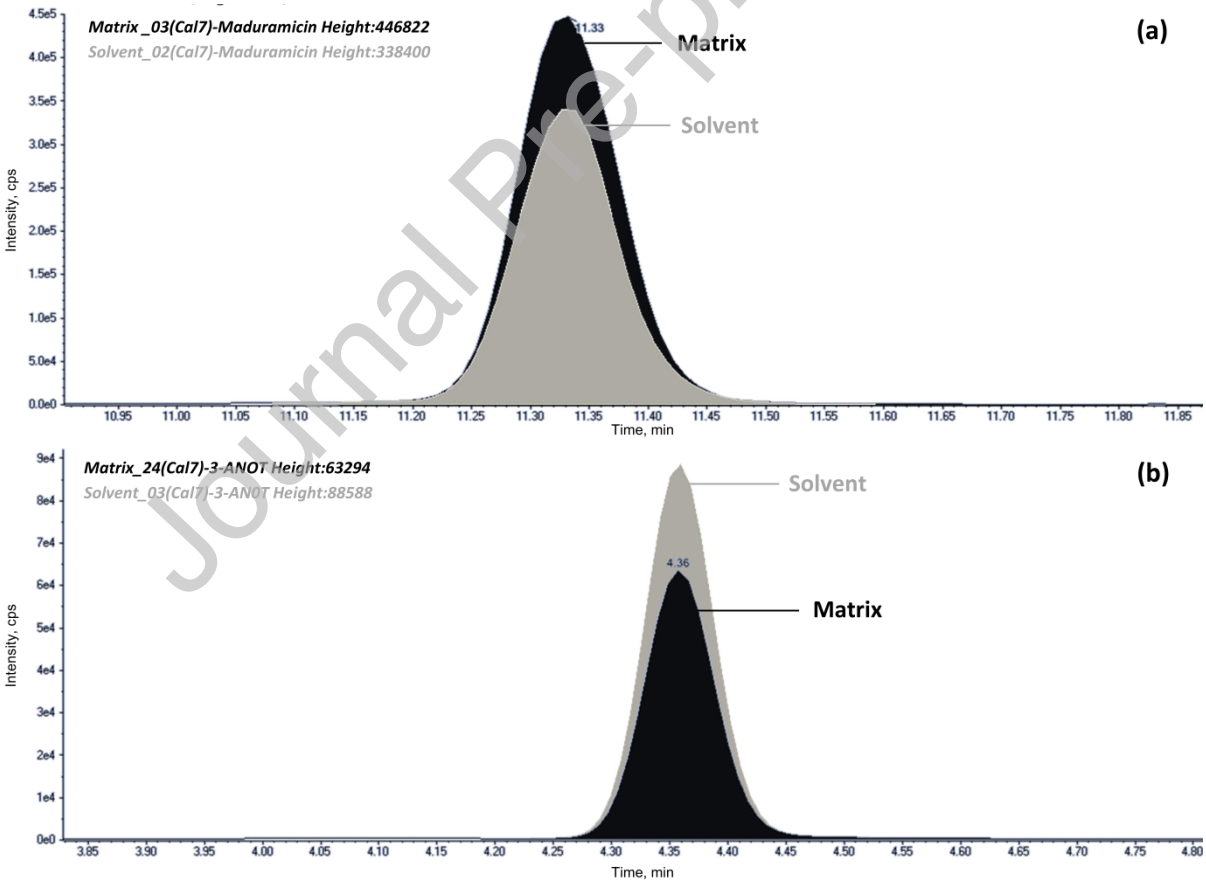


Figure 4. Extracted ion chromatogram (EIC) overlay of quantifier ions for a spiked solvent standard (grey) and spiked matrix sample (black), spiked at concentration equivalent to calibrant level L7 (125/200 ng L^{-1}), demonstrating (a) response enhancement for maduramicin and (b) suppression for 3-ANOT, due to matrix effects.

Appendix A

Table A1. Preparation of matrix matched calibration, with corresponding sample concentrations

Spiking Vol. (µL)	Calibration Level	Concentration Ranges (ng L^{-1}) for Analyte Group[a]:					
		A	B	C**	D	E	F
100	0.2 × L1	0.1	0.1	0.1	1	2	4
100	L1	0.5	0.5	0.5	5	10	20
100	L2	2.5	2.5	2.5	7.5	20	25
100	L3	10	10	10	10	40	50
100	L4	50	50	50	50	50	75
100	L5	100	75	75	100	75	100
100	L6	150	100	100	150	100	150
100	L7	200	125	125	200	125	200
100	L8	250	150	150	250	150	250
200	2 × L8	500	300	300	500	300	500

[a] Analytes within each concentration range group are as specified in Table 1 ** group C analytes were spiked from a separate set of calibrants (1-8) which were prepared in MeCN +10% formic acid

Table 1. UHPLC–MS/MS conditions for anticoccidial residues and respective internal standards

Analyte	Abbreviation	Std. Group	t_R (min)	Pre-ion (m/z)	Product Ions[a] (m/z)	[M]	DP (V)	CE (V)	CXP (V)	ESI Polarity	IS
Cyromazine-d_4*	CYROM-d_4	IS	2.53	171.1	86.0	$[M+H]^+$	60	27	10	+	None
Cyromazine*	CYROM	D	2.56	166.9	**84.8**/124.9	$[M+H]^+$	30	25	12	+	Cyromazine-d_4
Roxarsone*	ROX	F	2.90	263.8	**217.8**/90.9	$[M-H]^-$	26	29	20	-	None
Amprolium*	AMP	A	3.61	242.8	**149.9**/94.0	$[M+H]^+$	60	17	14	+	None
Imidocarb-d_8	IMIDO-d_8	IS	4.00	357.1	191.9	$[M+H]^+$	26	39	22	+	None
Imidocarb	IMIDO	E	4.03	349.0	**187.9**/162.0	$[M+H]^+$	120	33	14	+	Imidocarb-d_8
Nafamostat*	NAFAM	E	4.16	348.1	**162.0**/186.9	$[M+H]^+$	120	23	8	+	None
Clopidol	CLOP	B	4.20	191.9	**100.9**/86.9	$[M+H]^+$	131	39	10	+	None
ANOT*	ANOT	E	4.35	196.0	**106.9**/153.0	$[M+H]^+$	26	23	12	+	None
Diaveridine	DIAV	B	4.37	261.1	**122.9**/244.9	$[M+H]^+$	1	29	14	+	None
Pentamidine*	PENT	F	4.56	341.1	**324.1**/120.0	$[M+H]^+$	121	43	12	+	None
Aklomide*	AKLO	F	5.11	201.0	**137.9**/154.8	$[M+H]^+$	36	37	16	+	None
Isometamidium*	ISOMET	F	5.13	461.1	**313.0**/298.0	$[M+H]^+$	36	29	16	+	None
Halo-HBr-$^{13}C_6$	HALO-$^{13}C_6$	IS	5.26	419.9	138.0	$[M+H]^+$	60	25	16	+	None
Halofuginone-HBr	HALO	A	5.27	414.3	**120.1**/100.1	$[M+H]^+$	61	27	14	+	Halo-HBr-$^{13}C_6$
Arprinocid	ARPRIN	B	5.30	278.0	**142.9**/106.9	$[M+H]^+$	20	79	12	+	None
Nitromide*	NITRO	F	5.48	209.9	**166.9**/62.9	$[M-H]^-$	-20	-20	-17	-	None
Dinitolmide*	DINITOL	E	5.52	223.9	**181.0**/77.0	$[M-H]^-$	-15	-14	-21	-	None
Ethopabate- d_5	ETHO-d_5	IS	5.57	243.1	211.0	$[M+H]^+$	35	15	12	+	None
Ethopabate	ETHO	A	5.61	238.0	**135.9**/206.0	$[M+H]^+$	35	35	14	+	Ethopabate- d_5
Robenidine-d_8	ROB-d_8	IS	6.88	342.0	342.0	$[M+H]^+$	100	63	12	+	None
Robenidine	ROB	A	6.92	334.0	**154.9**/137.9	$[M+H]^+$	100	27	18	+	Robenidine-d_8
Toltrazuril-SO	TOL-SO	F	7.19	440.0	440.0	$[M-H]^-$	-15	-6	-11	-	None
Buquinolone*	BUQUIN	C	7.40	362.0	**316.0**/203.9	$[M+H]^+$	20	47	22	+	None

Analyte	Abbreviation	Std. Group	t_R (min)	Pre-ion (m/z)	Product Ions[a] (m/z)	[M]	DP (V)	CE (V)	CXP (V)	ESI Polarity	IS
Nequinate*	NEQUIN	C	7.50	366.0	**200.9**/144.9	$[M+H]^+$	20	61	16	+	None
DNC-D_8	DNC-d_8	IS	7.66	308.9	140.8	$[M-H]^-$	-30	-16	-15	-	None
4'4''-dinitrocarbanilide**	NICARB**	D	7.69	300.9	106.8	$[M-H]^-$	-35	-52	-13	-	DNC-D_8
Toltrazuril-SO_2	TOL-SO_2	F	7.86	455.9	455.9	$[M-H]^-$	-50	-12	-23	-	None
Diclazuril	DICLAZ	A	8.32	404.8		$[M-H]^-$	-10	-28		-	None
				406.8	**333.7**	$[M-H]^-$	-10	-28	-35		
					335.7		-10	-28	-25		
Toltrazuril	TOL	D	8.38	423.9	**423.9**	$[M-H]^-$	-20	-10	-5	-	None
Deco-d_5	DECO-d_5	IS	8.73	423.1	377.1	$[M+H]^+$	130	33	20		None
Decoquinate	DECO	C	8.75	418.1	**372.1**/203.9	$[M+H]^+$	130	55	22	+	None
Semduramicin	SEMD	A	9.43	890.4	**629.3**/727.2	$[M-Na+NH_4]^+$	80	37	4	+	None
Lasalocid	LAS	A	10.03	613.2	**377.1**/595.1	$[M+Na]^+$	130	53	20	+	None
Monensin	MON	A	10.33	693.0	**675.2**/461.1	$[M+H]^+$	80	55	36	+	None
Salinomycin	SAL	F	10.83	773.1	**431.1**/531.1	$[M+H]^+$	120	69	22	+	None
Maduramicin	MAD	D	11.33	934.4	**629.4**/647.4	$[M+H]^+$	60	41	20	+	None
Narasin	NAR	A	11.75	787.3	**431.0**/531.1	$[M+Na]^+$	91	71	22	+	None

t_R= Retention time, M Wt = molecular weight, Pre-ion = precursor ion, *m/z* = mass to charge ratio, [M] = molecular ion, DP = declustering potential, CE= collision energy, CXP = collision cell exit potential, ESI polarity mode; (+) = positive mode and (-) = negative mode IS= internal standard [a] Quantification Ion (bold) / Qualifier Ion * denotes additional compounds included in this method, that were not included in the Moloney et al. method [1], ** Nicarbazin (NICARB) detected as 4'4''-dinitrocarbanilide

Table 2. Retention time, calibration range, mean linearity (of *n* = 5 runs) and results of matrix effects (ME) at two concentrations (*n* = 25) for each of the 26 anticoccidials

Analyte	t_R ±SD min	Calibration Range (ng L^{-1})	Linearity R^2	Mean ME (%) (n-25)		ME RANGE (%)				RSD (n=25) (%)	
						[Low]		[High]			
				[Low]	[High]	Min	Max	Min	Max	No IS	With IS
Aklomide	5.11 ± 0.01	20.0 - 250	0.9966	8.6	1.2	-5.9	19.9	-13.3	13.0	6.8	-
Amprolium	3.61 ± 0.03	0.5 - 250	0.9995	3.3	2.5	-0.3	10.2	-3.9	8.7	2.8	-
ANOT	4.35 ± 0.01	10.0 - 150	0.9982	0.7	-2.9	-7.8	10.4	-21.9	11.8	7.2	-
Arprinocid	5.30 ± 0.00	0.5 - 150	0.9996	8.9	2.1	2.6	15.5	-4.2	7.4	2.9	-
Buquinolone	7.40 ± 0.00	0.5 - 150	0.9993	7.1	-0.2	0.1	12.0	-4.8	3.7	2.1	-
Clopidol	4.20 ± 0.00	0.5 - 150	0.9996	7.3	2.6	-1.2	13.8	-3.3	7.2	2.7	-
Cyromazine	2.56 ± 0.01	1.0 - 250	0.9997	14.1	-1.3	0.0	21.0	-5.8	3.7	2.6	0.9
Decoquinate	8.75 ± 0.01	0.5 - 150	0.9977	10.1	-1.7	1.2	17.3	-5.9	2.5	2.1	1.0
Diaveridine	4.37 ± 0.00	0.5 - 150	0.9995	7.0	2.6	-1.0	12.1	-1.8	8.6	2.5	-
Diclazuril	8.32 ± 0.00	0.1 - 250	0.9989	-5.9	1.1	-20.1	6.6	-5.3	11.9	3.9	-
Dintolmide	5.52 ± 0.01	10.0 - 150	0.9989	-7.2	-12.4	-22.0	3.4	-19.3	0.1	7.0	-
Ethopabate	5.61 ± 0.00	0.1 - 250	0.9985	6.4	-1.7	-0.7	14.8	-7.2	1.8	2.3	1.8
Halofuginone	5.27 ± 0.00	0.1 - 250	0.9993	14.9	1.0	-2.1	24.0	-3.9	6.8	2.8	2.5
Imidocarb	4.03 ± 0.02	-	-	4.9	-2.5	-0.9	10.8	-7.8	2.7	2.6	1.4
Isometamidium	5.13 ± 0.08	-	-	-6.8	2.5	-14.1	1.8	-2.8	8.8	3.0	-
Lasalocid*	10.03 ± 0.00	0.1 - 250	0.9953	-5.0	-1.1	-14.1	1.3	-5.8	4.3	3.2	-
Maduramicin*	11.33 ± 0.01	1.0 - 250	0.9981	1.1	5.0	-16.2	20.9	-7.8	18.4	5.7	-
Monensin*	10.33 ± 0.01	0.1 - 250	0.9987	-4.8	3.8	-13.5	3.7	-3.5	11.5	3.8	-
Nafamostat	4.16 ± 0.02	-	-	9.2	-2.2	0.7	17.3	-9.0	2.4	2.9	-
Narasin*	11.75 ± 0.00	0.1 - 250	0.9985	-5.8	2.1	-16.2	3.1	-2.0	8.7	2.7	-
Nequinate	7.50 ± 0.00	0.5 - 150	0.9991	8.9	0.6	-0.4	14.7	-3.6	3.3	1.8	-
Nicarbazin	7.69 ± 0.00	1.0 - 250	0.9988	11.1	0.6	-1.1	20.3	-1.7	2.6	1.3	1.0

Analyte	t_R ±SD min	Calibration Range (ng L^{-1})	Linearity R^2	Mean ME (%) (n-25)		ME RANGE (%)				RSD (n=25) (%)	
						[Low]		[High]			
				[Low]	[High]	Min	Max	Min	Max	No IS	With IS
Nitromide	5.48 ± 0.00	20.0 - 250	0.9990	-5.3	-3.2	-16.7	7.2	-9.0	0.7	2.5	-
Pentamidine	4.56 ± 0.02	-	-	-9.6	0.1	-3.8	19.7	-6.6	6.8	3.3	-
Robenidine	6.92 ± 0.02	0.1 - 250	0.9973	-13.0	0.0	-1.1	19.9	-3.4	4.0	2.1	1.0
Salinomycin*	10.83 ± 0.00	0.1 - 250	0.9985	-7.3	-1.8	-18.7	0.1	-7.2	5.4	3.6	-
Semduramicin*	9.43 ± 0.00	1.0 - 250	0.9970	-4.0	4.2	-16.9	17.3	-6.1	13.0	5.2	-
Toltrazuril	8.38 ± 0.00	20.0 - 250	0.9992	-6.8	-3.7	-1.9	13.0	-10.3	2.0	3.0	-
Toltrazuril sulphone	7.86 ± 0.00	20.0 - 250	0.9988	-1.2	3.3	-14.3	11.0	-6.4	16.8	4.6	-
Toltrazuril sulphoxide	7.19 ± 0.00	20.0 - 250	0.9986	-0.7	-0.4	-3.7	5.7	-8.9	11.2	3.5	-

* denotes ionophore compounds, t_R = retention time, SD = standard deviation, R^2 = coefficient of determination, ME = matrix effect, RSD = relative standard deviation, IS = internal standard, [Low] = concentration equivalent to calibrant 2 (2.5 ng L^{-1} for groups A, B and C and 7.5, 20 and 25 ng L^{-1} for D, E and F) [High] = concentration equivalent to calibrant 7 (125 ng L^{-1} for standard groups B, C and E and 200 ng L^{-1} for groups A, D, and F.

Table 3. Validation trueness and precision (RSD) under repeatability conditions (WL_r) (n = 8) and reproducibility conditions(WL_R) (n = 29) at four concentration levels for the 26 anticoccidial compounds, with their respective limit of detection (LOD) and limit of quantification (LOQ) values (ng L^{-1})

Analyte	Validated levels L1, L2, L3, L4 (ng L^{-1})	WL_r Trueness (RSD_r) (%)[a]				WL_R Trueness (RSD_{wR}) (%)[b]				LOD[c] (ng L^{-1})	LOQ[d] (ng L^{-1})
		L1	L2	L3	L4	L1	L2	L3	L4		
Aklomide	25, 75, 150, 200	102 (6)	110 (5.9)	98 (8.2)	93 (1.5)	97 (9.1)	102 (7.6)	97 (7.7)	96 (6.3)	5	20
Amprolium	2.5, 50, 150, 200	102 (4.5)	109 (3.5)	109 (3.5)	106 (3.6)	105 (8.6)	104 (5.4)	102 (6.1)	99 (8.8)	0.1	0.5
ANOT	20, 50, 100, 125	105 (6.5)	101 (3.4)	98 (4.1)	92 (5.2)	100 (7.0)	99 (3.3)	94 (8.1)	91 (7.4)	2.5	10
Arprinocid	2.5, 50, 100, 125	101 (3.4)	99 (1.9)	94 (3.6)	91 (1.9)	104 (9.6)	98 (5.6)	95 (6.5)	93 (7.4)	0.1	0.5
Buquinolone	2.5, 50, 100, 125	101 (1.3)	90 (1.8)	86 (3.7)	88 (2.6)	101 (6.8)	91 (4.3)	88 (5.1)	88 (8.0)	0.1	0.5
Clopidol	2.5, 50, 100, 125	104 (2.8)	100 (1.6)	96 (2.4)	93 (1.8)	103 (8.9)	98 (5.3)	94 (4.3)	91 (4.9)	0.1	0.5
Cyromazine	7.5, 50, 150, 200	100 (1.2)	103 (0.8)	101 (0.5)	100 (1)	101 (2.9)	101 (1.4)	100 (0.9)	99 (1.0)	0.1	1
Decoquinate	2.5, 50, 100, 125	113 (1)	105 (1.4)	97 (1.5)	93 (0.9)	111 (3.3)	103 (3.2)	97 (3.0)	95 (4.3)	0.1	0.5
Diaveridine	2.5, 50, 100, 125	100 (2.1)	96 (1.5)	93 (3.9)	92 (2.8)	103 (9.3)	95 (5.1)	91 (5.7)	89 (7.2)	0.15	0.5
Diclazuril	2.5, 50, 150, 200	100 (3.8)	102 (3.7)	97 (1.6)	98 (4.7)	105 (7.5)	104 (5.5)	100 (5.6)	99 (5.8)	0.02	0.1
Dinitolmide	20, 50, 100, 125	103 (3.1)	105 (2.1)	102 (2.4)	102 (2.4)	102 (4.4)	102 (4.9)	99 (8.0)	99 (8.6)	2	10
Ethopabate	2.5, 50, 150, 200	106 (1.9)	106 (2.3)	100 (1.5)	96 (1.8)	110 (9.1)	105 (2.0)	100 (1.9)	97 (2.3)	0.02	0.1
Halofuginone	2.5, 50, 150, 200	96 (3)	104 (2.1)	103 (1.9)	102 (2.1)	104 (8.1)	102 (2.7)	102 (2.6)	101 (2.2)	0.05	0.1
Lasalocid	2.5, 50, 150, 200	112 (1.2)	109 (1.3)	94 (2.6)	88 (1)	110 (3.6)	107 (3.8)	94 (4.7)	88 (4.8)	0.01	0.1
Maduramicin	7.5, 50, 150, 200	106 (3.7)	101 (5.4)	94 (8)	95 (5.9)	102 (6.6)	106 (7.1)	97 (8.1)	95 (9.4)	0.5	1
Monensin	2.5, 50, 150, 200	102 (2.4)	108 (3.6)	104 (3.3)	102 (3.3)	103 (5.6)	106 (7.0)	100 (7.5)	101 (10.3)	0.005	0.1
Narasin	2.5, 50, 150, 200	100 (3)	100 (3.3)	95 (3.7)	91 (2.3)	101 (4.7)	101 (5.2)	93 (3.9)	91 (4.5)	0.005	0.1
Nequinate	2.5, 50, 100, 125	96 (1.2)	87 (1.8)	86 (3.4)	87 (2.5)	101 (3.9)	90 (3.8)	88 (5.5)	89 (7.6)	0.1	0.5
Nicarbazin	7.5, 50, 150, 200	104 (0.7)	104 (1.8)	102 (1.5)	99 (1.9)	103 (1.7)	105 (1.5)	101 (1.5)	100 (1.5)	0.1	1
Nitromide	25, 75, 150, 200	103 (2.3)	101 (1.5)	101 (1.9)	99 (2.3)	98 (7.5)	100 (3.9)	99 (4.9)	98 (4.5)	5	20
Robenidine	2.5, 50, 150, 200	107 (1.5)	104 (0.9)	103 (1.3)	104 (0.6)	107 (2.9)	101 (1.9)	101 (1.4)	101 (1.6)	0.03	0.1
Salinomycin	2.5, 50, 150, 200	98 (3.4)	100 (3.1)	96 (4.6)	93 (2.4)	100 (5.6)	99 (5.6)	93 (5.8)	91 (6.4)	0.02	0.1
Semduramicin	7.5, 50, 150, 200	104 (4.4)	106 (7.7)	93 (4.7)	90 (4.2)	100 (5.6)	99 (6.2)	93 (9.6)	91 (8.5)	0.25	1
Toltrazuril	25, 75, 150, 200	102 (2.1)	101 (1.3)	102 (1.7)	99 (2)	99 (4.7)	99 (2.6)	100 (2.8)	99 (3.1)	4	20
Toltrazuril	25, 75, 150,	97	100	100	102	98	99	99	99	10	20

Analyte	Validated levels L1, L2, L3, L4 (ng L^{-1})	WL_r Trueness (RSD_r) (%)[a]				WL_R Trueness (RSD_{wR}) (%)[b]				LOD[c] (ng L^{-1})	LOQ[d] (ng L^{-1})
		L1	L2	L3	L4	L1	L2	L3	L4		
sulphone	200	(2.3)	(3)	(3.1)	(2.4)	(3.1)	(3.2)	(3.1)	(3.0)		
Toltrazuril	25, 75, 150,	97	98	99	98	99	100	99	98		
sulphoxide	200	(2.1)	(1.9)	(2.2)	(1.5)	(5.4)	(2.1)	(3.5)	(4.0)	4	20

[a] WL_r =Within-laboratory repeatability while RSD_r = Relative standard deviation under repeatability conditions, [b] WL_R=Within-laboratory reproducibility, while RSD_{wR} = Relative standard deviation under reproducibility conditions [c] LOD = Limit of Detection based on S/N = 5, [d] LOQ = Limit of Quantitation based on S/N = 10., L1, L2 ,L3,and L4 refer to each of the four
levels at which the validation was performed, equivalent to calibration points 2, 4, 6 and 7 respectively.

Table 4. Summary of the seven anticoccidial compounds, and respective concentration ranges, detected during a spatial sampling campaign throughout the Republic of Ireland in 2018

Anticoccidial Compound	Detected Concentration Range (ng L^{-1})*
Ionophores	
Lasalocid	≥LOQ – 56
Monensin	≥LOQ – 386
Narasin	≥LOQ – 47
Salinomycin	≥LOQ – 19
Chemical coccidiostats	
Amprolium	≥LOQ – 50
Diclzauril	≥LOQ – 66
Nicarbazin	≥LOQ – 135

* See Table 3 for LOQ of each individual compound