# MASTERARBEIT | MASTER'S THESIS

Titel | Title

Development and validation of an LC-MS/MS method for the determination of coccidiostats and antimicrobial agents in chicken and rabbit feed

verfasst von | submitted by

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# Abstract

Given the increasing demand for food and the intensification of animal farming, the monitoring of feed additives such as coccidiostats and antibiotics is becoming increasingly important to safeguard both animal and human health. In this study, an LC-MS/MS method was developed for the detection and quantification of eleven authorized coccidiostats according to Regulation (EC) No 1831/2003 and two unauthorized substances in poultry and rabbit feed. Additionally, selected tetracyclines were included to extend the method's applicability to antibiotics of high relevance in veterinary medicine. A key focus of this study was the reliable detection of low-level carry-over contamination at 1 % and 3 % in feed not intended to contain the target compounds, such as rabbit feed (non-target feed). Monitoring such cross-contamination is essential to improving feed safety and combating the spread of antimicrobial resistance. The method was optimized with respect to both sample extraction and chromatographic separation. Among the tested extraction solvents, a mixture of 80 % acetonitrile, 10 % water, and 10 % methanol, followed by a dilute-and-shoot approach, showed superior results, and was selected for method development. Chromatographic separation was carried out using a CORTECS T3 column, with further adjustments to retention time, peak resolution, and mobile phase composition. For quantification, at least two intensive product ions were selected per analyte while the use of target enhancement improved signal intensity. Method validation including linearity, limit of detection (LOD) and limit of quantification (LOQ), recovery and trueness was carried out according to EURACHEM (2025) guidelines. The method demonstrated excellent linearity across relevant concentration ranges, with coefficients of determination ($R^2$) greater than or equal to 0.99. LOD and LOQ were sufficiently low to allow the measurement of 3 % and 1 % carry-over levels in feed matrices. Quantification was performed by internal standard calibration based on response factors. The applicability of the method was confirmed through successful participation in a proficiency test for narasin, where results fell well within the accepted range. Furthermore, the method was applied to six commercial rabbit feed samples. Salinomycin, narasin, and monensin were detected at carry-over levels in three out of six samples, illustrating the method's practical relevance and providing new data on the occurrence of coccidiostats in rabbit feed, a topic for which only limited data is currently available.



Keywords: LC-MS/MS, coccidiostats, tetracyclines, carry-over, rabbit feed, poultry feed, validation, feed inspection, quality control

# Zusammenfassung

Angesichts der steigenden Nachfrage nach Lebensmitteln und der Intensivierung der Tierhaltung gewinnt die Überwachung von Futtermittelzusatzstoffen wie Kokzidiostatika und Antibiotika zum Schutz der Gesundheit von Mensch und Tier zunehmend an Bedeutung. In dieser Studie wurde eine LC-MS/MS-Methode zur Bestimmung und Quantifizierung von elf zugelassenen Kokzidiostatika gemäß Verordnung (EG) Nr. 1831/2003 sowie zwei nicht zugelassenen Substanzen in Geflügel- und Kaninchenfutter entwickelt. Zusätzlich wurden ausgewählte Tetrazykline integriert, um die Anwendbarkeit der Methode auf veterinärmedizinisch relevante Antibiotika zu erweitern. Ein zentrales Ziel der Arbeit war der zuverlässige Nachweis geringer Carry-Over-Kontaminationen (1 % und 3 %) in Futtermitteln, die nicht gezielt mit Wirkstoffen versetzt wurden, wie etwa Kaninchenfutter. Die Überwachung solcher Verschleppungen ist entscheidend für die Futtermittelsicherheit und die Eindämmung der Ausbreitung antimikrobieller Resistenzen.

Die Methode wurde sowohl hinsichtlich der Probenextraktion als auch der chromatographischen Trennung optimiert. Unter den getesteten Extraktionsmitteln erzielte ein Gemisch aus 80 % Acetonitril, 10 % Wasser und 10 % Methanol in Kombination mit einem „dilute-and-shoot“-Ansatz die besten Ergebnisse und wurde für die Methodenentwicklung ausgewählt. Die chromatographische Trennung erfolgte über eine CORTECS T3-Säule. Dabei wurden Retentionszeiten, Peak-Auflösung und Zusammensetzung der mobilen Phase gezielt angepasst. Für die Quantifizierung wurden pro Analyt mindestens zwei intensive Produkt-Ionen ausgewählt und die Signalintensität durch den Einsatz von „Target Enhancement“ verbessert. Die Validierung der Methode erfolgte anhand von Linearität, Bestimmungs- und Nachweisgrenze, Wiederfindung und Richtigkeit gemäß den Richtlinien von EURACHEM (2025). Die Methode zeigte eine ausgezeichnete Linearität über die relevanten Konzentrationsbereiche hinweg, mit Bestimmtheitsmaßen ($R^2$) von größer oder gleich 0,99. Die Nachweis- und Bestimmungsgrenzen waren ausreichend niedrig, um Carry-Over-Gehalte von 3 % bzw. 1 % in Futtermittelmatrizes sicher nachzuweisen. Die Quantifizierung erfolgte mittels interner Standardkalibration basierend auf Responsefaktoren.

Die Anwendbarkeit der Methode wurde durch die erfolgreiche Teilnahme an einem Ringversuch für Narasin bestätigt, bei dem die Ergebnisse innerhalb des akzeptierten Bereichs lagen. Darüber hinaus wurde die Methode auf sechs kommerzielle Kaninchenfutterproben angewendet.

Salinomycin, Narasin und Monensin wurden in drei von sechs Proben auf Carry-Over-Niveau nachgewiesen. Die Ergebnisse zeigen die Relevanz der Methode und ergänzen die bislang geringe Datenlage zu Kokzidiostatika in Kaninchenfutter.

Schlüsselwörter: LC-MS/MS, Kokzidiostatika, Tetrazykline, Carry-Over, Kaninchenfutter, Geflügelfutter, Validierung, Futtermittelkontrolle, Qualitätssicherung

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# List of abbreviations

| | |
|---|---|
| AMR | Antimicrobial resistance |
| DDA | Data dependent acquisition |
| DIA | Data independent acquisition |
| ESI | Electrospray ionization |
| EU | European union |
| GC | Gas chromatography |
| HILIC | Hydrophilic interaction liquid chromatography |
| HPLC | High performance liquid chromatography |
| IS | Internal Standard |
| LC-MS/MS | Liquid chromatography tandem mass spectrometry |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| MALDI | Matrix assisted laser desorption ionization |
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometry |
| m/z | Mass-to-charge |
| PRM | Parallel reaction monitoring |
| QuEChERS | Quick easy cheap effective rugged and safe |
| RF | Response Factor |
| SPE | Solid Phase Extraction |
| SRM | Selected reaction monitoring |
| ToF | Time of flight |

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# 1 Introduction

## 1.1 Poultry and rabbit farming

Poultry farming is one of the most important parts of animal farming, primarily focusing on the production of meat and eggs (Mottet & Tempio, 2017). In 2022, the number of poultry kept for agriculture worldwide was around 28.3 million poultry birds (FAOSTAT, 2022). Due to the growing world population, the livestock sector of poultry production will continue to increase worldwide (Mottet & Tempio, 2017). The global production of rabbit meat reached 681995.64 tonnes in 2023 with China being the largest producer (FAOSTAT, 2023).

To meet the rising demand and to secure protein supply as well as food and nutritional security, intensive farming techniques are increasingly being used. Intensive farming is a method of raising large numbers of animals in small spaces to maximize production efficiency which can cause environmental and health risks. (Gržinić et al., 2023) It contributes for example to an increase in nitrogen and phosphorus in the water or soil due to high amount of poultry manure and litter. Also, the spread of pathogenic microorganisms and the development and spread of multi resistant pathogens are significant problems. This mainly concerns broilers which are chickens raised for meat production and layers which are the chickens kept for egg production. To prevent the spread of disease in confined spaces, a variety of pharmaceuticals and antibiotics are used. These include for example tetracyclines, macrolides penicillins and coccidiostats. (Gržinić et al., 2023) These are fed in the form of medicated feed which is a mixture of feed and veterinary medicine (Regulation (EU) 2019/4, 2019).

## 1.2 Coccidiosis

Coccidiosis is a disease caused by parasites of the genus Eimeria mainly affecting the intestinal tract of young and weak animals leading to health problems such as intestinal lesions, diarrhoea, poor weight gain, poor feed conversion and in some cases death (Clarke et al., 2014). It is one of the most common infections in poultry farming mainly due to the high density and large numbers of birds kept together facilitating the spread of the disease by contact with infected faeces (Peek & Landman, 2011). Nevertheless, the disease can also occur in other intensively farmed species such as pigs, poultry, cattle, sheep and rabbits especially when there are poor hygiene practices and infected animals are not separated form healthy ones (Clarke et al., 2014). To avoid the spread of coccidiosis a good farm management is crucial which involves the use of quality

ventilation systems, good litter hygiene and fast removal of contaminated faeces (Martins et al., 2022). Also feeding the animals with prebiotics, probiotics, plant extracts and essential oils can contribute to a healthier intestinal microbiota and improve animal health to make them less susceptible to coccidiosis (Martins et al., 2022). However, to control the spread of coccidiosis and prevent the financial loss from diseased animals, coccidiostats are mostly added to the animal feed as feed additives (Peek & Landman, 2011).

### 1.2.1 Coccidiostats

The coccidiostats used as feed additives can be divided into two different categories including synthetic compounds and ionophores. They differ in their production techniques and mechanisms of action. The synthetic compounds are formed by chemical synthesis while the ionophores are produced by different bacteria of the Streptomycetaceae family. (Peek & Landman, 2011) The structural formulas of the chemical coccidiostats are shown in *figure 1.*

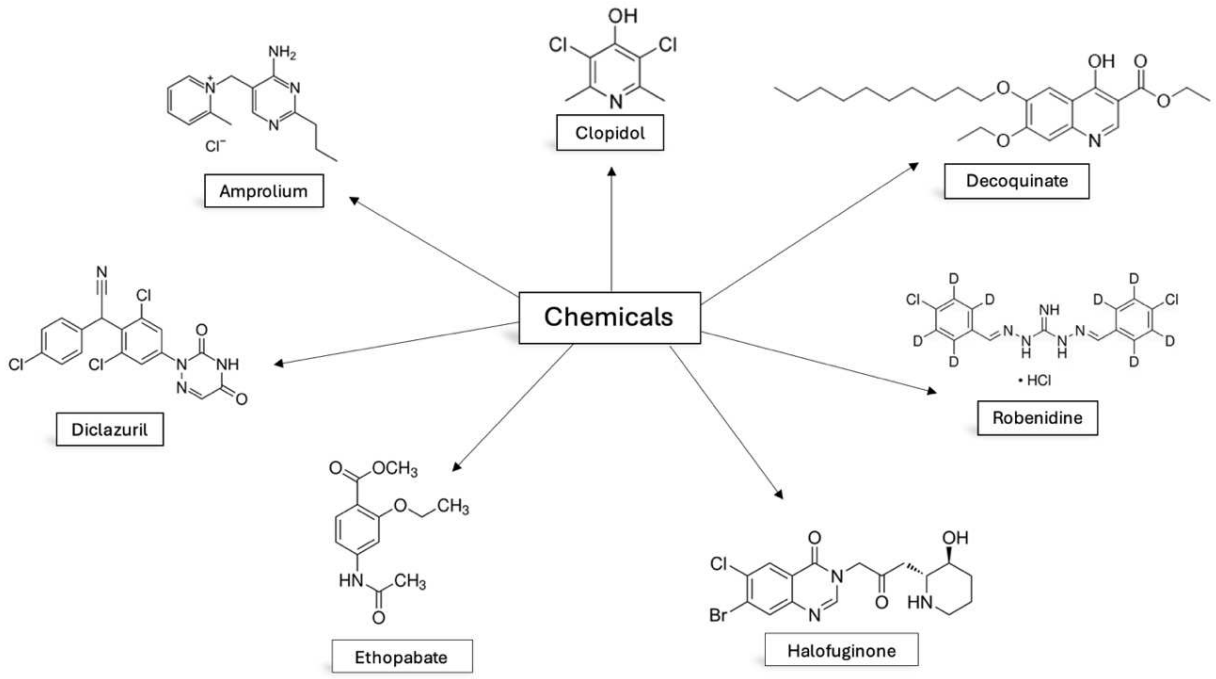



***Figure 1:*** *Structural formulas of authorized and unauthorized chemical coccidiostats.*

The chemical coccidiostats share structural similarities and they all contain aromatic rings like benzol or pyrimidine rings. These aromatic rings are often substituted with functional groups like halogens, hydroxyl groups and nitro groups. Additionally, they contain at least one secondary, but usually tertiary or even quaternary amino groups. (Clarke et al., 2014)

They show different modes of action against the parasites but most of them interact with the parasite metabolism. Amprolium for example blocks the absorption of thiamine while clopidol, robenidine and decoquinate inhibit the energy production in the mitochondria of the parasites. Ethopabate and diclazuril interfere with the synthesis of nucleic acids. (Peek & Landman, 2011)

The second important group represents the ionophores which are produced by the fermentation of Streptomyces spp. They can be divided into monovalent ionophores (salinomycin, narasin and monensin), monovalent glycosidic ionophores (maduramicin and semduramicin) and divalent ionophores (lasalocid). They all interfere with the parasites cell membrane function because they can bind and transport important ions such as natrium, calcium and potassium by formation of complexes. (Peek & Landman, 2011) Due to the increase of ions in the parasite inside, water uptake by osmosis increases, leading to an osmotic imbalance, and the cells swell and burst. This automatically leads to the death of the parasites. (Martins et al., 2022) Monensin for example forms lipid soluble complexes with sodium and potassium while salinomycin shows a high preference for potassium also promoting the efflux of K+ ions from mitochondria and cytoplasm. Lasalocid binds magnesium and calcium ions. (Noack et al., 2019) The structural formulas of the ionophores are demonstrated in *figure 2*. They are mostly characterized by multiple tetrahydrofuran rings (Noack et al., 2019).

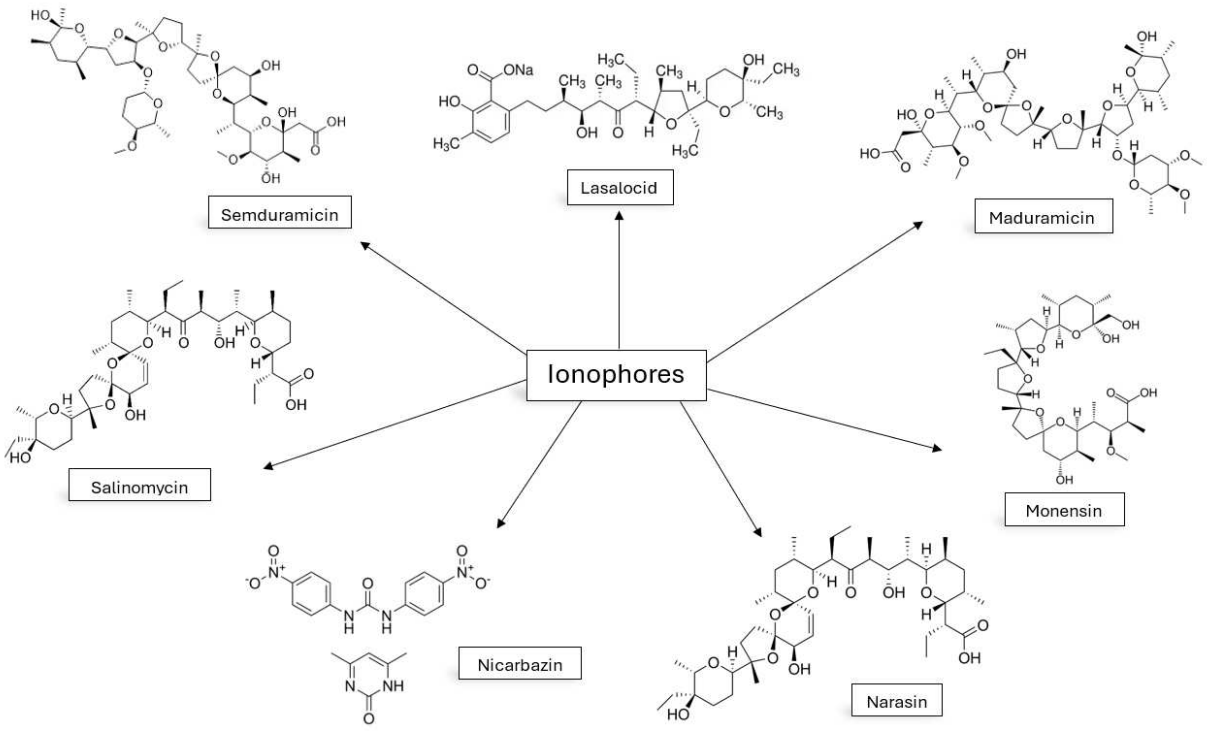



***Figure 2:*** *Structural formulas of authorized ionophore coccidiostats.*

### 1.2.2 Legislation of Coccidiostats

Coccidiostats are authorized for use as feed additives in accordance with Regulation (EC) No 1831/2003. However, they are only authorized under certain conditions such as the target animal species or the intended use of the animals. These conditions include the maximum permitted concentration in feed, which varies depending on the animal species and the production purpose. For example, requirements differ between chickens for fattening and chickens reared for laying. Specific withdrawal periods before slaughter are also considered when determining acceptable carry-over levels. (Commission Directive 2009/8/EC, 2009).

The maximum content is listed as mg/kg (ppm) relative to a feedingstuff with a moisture content of 12 %. The eleven approved coccidiostats are lasalocid sodium, narasin, salinomycin sodium, monensin sodium, semduramicin sodium, maduramicin ammonium alpha, robenidine hydrochloride, decoquinate, halofuginone hydrobromide, nicarbazin and diclazuril (Commission Directive 2009/8/EC, 2009). The maximum contents of the coccidiostats in mg/kg (ppm) relative to a feedingstuff with a moisture content of 12 % are listed in *table 1*.

***Table 1:*** *Maximum permitted levels of coccidiostats in target and non-target feed (mg/kg, 12% moisture) including carry-over of 1 % and 3 % according to Commission Directive 2009/8/EC.*

| | **Maximum content (mg/kg) relative to feedstuff (12 % moisture)** | | |
|---|---|---|---|
| | Non-target feed | | Target feed |
| | Carry-over 1 % | Carry-over 3 % | 100 % |
| Lasalocid sodium | 1.25 | 3.75 | 125 |
| Narasin | 0.7 | 2.1 | 70 |
| Salinomycin sodium | 0.7 | 2.1 | 70 |
| Monensin sodium | 1.25 | 3.75 | 125 |
| Semduramicin sodium | 0.25 | 0.75 | 25 |
| Maduramicin ammonium | 0.05 | 0.15 | 5 |
| Robenidine hydrochloride | 0.7 | 2.1 | 70 |
| Decoquinate | 0.4 | 1.2 | 40 |
| Halofuginone hydrobromide | 0.03 | 0.09 | 3 |
| Nicarbazin | 0.5 | 1.5 | 50 |
| Diclazuril | 0.01 | 0.03 | 1 |

Animal feed producers might also manufacture different animal feed types after each other in the same production line. Therefore, it is quite possible that coccidiostats may end up in non-target feed due to carry-over or cross contamination after the production of target feed. This process can happen during all stages of production, processing, storage, and transport and can also affect animal feed of different species. (Commission Directive 2009/8/EC, 2009)

Under good manufacturing practices, carry-over should be minimized according to the as low as reasonably achievable principle. The maximum carry-over levels for non-target animal feed allows up to 1 % of the maximum content for sensitive species and up to 3 % for less sensitive species. (Commission Directive 2009/8/EC, 2009) Young animals, horses, dogs, cat, rats, and rabbits may experience adverse effects from coccidiostats especially from ionophores and are considered sensitive species due to their specific physiology. This is because ionophores interact with the cellular ion balance. Monensin for example is one of the most toxic ones with a lethal dose of 1-3 mg/kg body weight in horses while salinomycin and lasalocid are described as the least toxic of the ionophore coccidiostats. (Noack et al., 2019)

## 1.3 Use of coccidiostats in poultry and rabbit farming

The administration of coccidiostats is achieved through incorporation into the animal feed. For poultry farming three different drug programs are used: single drug, shuttle drug and rotation programs. In the single drug program only one substance is administered, which has the disadvantage that resistance can develop over time. To prevent this, different coccidiostats are used in the shuttle program depending on the age of the animals. (Martins et al., 2022) In the rotation program the coccidiostats are replaced after a period of two months and a new category is used. Mostly two different substances are administered at the same time. (Peek & Landman, 2011) In poultry farming all the substances listed in *table 1* are used (Commission Directive 2009/8/EC, 2009). When ionophores and chemical coccidiostats such as nicarbazin and narasin are used together, synergistic effects can be observed (Noack et al., 2019). For example, Maxiban® G160 is a product used at levels of 40–50 mg of narasin per kg and 40–50 mg of nicarbazin per kg of complete feed (EFSA FEEDAP Panel, 2010).

In addition to poultry, rabbits are also affected by coccidiosis. The disease shows the same symptoms as in poultry leading to reduced feed intake, weight loss, diarrhea and even death, thereby mostly affecting young and weak animals kept in groups. (Lohkamp et al., 2024)

However currently only the two coccidiostats diclazuril and robenidine are authorized as feed additives for rabbits (European Commission, 2021). This does not allow rotation programs to reduce the development of anticoccidial resistance making it challenging for farmers and veterinarians to manage coccidiosis effectively (Lohkamp et al. 2024).

## 1.4 Antimicrobial active substances

Besides coccidiosis also other diseases can occur in animal farming. To treat these diseases and prevent their spread, various antimicrobial active substances are used. The manufacture and the use of medicated feed are regulated in the Regulation (EU) 2019/4. By controlling the use of these substances, antibiotic resistance, and carry-over of medications into non-target feed can be avoided. Non-target feed refers to any feed, medicated or not, that is not meant to include a particular active substance (Regulation (EU) 2019/4, 2019). A list of the allowed antimicrobial active substances is shown in *table 2.* Due to the frequent use of tetracycline, chlortetracycline, and doxycycline this work focuses on their determination.

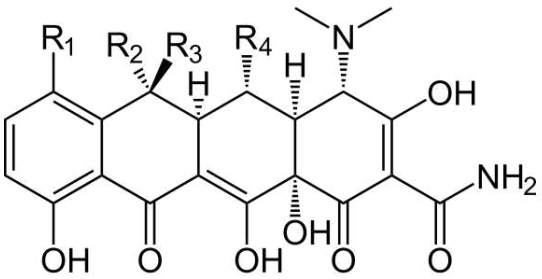
***Table 2:*** *List of authorized antimicrobial active substances according to Regulation (EU) 2019/4.*

| **Antimicrobial active substances** | | | |
|---|---|---|---|
| Amoxicillin | Florfenicol | **Tetracycline** | Tiamfenicol |
| Amprolium | Flumequin | Oxytetracycline | Tilmicosin |
| Apramycin | Lincomycin | Oxolinix Acid | Trimethoprim |
| **Chlortetracycline** | Neomycin | Paromomycin | Tylosin |
| Colistin | Spectinomycin | Penicillin V | Valnemulin |
| **Doxycycline** | Sulfonamides | Tiamulin | Tylvalosin |

The antibiotics listed in *table 2* are often used for the treatment of intestinal infections like colibacillosis, necrotic enteritis and other diseases caused by Salmonella, E. coli or Clostridium spp as these pathogens are known for the largest disease outbreaks and economic losses in poultry farming (Roth et al., 2019). In 2020 the global usage of antimicrobial agents in food producing animals was approximately 99 502 tonnes with projections indicating an 8.0 % increase by 2030. The region with the most antibiotic use was Asia with about 67 % of the total whereas less than 1 % were used in Africa. (Mulchandani et al., 2023)

### 1.4.1 Tetracyclines

Tetracyclines are one of the most used drugs in food producing animals and are utilized for the treatment against gram-positive and gram-negative bacteria to treat respiratory diseases. They work by binding reversibly to the 16S rRNA of 30S ribosomal subunit, blocking protein synthesis of the bacteria. The most common used tetracyclines are tetracycline, chlortetracycline, doxycycline and oxytetracycline. (Koutsoumanis et al., 2021) The structures of the tetracyclines are shown in *figure 3.*




| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| Tetracycline | H | OH | $CH_3$ | H |
| Chlortetracycline | Cl | OH | $CH_3$ | H |
| Doxycycline | H | H | $CH_3$ | OH |
| Oxytetracycline | H | OH | $CH_3$ | OH |

***Figure 3**: Chemical structure of tetracycline derivatives.*

The monitoring and regulation of the presence of tetracyclines in animal feed are of utmost importance to prevent excessive consumption and the development of antimicrobial resistance (AMR). AMR occurs when an antimicrobial agent loses its ability to inhibit the growth of a bacterium leading to therapy failure in case of pathogenic organisms. The main mechanisms of tetracycline resistance are increased efflux, reduced uptake, ribosomal protection and enzymatic inactivation. (Koutsoumanis et al., 2021)

The tetracyclines and other antimicrobial substances are administered in form of medicated feed which is a homogeneous mixture of feed and veterinary medicinal products. Cross contamination in non-target feed may occur during different manufacturing processes and should be avoided or kept as low as possible. (Regulation (EU) 2019/4, 2019)

The EFSA has set feed antimicrobial resistance selection concentrations (FARSC) values for the tetracyclines which are the maximum levels of antimicrobials in animal feed that prevent the development of antibiotic resistance. The FARSC corresponding to the concentration of chlortetracycline, oxytetracycline and tetracycline for feed intended for fattening chickens range from 0.17-0.84 µg/kg and for laying hens from 0.30 – 1.51 µg/kg feed. For doxycycline, the values for feed intended for fattening chickens lie between 0.17–1.12 µg/kg feed, and for laying hens, between 0.30–2.01 µg/kg feed. No specific FARSC values have been determined for rabbits. (Koutsoumanis et al., 2021) Moreover, tetracyclines are not allowed to be administered for prophylactic purpose or as growth enhancers they may only be used therapeutically to treat infected animals (Regulation (EU) 2019/4, 2019).

## 1.5 Aims

Given the increasing demand for food and the need for intensive animal farming the monitoring of animal feed additives like coccidiostats and antibiotics is becoming increasingly important to protect the health of humans and animals. This master thesis therefore aims to develop a LC-ESI-QTOF-MS/MS method to determine the eleven authorized coccidiostats lasalocid sodium, narasin, salinomycin sodium, monensin sodium, semduramicin sodium, maduramicin ammonium, robenidine hydrochloride, decoquinate, halofuginone hydrobromide, nicarbazin and diclazuril listed in the Commission Directive 2009/8/EG. Also, the authorized antimicrobial active substances tetracycline, chlortetracycline, doxycycline and tilmicosin registered in the Regulation (EU) 2019/4 are measured. In addition, two unauthorized coccidiostats, ethopabate and dimetridazole are determined to secure safety in animal feed.

A significant benefit of using TOF-MS/MS is its ability to provide high mass resolution measurements, which allows for the accurate identification of compounds with very similar m/z ratios. The main advantage lies in the higher selectivity achieved through PRM, as this approach enables the simultaneous detection of multiple fragment ions per analyte without loss of sensitivity. For this reason, MRM measurements with triple quadrupole (QQQ) instruments are often limited to two transitions per analyte, whereas PRM measurements on high-resolution mass spectrometers allow for the acquisition of several fragment ions. Although duty cycles and thus sensitivity are generally distinctly lower than in triple quadrupole systems, the application of target enhancement can boost signal intensity and partially compensate this limitation.
Moreover, the method involves the measurement of carry-over in non-targeted feed in the range of 1 % and 3 %, enhancing overall feed safety and quality control. Another notable aspect of this master thesis is the determination of coccidiostats in rabbit feed, as these substances are also used in this context, yet data on their occurrence and monitoring are still lacking. Therefore, the focus of this master thesis will be on chicken and rabbit feed.
To optimize chromatographic separation various LC gradients are tested with different mobile phases and additives. Initially targeted LC-MS/MS measurements are performed to enable the selection of fragments for each compound and the determination of the retention time at the given LC gradient. Based on the information obtained at least two fragments are selected for each compound and a targeted method is developed. To optimize sample preparation different solvents are tested for matrix effects. Three different purification methods are evaluated, and the most appropriate one is selected. Following this the MS parameters such as cone voltage,

collision energy and target enhancement are optimized. To ensure reliability, the method is validated using limit of detection (LOD), limit of quantification (LOQ), recovery and trueness.

# 2 Material and methods

## 2.1 Mass spectrometry

Mass spectrometry is a method used to determine the masses of atoms, molecules or fragments of molecules. A mass spectrometer as seen in *figure 4* includes a sample inlet system, an ion source, a mass analyser, and a detector. The molecules are ionized and then analysed according to their mass-to-charge (m/z) ration. There are different ionization techniques as for example electrospray ionization (ESI) or matrix assisted laser desorption ionization (MALDI). The use is dependent on the sample and analysis type. The generated ions provide a unique mass spectrum for the analysis of individual compounds and complex mixtures. The mass analyser separates ions based on their mass-to charge ratios. Examples for mass analysers are quadrupole, time of flight or ion trap. The detector records the ions and generates a mass spectrum with peaks corresponding to m/z values. Mass spectrometry is used in many fields including chemistry, biology, pharmacology, and environmental science for the identification of compounds, quantification of substances and determination of molecular structures. (Harris, 2014)

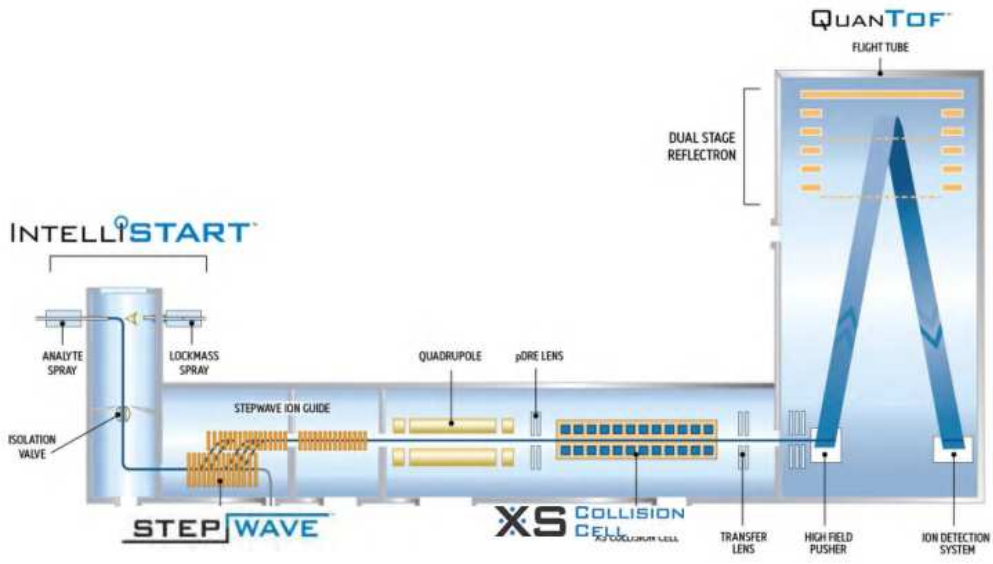



***Figure 4**: Construction of the Waters® Xevo G2-XS QTof mass spectrometer (Waters, 2014) with stepwave ion guide, quadrupole mass filter, collision cell and reflectron TOF analyser.*

MS analysis is mostly coupled with separation technique such as high-performance liquid chromatography (HPLC) or gas chromatography (GC). This combination allows for a separation of complex mixtures via chromatography followed by mass spectrometric analysis for identification and quantification of different compounds. (Otto 2019).

For the analysis of coccidiostats and antibiotics mostly reversed phase columns are used (Goessens et al., 2020; Hoff et al., 2020; Valese et al., 2017). Reversed phase chromatography is based on the different distribution of a substance between a nonpolar mobile phase and a polar stationary phase consisting of silica carrying different hydrophobic modifications like C18, C8 or phenyl groups. As mobile phase mostly a combination of acetonitrile, water or methanol is used. To enhance ionisation formic acid in concentrations of 0.1 % is mostly added. (Matissek & Fischer, 2021)

### 2.1.1 Electrospray ionization

The electrospray ionization is one of the most common used ionization methods and employed in this work. It is a soft ionization technique meaning that the molecules are not fragmented by the ionization making it ideal for analysing complex molecules. ESI works by applying a high voltage to a liquid sample in a fine capillary which creates a mist of charged droplets that can evaporate and form the so-called Taylor cone. The charge intensity increases leading to several coulomb explosions that produce even smaller droplets and at the end gas phase ions. These ions are then directed into the mass spectrometer and are sorted by their mass-to-charge ratio. (Otto, 2019) A schematic illustration of the ESI technique is demonstrated in *figure 5.*

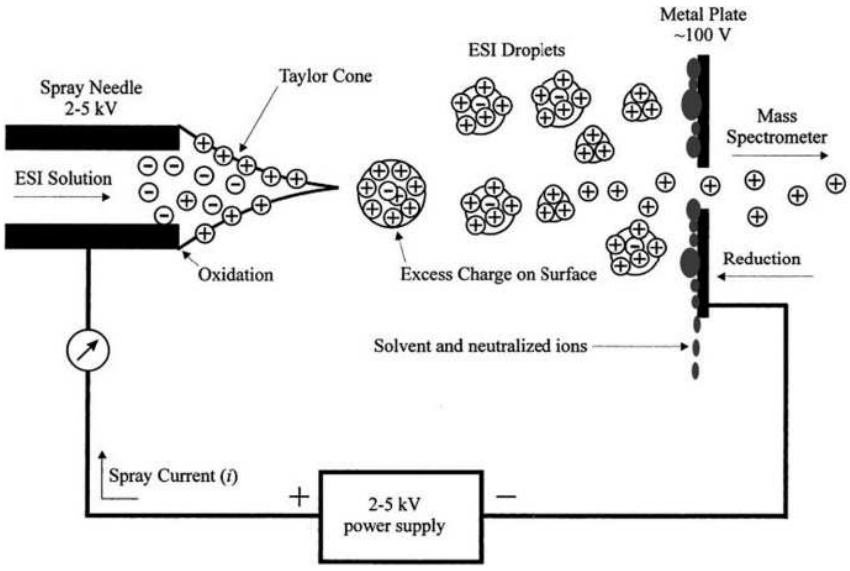



***Figure 5:*** *Schematic illustration of electrospray ionisation process (Cech & Enke, 2001).*

### 2.1.2 Mass analyser

The mass analyser separates ions based on their mass-to charge ratios. The mass analyser used in this work is a quadrupole combined with a time of flight (ToF) tube. A quadrupole mass analyser consists of four parallel metal rods. These rods are applied with a radio frequency and direct current voltage to create an oscillating electric field. Only ions with a specific m/z ratio

reach the detector and others are deflected and removed from the path. By adjusting the voltages of the radio frequency and the direct current voltage ions can be filtered selectively. (Otto, 2019) The ToF analyser separates ions based on the time it takes them to reach the detecting system. At the beginning all ions receive the same kinetic energy however the flight time then differs as lighter ions with lower m/z rations reach the detector sooner than heavier ions. ToF analysers can achieve high resolution and fast data acquisition which makes them suitable for a broad mass range and the analysis of large biomolecules. (Otto, 2019)

Additionally target enhancement can be used to improve sensitivity and increase the signal-to-noise ratio. It works by synchronizing the pusher at the detector entrance with the targeted mass, thereby increasing the duty cycle for ions of interest and allowing a greater number of ions to reach the detector. (Alelyunas et al., 2014)

### 2.1.3 Methodologies in mass spectrometry

There are two different measurement modes in liquid chromatography tandem mass spectrometry (LC-MS/MS) which are targeted and non-targeted. The targeted approach detects and quantifies specific and known molecules within a sample making it a method with high sensitivity and selectivity. Techniques include Multiple Reaction Monitoring (MRM), Selected Reaction Monitoring (SRM) and Parallel Reaction Monitoring (PRM). (Li et al., 2021). Non-targeted methods on the other side aim to detect and identify a wide range of compounds in a sample. The two untargeted methods are data dependent acquisition (DDA) and data independent acquisition (DIA) (Li et al., 2021). In this master thesis, data independent acquisition was carried out in MSE mode, using a low collision energy of 6 V for precursor detection and a high-energy collision energy ramp of 20–40 V for fragmentation of all ions. For the determination of coccidiostats and antibiotics in animal feed mostly selected reaction monitoring or multiple reaction monitoring with at least two m/z transitions for each compound is used (Borràs et al., 2013; Hoff et al., 2020; Valese et al., 2017). In selected reaction monitoring a specific precursor ion is selected and fragmented while multiple reaction monitoring involves monitoring multiple precursor-product ion transitions (Li et al., 2021).

In this work a hybrid approach is employed, combining the targeted with the non-targeted analysis by performing a non-targeted analysis to get an overview of all compounds present including retention time and the main fragments. Based on these results a targeted analysis is performed using the two or three most abundant fragments.

## 2.2 Analytical instrumentation

Chromatographic separation was performed by a Waters UPLC-System ACQUITY Bio H-Class instrument equipped with a CORTECS T3 Column (1.6 µm, 2.1 mm × 100 mm, Waters) and a CORTECS T3 VanGuard pre-column (1.6 µm, 2.1 mm × 5 mm). The mobile phase consisted of water with 0.1 % formic acid (A), acetonitrile with 0.1 % formic acid (B), 50 mM ammonium formate solution in methanol/water (80/20, v/v) with 0.1 % formic acid (C) and acetonitrile (D). The column temperature was set at 40 °C and the sample temperature in the autosampler was set at 10 °C. The flow was set at 0.3 mL/min. The elution programme for the positive mode was run for 45 minutes as follows: 0.00: 98 % A and 2 % C; 0.00-1.00: 95 % A, 3 % B and 2 % C; 1.00-5.00: 55% A, 43 % B and 2 % C; 5.00-10.00: 35 % A , 2 % C and 36 % D; 10.00-35.00: 4 % A, 10 % C, 86 % D; 35.00-38.00: 100 % B; 38.00-45.00: 98% A and 2 % C. The gradient for the positive mode is shown in *figure 6.*

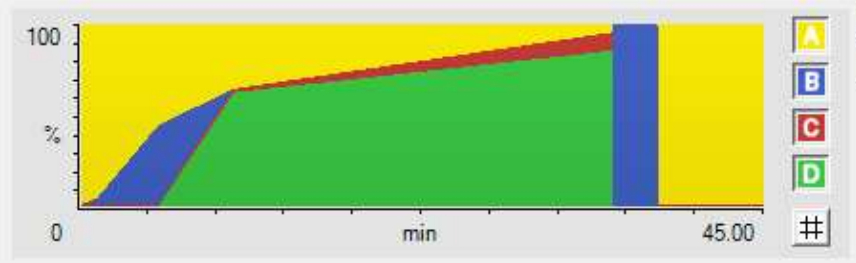



***Figure 6:*** *Applied LC solvent gradient for positive electrospray ionization mode.*

For the negative mode a shorter elution program with a duration of 25 minutes was used: 0.00: 98% A and 2 %B; 0.00-1.00: 95 % A and 5 % B; 1.00-5.00: 55 % A and 45 % B; 5.00-15.00: 5 % A and 95 % B; 15.00-18.00: 100 % B; 18.00-25.00 98 % A and 2 % B. The gradient is demonstrated in *figure 7.*

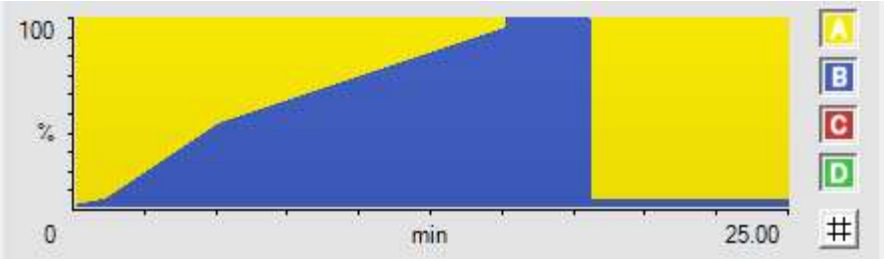



***Figure 7:*** *Applied LC solvent gradient for negative electrospray ionization mode.*

Detection was performed with a Xevo G2 XS QTOF mass spectrometer from Waters with an electrospray ionization interface. For MS analysis positive and negative ESI mode was used. The lock mass was calibrated with leucine-enkephalin every 180 seconds. The capillary voltage was set to 3.00 kV, the sampling cone voltage to 25 V, and the source offset to 60 V. The source temperature was maintained at 150 °C, while the desolvation temperature was set to 550 °C. The

cone gas flow rate was adjusted to 50 L/h and the desolvation gas flow rate to 800 L/h. For negative mode the same parameters were used except of a capillary voltage of 2.5 kV. The following MS parameters were set for the untargeted method: acquisition times start 0 min and end time 45 min, acquisition mode positive/negative, analyser mode sensitivity, range low mass 50 Da and high mass 3000 Da, scan time 0.3 sec, collision Energy 6 V, ramp collision energy from 20-40 V and cone voltage 25 V.

### 2.2.1 Creation of the targeted method

An initial untargeted screening was performed to identify the most abundant precursor ions and relevant product ions for each compound. Analysis of the untargeted measurements was performed with Skyline (version 24.1) where an isolation list was generated. The isolation list, including the optimized collision energies and retention times for each compound, was then transferred to the MassLynxTM software (version 4.2) to generate the targeted method. The retention time window for each compound was set to ± 2 min. The mass spectrometry parameters for the targeted method are listed in *table 3.*

***Table 3:*** *Mass spectrometric parameters for the eleven authorized coccidiostats (positive and negative ESI mode), two unauthorized coccidiostats, three antibiotics and their corresponding internal standards.*

| Compounds | Mass [g mol$^{-1}$] | $t_R$ [min] | Precursor ion [m/z] | Quantifier ion [m/z] | 1. Qualifier ion [m/z] | 2. Qualifier ion [m/z] | Cone voltage [V] | Collision energy [eV] | Target Enhanc. [m/z] |
|---|---|---|---|---|---|---|---|---|---|
| **ESI +** | | | | | | | | | |
| ***Coccidiostats*** | | | | | | | | | |
| Halofuginone | 414.7 | 5.6 | 416.0208 $[M + H]^+$ | 100.0738 | 120.0801 | 138.0905 | 50 | 16.5-22 | 100 |
| Robenidine | 334.2 | 8.2 | 334.0645 $[M + H]^+$ | 155.0366 | 138.0101 | 110.9983 | 20 | 14.1-19.2 | 140 |
| Decoquinate | 417.5 | 13.1 | 418.2609 $[M + H]^+$ | 372.2187 | 232.0613 | 204.0296 | 30 | 16.5-22.1 | 240 |
| Semduramicin | 873.1 | 22.4 | 895.5082 $[M + Na]^+$ | 851.5167 | 815.4954 | 727.4024 | 30 | 30.2-38.9 | 860 |
| Monensin | 670.9 | 24.4 | 693.4275 $[M + Na]^+$ | 675.4105 | 657.3988 | 461.2878 | 40 | 41.8-34.4 | 680 |
| Salinomycin | 751.0 | 25.9 | 773.4911 $[M + Na]^+$ | 431.3275 | 531.4259 | 487.3599 | 30 | 36.7-44.6 | 540 |
| Narasin | 765.0 | 29.5 | 787.5083 $[M + Na]^+$ | 769.4899 | 531.3304 | 431.2409 | 20 | 40-45 | 780 |
| Maduramicin | 917.1 | 27.8 | 939.5379 $[M + Na]^+$ | 895.5437 | 877.6602 | 859.5228 | 30 | 41.4-50 | 900 |
| Lasalocid | 590.8 | 32.8 | 613.3759 $[M + Na]^+$ | 577.3514 | 377.2611 | 595.3619 | 30 | 35-45 | 600 |
| ***Unauthorized Coccidiostats*** | | | | | | | | | |
| Dimetridazole | 141.1 | 3.9 | 142.0602 $[M + H]^+$ | 96.0652 | 95.0576 | 81.0414 | 30 | 8.6-12.4 | 100 |
| Ethopabate | 237.3 | 6.0 | 238.1659 $[M + H]^+$ | 206.0807 | 164.0696 | 136.0743 | 30 | 11.4-15.8 | 210 |
| ***Antibiotics*** | | | | | | | | | |
| Tetrazycline | 444.4 | 4.4 | 445.1599 $[M + H]^+$ | 410.1227 | 154.0491 | - | 30 | 17.3-23.1 | - |
| Chlortetracycline | 478.9 | 5.1 | 479.1212 $[M + H]^+$ | 445.1551 | 462.1216 | - | 30 | 18.3-24.3 | - |
| Doxycycline | 444.4 | 5.3 | 445.1589 $[M + H]^+$ | 428.1337 | 154.0491 | - | 30 | 17.3-23.1 | - |
| ***Internal standards*** | | | | | | | | | |
| Demelocycline | 464.9 | 4.9 | 465.1187 $[M + H]^+$ | 448.0863 | 430.0786 | - | 30 | 30-40 | - |
| Nigericin | 725.0 | 31.2 | 742.4856 $[M + NH_4]^+$ | 657.4376 | 675.4484 | 461.3264 | 30 | 30-30 | 680 |
| **ESI -** | | | | | | | | | |
| ***Coccidiostats*** | | | | | | | | | |
| Dinitrocarbanilide | 302.2 | 8.6 | 301.0651 $[M - H]^-$ | 137.0409 | 107.0403 | - | 30 | 16.4-22 | 115 |
| Diclazuril | 407.6 | 9.4 | 404.9723 $[M - H]^-$ | 335.9788 | 333.9810 | 298.9851 | 30 | 16.2-21.7 | 340 |
| ***Internal Standard*** | | | | | | | | | |
| Dinitrocarbanilide-d8 | 310.2 | 8.6 | 309.0884 $[M - H]^-$ | 141.0612 | 111.0609 | - | 30 | 16.4-22 | 150 |

For identification and quantification, at least the two most intensive product ions were chosen as quantifier ion and qualifier ion. Where possible, a third transition was included in addition to the primary and secondary transitions to improve analyte confirmation. The final developed transitions (precursor and product ions), retention times, and instrument settings were experimentally optimized and determined individually for each compound. The final targeted method covered sixteen analytes and three internal standards. To boost sensitivity different target enhancements for each compound were used. The target enhancement was configured to benefit as many fragments as possible by setting it slightly above the most intense or the specifically selected fragment intended to be enhanced.

## 2.3 Quantification

For quantification different internal standards (IS) were used. Nigericin was selected for the positive ESI mode authorized and unauthorized coccidiostats while dinitrocarbanilide-d8 was used for the quantification of the negative ESI mode coccidiostats. Demelocycline served as the internal standard for antibiotic quantification. Concentrations were calculated based on response factors (RF) using equation (1) and equation (2).

$$RF = \frac{\left(\frac{Peak\ Area\ Analyte}{Peak\ Area\ IS}\right)}{\left(\frac{Concentration\ Analyte}{Concentration\ IS}\right)} \quad (1)$$

$$Concentration\ Analyte = \left(\frac{Peak\ Area\ Analyte}{Peak\ Area\ IS}\right) * \left(\frac{Concentration\ IS}{RF}\right) \quad (2)$$

The use of these internal standards for quantification is specified in the ÖNORM EN 17299 and supported by their application in scientific publications such as those by Goessens et al. (2020) and Hoff et al. (2020). However according to ÖNORM EN 17299 additionally robenidine-d8, bis-diclazuril, decoquinate-d5, dimetridazole-d3 and ethopabate-d5 are recommended for quantification. These deuterated internal standards are considered the gold standard for quantitative LC-MS/MS analysis.

However, due to cost constraints or limited commercial availability, it is not always feasible to include deuterated standards for every compound in the method. For dinitrocarbanilide, dinitrocarbanilide-d8 was used as a deuterated internal standard. In this case, quantification was performed using the ion ratio, calculated as the peak area of dinitrocarbanilide divided by the peak area of dinitrocarbanilide-d8.

## 2.4 Chemicals and standards

Maduramicin ammonium, dimetridazole, robenidine hydrochloride, decoquinate, nicarbazin, halofuginone, monensin, ethopabate, tetracycline, chlortetracycline, doxycycline, demeclocycline, diclazuril and dinitrocarbanilide-d8 standards were purchased from Sigma-Aldrich (Bornem, Belgium). Salinomycin, narasin, lasalocid and nigericin were obtained from LGC Standards (Teddington, United Kingdom). Ultra-pure water type 1 (conductibility: 0.055 µS/cm, sterile and particle free: > 0.2 µm) was produced with a HALIOS system from EnviroFALK (Westerburg, Germany). Acetonitrile (LC-MS Chromasolv™, ≥ 99.9 %) was purchased from Honeywell (Morristown, New Jersey, USA). Isopropanol (LC-MS, ≥ 99.9 %), ammonium formate (for LC-MS, ≥ 99 %), methanol (LC-MS Chromasolv™, ≥ 99.9 %), and formic acid (≥ 99 %, for LC-MS) were obtained from VWR (Darmstadt, Germany). Dimethyl sulfoxide (HPLC grade, 99.9+ %) was sourced from Thermo Fisher Scientific (Dreieich, Germany). Sodium hydroxide solution (49–51 % in water), N,N-dimethylformamide, acetic acid (for LC-MS, ≥ 99.8 %), ammonia solution (25 %, for analysis), magnesium sulfate (anhydrous), sodium chloride (for analysis), and ethanol (absolute, for analysis) were purchased from Sigma-Aldrich (Bornem, Belgium). TOF G2-S Sample Kit -1 Leucin-Enkephalin was purchased from Waters (Milford, USA) as well as the SPE-purification extraction cartridges Oasis PRiME MCX 3cc (60 mg). To filter the samples syringe filters consisting of nylon/polyamid (0.22µM 25 MM) from ISOLAB (Eschau, Germany) were employed. For QTof calibration a 5 mM sodium formate solution in 2-propanol/water (90/10, v/v) was prepared according to introduction of the manufacturer.

In line with ÖNORM EN 17299 and the study by Hoff et al. (2020) the following stock solutions were prepared: ethopabate and nigericin were dissolved in methanol and for monensin, salinomycin, narasin and maduramicin a solution of acetonitrile/methanol (70/30, v/v) was used. Robenidine, decoquinate and dimetridazole standards were prepared in ethanol. For tetracycline, chlortetracycline, doxycycline and demelocycline a mixture of acetonitrile/methanol (50/50, v/v) was used. Stock solutions for dinitrocarbanilide-d8 were prepared in N-N-dimethylformamide and nicarbazin in dimethylsulfoxide. For lasalocid, acetonitrile was used, and for halofuginone, a 50:50 (v/v) mixture of water and acetonitrile. The stock solutions were prepared at a concentration of 1 mg/mL. Additionally, a mixed standard working solution was prepared in acetonitrile resulting in concentrations of 1.5 µg/mL for lasalocid, 5 µg/mL for dimetridazole, 0.1 µg/mL for maduramicin and decoquinate and 1 µg/mL for the remaining standards.

## 2.5 Sample preparation

Three different extraction solvents were evaluated using (A) acetonitrile/methanol/water (80/10/10, v/v) according to ÖNORM EN 17299, (B) acetonitrile/water with 0.1 % acetic acid (90/10, v/v) according to Valese et al. (2017) and (C) acetonitrile according to Rybicki et al. (2022). Three distinct sample preparation procedures were tested.

*Procedure 1:* Procedure 1 was based on ÖNORM EN 17299. Approximately 5 g of the sample was weighted into a 50 mL falcon tube and 40 mL of the extraction solution (A) was added. After the extraction solution was added the tubes were shaken vigorously followed by an extraction in an ultrasonic bath at 22 °C for 30 minutes. After the extraction the tubes were shaken on an overhead shaker at room temperature for one hour. Then the samples were centrifuged at 1850 g at 20 °C for 10 minutes. Before the injection the supernatant of the samples got filtered through a 0.2 µm polyamide filter.

*Procedure 2* was based on solid phase extraction (SPE), following the same extraction steps as Procedure 1, with an additional SPE cleanup. The SPE columns were conditioned with 2 mL methanol and centrifuged for 4 minutes at 3000 rpm. Subsequently, 2 mL of washing solution (0.18 M formic acid in methanol/water, 90/10, v/v) was added, followed by centrifugation for 4 minutes at 3000 rpm. After conditioning, 3 mL of the sample extract was loaded onto the columns and centrifuged for 10 minutes at 1000 rpm. The sample extract was prepared by mixing 1.5 mL of the sample with 1.5 mL of 0.2 M formic acid in water. The columns were then washed twice with 2 mL of washing solution, each wash followed by centrifugation for 4 minutes at 3000 rpm. Elution was performed by adding 1 mL of elution solution (5 % ammonium hydroxide in acetonitrile) and centrifuging for 1 minute at 500 rpm. Following SPE cleanup, the samples were vacuum-dried using a SpeedVac at 45 °C for around two hours and reconstituted in 200 µL of extraction solution (A) prior to MS analysis.

*Procedure 3:* Procedure 3 was adopted from Rybicki et al. (2022), which is a version of a quick, easy, cheap, effective, rugged and safe (QuEChERS) method using the same extraction method as procedure 1 adding a QuEChERS cleanup. 15 mL of the extract were transferred to a 50 mL extraction tube and dissolved in 1.62 g magnesium sulfate and 0.81 g sodium chloride. The tubes were then shaken on an overhead shaker for 15 minutes followed by a centrifugation process at 5000 rpm for 20 minutes. After that the supernatant of the samples got filtered through a 0.2 µm polyamide filter.

## 2.6 Samples

Rabbit feed samples were collected at the Austrian Agency for Health and Food Safety between March and April 2025 and grinded to 0.5 mm. The used samples are listed in *table 4.*

***Table 4:*** *List of rabbit feed samples collected at the Austrian Agency for Health and Food Safety.*

| Sample-ID | Sample Name |
|---|---|
| 24134081-001 | Kanin Korn Classic (Batch No: 198871) |
| 24062190-001 | TK775-4 Zuchtkaninchenfutter |
| 24065374-001 | Kanin Korn Classic (Batch No: 61036975) |
| 24153645-001 | Kanin Korn Classic (Batch No: 200058) |
| 24070187-001 | Alpenkorn Kanin |
| 24138488-001 | K-75 Kaninchenfutter |

## 2.7 Validation procedure

The validation of the LC-MS/MS method was conducted with reference to the principles of Eurachem Guide (Cantwell, 2025). Selected parameters such as LOD, LOQ, linearity, and recovery were assessed following adapted procedures. In addition, the method's performance was evaluated through participation in a proficiency test.

LOD, LOQ and linearity were determined by using a matrix matched calibration (10 % matrix) with concentrations ranging between 0.5 and 3000 pg on column for each compound and 13 calibration points. Based on the evaluation of linearity, at least five calibration points within the linear range were selected for the final calibration curve. LOD and LOQ were calculated using the software Valoo (analytic-software, 1999-2024), which is based on ÖNORM DIN 32645. Due to the unavailability of a certified matrix-free feed sample, a mixture of soybean and maize was prepared and used as a surrogate matrix for validation purposes.

Recovery was evaluated in spiked samples at two concentration levels, one at 3 % carry-over from non-targeted feed individually adjusted for each analyte (*see table 1*) and the other corresponding to concentrations close to the LOD. All samples for the validation process were extracted with acetonitrile/methanol/water (80/10/10, v/v) and prepared according to sample preparation procedure 1. All samples were carried out as biological triplicates. Samples from the international analytical group section feedingstuff proficiency test were analysed in biological triplicates using the same sample preparation as mentioned above. The mean value of the three measurements was calculated and compared against the assigned target range provided by the proficiency test organizer.

## 2.8 Statistical analysis

All experiments were performed with at least three independent replicates ($n \geq 3$). Results are generally reported as mean values ± standard deviation (SD), unless otherwise specified (e.g., recovery and LOD/LOQ values are given as mean only). Normal distribution (Shapiro–Wilk test) and equal variance (Levene's test) were assessed prior to analysis. One-way ANOVA was applied to evaluate statistical differences between groups, followed by post hoc test for pairwise comparisons. A significance level of $p < 0.05$ was considered statistically relevant. All analyses were performed using RStudio (version 2025.05.0+496, R Foundation for Statistical Computing, Vienna, Austria).

# 3 Results and Discussion

## 3.1 Evaluation of matrix effects using different extraction solvents

The results of the matrix effect evaluation for extraction solvents A, B and C are presented below. The composition of each solvent is described in detail in the materials and methods section. The focus of this evaluation was to assess matrix effects such as ion suppression or ion enhancement. For this purpose, different extraction solvents were tested using standard solutions of the coccidiostats that were mixed with 10 % analyte-free matrix (maize and soy) and the respective solvents. The extent of ion suppression or enhancement was evaluated by comparing signal intensities to those obtained from standard solutions prepared in pure acetonitrile without matrix. The reported signal intensities refer to the quantifier ion (strongest transition, see *table 3*). The results are expressed as the percentage of the signal intensity relative to the pure ACN standard and are illustrated in *figure 8.* In this figure, the red line represents the pure standards in ACN (100 %), while the bars show a selection of analytes. This selection was made to allow for clearer comparison and improved presentation of the results because of different signal intensities although all analytes were tested and included in the overall evaluation.

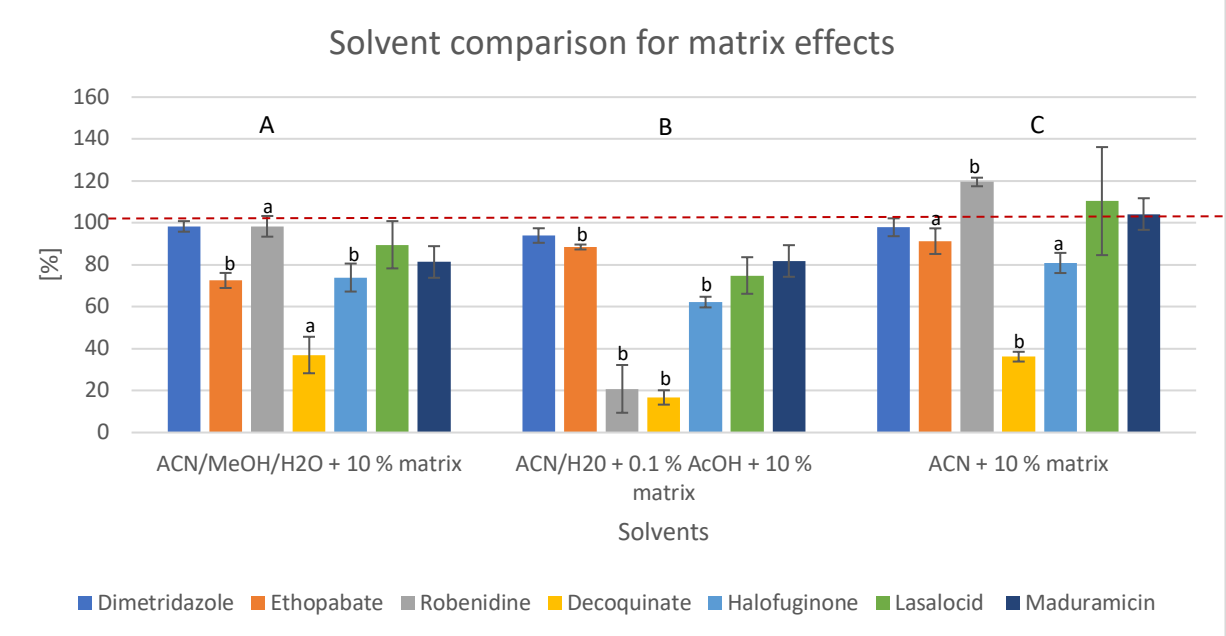



***Figure 8:*** *Comparison of different solvent systems for evaluating matrix effects (ion enhancement or suppression) in selected anticoccidial compounds, relative to 100 % ACN without matrix. A: acetonitrile/methanol/water (80/10/10, v/v); B: acetonitrile/water with 0.1 % acetic acid (90/10, v/v); C acetonitrile (100%). Different letters indicate significant differences between solvents (ANOVA with post-hoc test (pairwise Welch's t-test) (, $p < 0.05$). Letters are only shown for analytes with significant differences.*

The comparison of the three solvent systems revealed differences in matrix effects across the tested analytes. Solvent A showed no ion enhancements and consistent values close to the 100 % reference line for most compounds. The values ranged between 70 % and 98 %, except for

decoquinate (36%), which showed a relatively low response. Good results were observed for dimetridazole (98 %), robenidine (98 %) and lasalocid (89 %). Solvent B showed a more variable performance. While dimetridazole and ethopabate showed good results compared to the reference, the remaining analytes, particularly robenidine (21 %) and decoquinate (17 %), suffered from ion suppression. These findings suggest that the acidic environment may have negatively influenced the ionization efficiency of certain compounds. This is also reflected in the study by Boscher et al. (2010), where the addition of an acid to the extraction solvent led to increased ion suppression for ionophoric coccidiostats. However, the study by Valese et al. (2017) identified the best extraction results using a mixture of acetonitrile and water (90:10, v/v) containing 0.05% acetic acid. This suggests that the effect of acid on matrix interferences may be concentration-dependent, with lower acid levels potentially reducing ion suppression.

In contrast solvent C resulted in ion enhancement for several analytes including robenidine (120 %), lasalocid (110 %) and maduramicin (104 %), while also showing strong suppression for decoquinate (36 %). According to the literature, acetonitrile is a widely applied extraction solvent for the analysis of coccidiostats however it is mostly mixed with different solvents or additional additives (Boscher et al., 2010; Kenjeric et al., 2024; Rybicki et al., 2022; Valese et al., 2017). The absence of acids, additives, and solvents such as methanol or water may allow matrix components to interact more freely with the ionization process, potentially enhancing signal intensities for certain analytes as seen in this case.

In the context of a multi-analyte method, it is often not possible to select a single extraction approach that is optimal for all compounds. Nevertheless, ACN/MeOH/$H_2O$ (solvent A) exhibited the lowest overall matrix effects and was therefore selected for further method development. This choice was supported by a comparison of the mean deviation from the reference (100 % ACN without matrix) for each analyte, evaluated using ANOVA with a post-hoc test. ACN/MeOH/$H_2O$ showed the lowest mean deviation for four out of seven analytes, with significant differences for Decoquinate ($p = 0.032$) and Robenidine ($p = 0.041$), while ACN with 10 % matrix was superior for three analytes, with significant differences for Ethopabate ($p = 0.027$) and Halofuginone ($p = 0.039$). The ACN/$H_2O$ mixture consistently showed higher deviations from the reference and was not superior for any analyte. Since both ACN/MeOH/$H_2O$ and pure ACN were statistically superior in two cases each, ACN/MeOH/$H_2O$ was chosen as a compromise, considering the overall number of analytes with the lowest deviation and its application in ÖNORM EN 17299, and was used as the extraction solvent for further validation.

It can be assumed that the mixture of acetonitrile, methanol and water covers a broad range of analyte polarities and allows for adequate ionisation of the compounds, thereby minimizing strong matrix effects such as ion suppression or enhancement. This principle of combining solvents with different properties to cover a wide range of analyte polarities was also applied in the study by Boscher et al. (2010). They employed a mixture of methanol, acetonitrile, and McIlvaine buffer (pH 4.6), which is a water-based phosphate–citrate buffer supplemented with 0.3% EDTA, to reduce matrix effects during LC–MS/MS analysis.

## 3.2 Feed sample preparation optimisation

For sample preparation, dilute and shoot, QuEChERS and SPE methods were tested. The aim of the comparison of sample preparation methods was to determine which of the three approaches was best suited for the determination of coccidiostats in chicken and rabbit feed. The extraction procedure was identical for all methods and followed the specification of the ÖNORM EN 17299. The QuEChERS method was developed by Rybicki et al. (2022) and adapted to the requirements of the feed matrix and experimental setup. The SPE cleanup procedure was adapted to the manufactures (Waters: Milford, USA) recommendations. A detailed description of the sample preparation steps for each method is provided in the materials and methods section. To compare the three methods, a feed matrix (maize and soy) was spiked with coccidiostats standards, processed according to each sample preparation procedure, and subsequently analysed by LC-MS/MS. Comparison between methods was based on the peak areas obtained for each analyte. The results are demonstrated in *figure 9*.

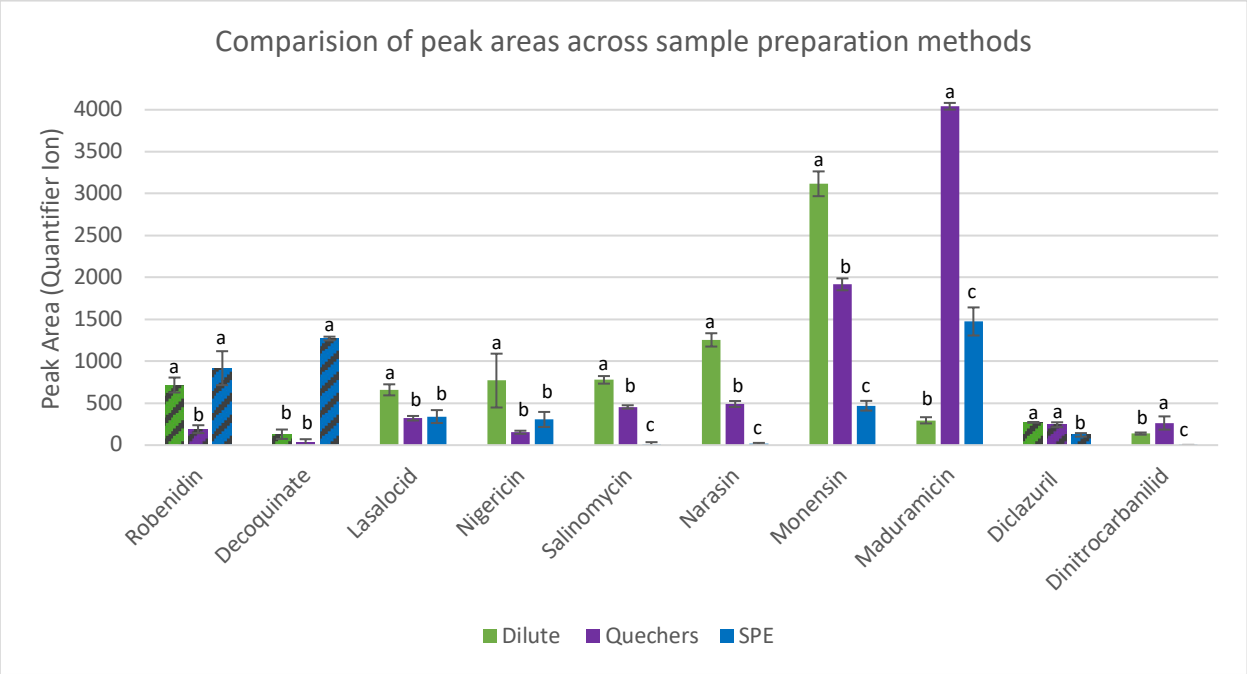



***Figure 9:*** *Comparison of sample preparation methods (Dilute, QuEChERS, and SPE) based on peak areas (quantifier ion) of selected coccidiostats spiked into feed matrix (maize and soy). Ionophores are shown as dashed bars, synthetic coccidiostats as solid bars. Different lowercase letters (a–c) above the bars indicate statistically significant differences between methods for each analyte (ANOVA followed by Tukey's HSD test, $p < 0.05$). Bars sharing the same letter are not significantly different.*

*Figure 9* presents the peak areas of ten coccidiostats, including both ionophore and synthetic compounds, analysed in both positive and negative ESI mode. Peak areas obtained from the three sample preparation strategies were statistically compared using ANOVA followed by Tukey's HSD test (n = 6 per method). Overall, the dilute-and-shoot approach yielded the highest peak areas for five out of ten analytes: lasalocid, nigericin, salinomycin, narasin and monensin (all $p < 0.01$).

For diclazuril, dilute-and-shoot also provided higher peak areas than SPE ($p < 0.01$), but did not differ significantly from QuEChERS ($p > 0.05$). For robenidine and decoquinate, SPE resulted in the highest peak areas, indicating efficient extraction and clean-up for these relatively non-polar analytes. The advantage of SPE was statistically significant for decoquinate ($p < 0.001$), while the difference between dilute-and-shoot and SPE was not statistically significant for robenidine ($p > 0.05$). However, for most other analytes SPE yielded clearly reduced signals compared to the other approaches, particularly for salinomycin, narasin, and dinitrocarbanilide. The QuEChERS method resulted in the highest peak areas for maduramicin and dinitrocarbanilide ($p < 0.001$) but showed lower performance for the remaining analytes compared to dilute-and-shoot.

Overall, dilute and shoot achieved the best results for most compounds, providing a strong basis for its selection as the preferred sample preparation technique. This outcome is in line with ÖNORM EN 17299, which also recommends dilute and shoot for the determination of coccidiostats in feed without any additional sample preparation technique. A likely explanation for the good performance of this method lies in the high degree of dilution applied prior to LC-MS/MS analysis, which helps to minimize matrix effects and enhances signal stability. During sample preparation, 5 g of feed were extracted with 40 mL of solvent, corresponding to a solid-to-liquid ratio of 1:8 (w/v). Additionally, depending on the analyte, the extract was further diluted by a factor of 1:100 or 1:1000 before injection which led to a strong dilution of matrix compounds in the sample. The study by Vincent et al. (2011) further supports the fact that SPE cleanup is not always necessary, as matrix interferences were effectively minimized through simple dilution and filtration steps. Pietruk et al. (2015) also demonstrated that reliable results can be achieved without SPE, using only solvent extraction, evaporation, and filtration prior to LC–MS/MS. Additionally dilute and shoot is a more time effective method compared to other more lab intensive sample preparation processes which is particularly relevant in routine laboratories handling larger amounts of samples where time efficiency plays an important role. In the context of this study, the SPE-based sample preparation was found to be about twice as time-consuming as the dilute and shoot approach, further supporting the choice of dilute and shoot for routine analysis.

The QuEChERS method is considerably faster compared to SPE cleanup but also includes a sample clean-up step. It was described by Cronly et al. (2011) as a time-effective and low-cost alternative to conventional SPE procedures. Nevertheless, although it produced the highest peak areas for maduramicin and dinitrocarbanilide, its overall performance was still lower than that of the dilute

and shoot method. This indicates that a clean-up step is not required and may even be counterproductive by introducing additional interferences using salts such as $MgSO_4$ and NaCl for the cleanup process. These findings are further supported by Kenjeric et al. (2024) who evaluated various clean-up approaches including QuEChERS and modified QuEChERS with PSA and C18. Their results also showed no notable improvement in matrix effect reduction when compared to the dilute and shoot approach. Specifically, for coccidiostats strong signal enhancement was observed which could also explain the relatively high peak areas using QuEChERS for maduramicin compared to the dilute and shoot method.

The SPE method using Oasis PRiME MCX 3 cc (60 mg) extraction cartridges designed for the retention and purification of basic compounds delivered the highest peak areas for robenidine and decoquinate. This suggests that specific interactions with the mixed-mode cation-exchange sorbent allowed for efficient extraction of these compounds. However, for the remaining analytes, particularly the ionophores, SPE resulted in lower signals. This indicates possible losses during the loading, washing, or elution steps, likely due to poor retention or analyte binding to the sorbent as ionophores such as monensin, narasin and salinomycin are only weakly basic and lack strongly protonatable groups. Retention could potentially be improved by adjusting the pH or using alternative sorbets. However, these changes may negatively affect the recovery of other analytes in the method. This highlights the challenge of developing multimethods for coccidiostats determination because of the strong chemical diversity in between the ionophores and chemicals which vary in polarity, pKa and solubility (Clarke et al., 2014). Developing a single SPE protocol that provides a good cleanup is therefore quite challenging. In contrast, the dilute and shoot approach avoids selective analyte losses, as it does not rely on compound-specific retention mechanisms such as those used in SPE sorbents. Furthermore, the SPE procedure was considerably more time-consuming and resource-intensive compared to the other two methods. This supports the suitability of the dilute and shoot method as a time-efficient and broadly applicable sample preparation method for the analysis of coccidiostats.

The testing of different sample preparation methods not only identified the most suitable approach for multi-analyte determination, but also revealed which method performed best for each individual coccidiostat. This information can be used in analyses where only a single compound is of interest. In such cases, the sample preparation protocol could be further optimized and adapted for the specific analyte, even if this would not be suitable for all analytes tested.

## 3.3 LC-MS/MS method optimisation

The LC-MS/MS method was developed for the quantification of the eleven authorized coccidiostats according to Commission Directive 2009/8/EC, two unauthorized coccidiostats and three antibiotics along with their respective internal standards *(table 3).* Chromatographic separation was achieved using a CORTECS T3 (1.6 µm, 2.1 mm × 100 mm, Waters) reversed-phase column, which provided satisfactory retention and resolution for all analytes. The selection was based on its compatibility with both polar and moderately nonpolar compounds which is supported by its application in previous studies using C18 columns for the determination of coccidiostats in feed. (Goessens et al., 2020; Hoff et al., 2020; Pietruk et al., 2015)

To illustrate the method development, *figure 10* shows an untargeted approach for the detection of coccidiostats in positive mode, based solely on precursor m/z identification. As mobile phase, a gradient elution of acetonitrile with 0.1% formic acid and water with 0.1% formic acid was used.

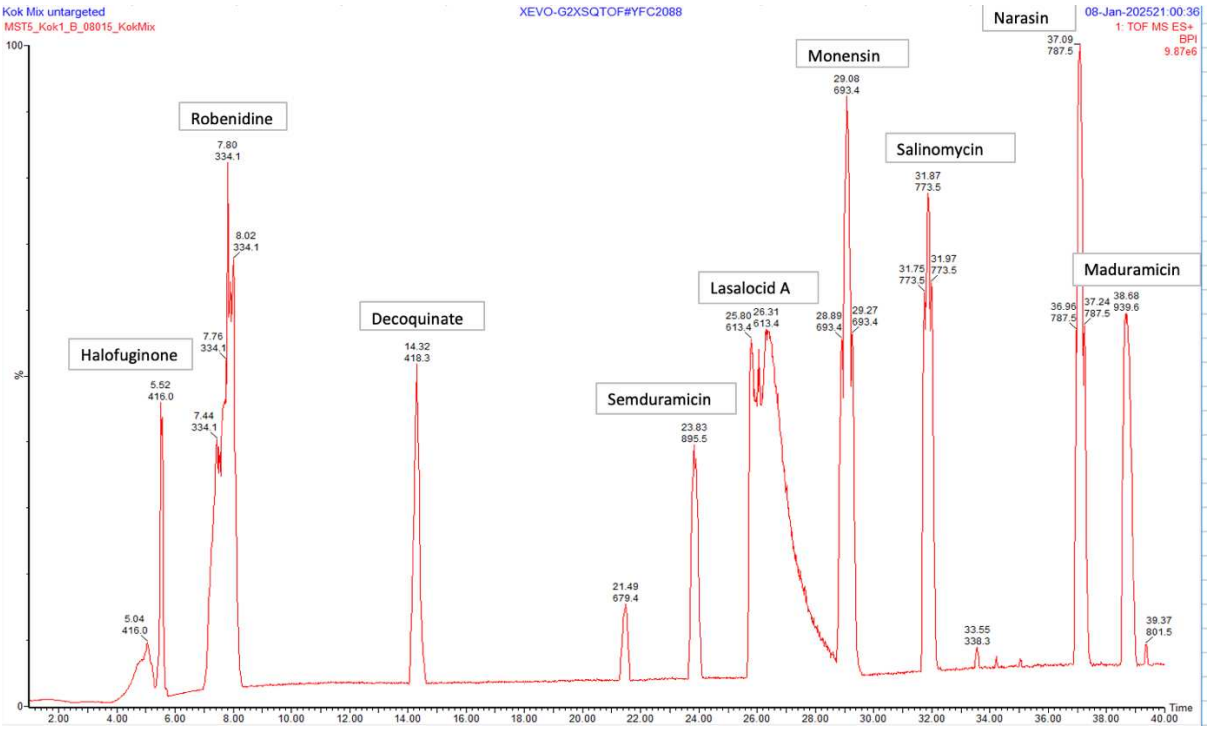



***Figure 10:*** *Untargeted LC-MS/MS approach for the detection of coccidiostats in positive mode, relying on precursor m/z identification without optimisation of gradient, mobile phase, cone voltage, collision energy, or quantifier ion selection.*

To improve the method several mobile phase compositions were evaluated, including water, methanol, and acetonitrile with and without the addition of buffers and acid. Based on literature and experimental performance, a mixture of water with 0.1 % formic acid, acetonitrile with 0.1 % formic acid, 50 mM ammonium formate solution in methanol/water (80/20, v/v) with 0.1 % formic acid and pure acetonitrile was selected as it provided the best peak shape and retention

for all analytes. The use of ammonium formate enhanced ionization efficiency and was frequently reported in other developed LC-MS/MS methods (Goessens et al., 2020; Pietruk et al., 2015). *Figure 11* presents the optimized targeted method developed in this work, including the use of quantifier ions, adapted cone voltage, optimized collision energies, and target enhancement. Compared to the untargeted measurement, the selection of quantifier and qualifier ions enabled more accurate analyte assignment, with clear improvements in peak shape and integrability. In addition, the targeted approach provided higher sensitivity, improved reproducibility, and allowed reliable quantification by minimizing interferences and focusing on predefined transitions.

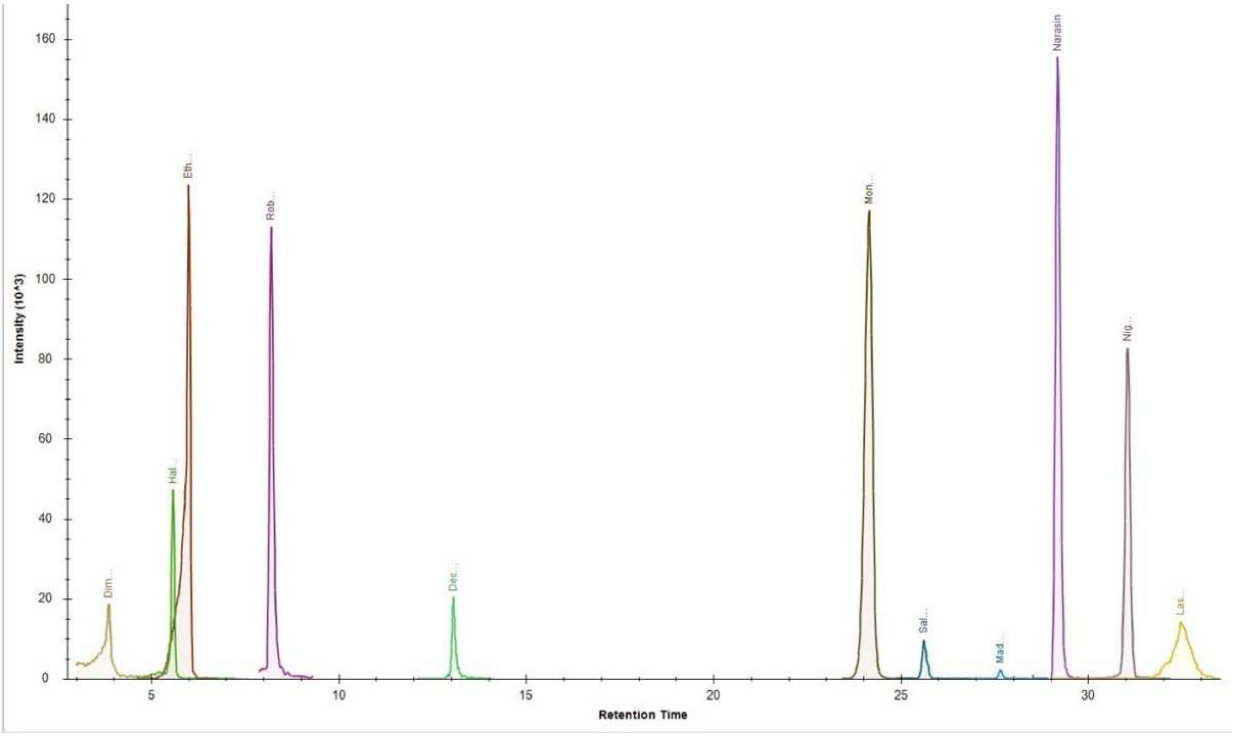



***Figure 11:*** *Representative chromatogram of coccidiostats and the internal standard nigericin in positive ESI mode using a CORTECS T3 Column (1.6 µm, 2.1 mm × 100 mm, Waters) and the optimized gradient conditions described above.*

The gradient was optimized to achieve good peak distribution, ensuring reliable quantification, and allowing the inclusion of additional analytes without major modifications. The final method duration was set to 45 minutes.

To measure analytes in both positive and negative ESI mode, two separate gradients were developed. For negative ion mode acetonitrile and water with 0.1 % formic acid were used and no buffering agents were needed. As the negative mode only included three analytes the run time was set to 25 minutes to reduce time. The chromatogram for negative ESI mode coccidiostats is shown in *figure 12*.

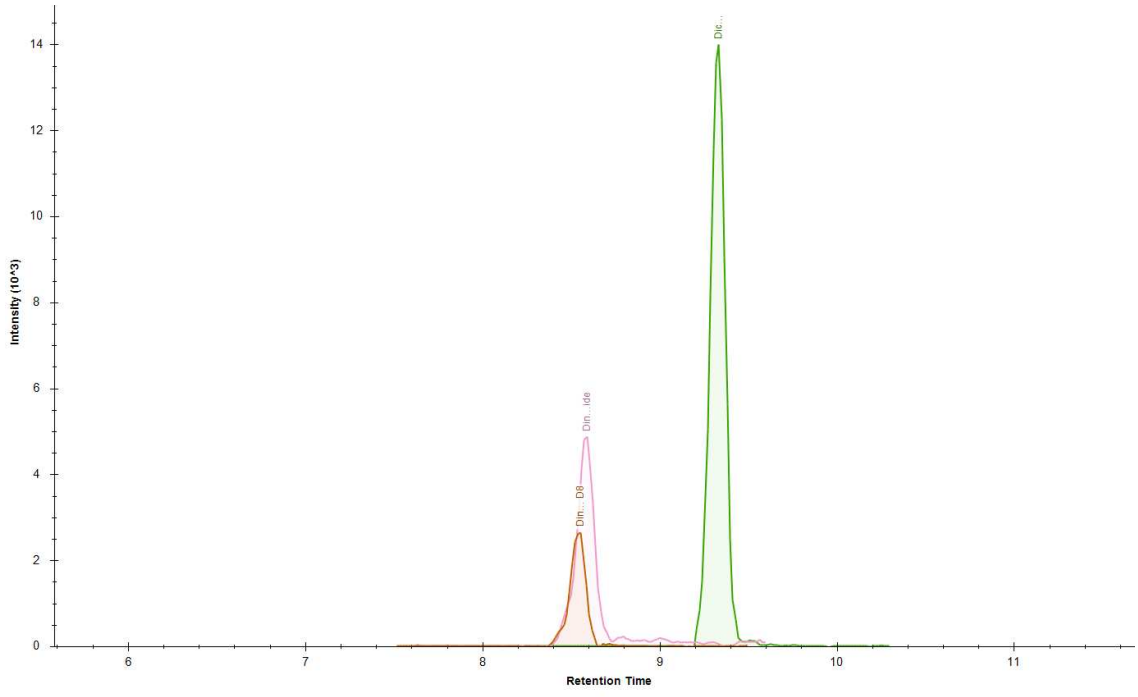



***Figure 12:*** *Representative chromatogram of coccidiostats and the internal standard dinitrocarbanilide-d8 in negative ESI mode using a CORTECS T3 Column (1.6 µm, 2.1 mm × 100 mm, Waters) and the optimized gradient conditions described above.*

For the mass spectrometric analysis a Xevo G2 XS QTof with electrospray ionization was used. ESI provided to be a suitable ionization technique, enabling efficient ionization of coccidiostats and antibiotics. For method development the precursor and at least two product ions were selected for each analyte. The most intense transitions were chosen as quantifier and qualifier ions. The selected adducts were [M + H] +, [M + Na] +, [M + NH4]+ and [M - H]-. Where applicable, transitions were aligned with those reported in literature to ensure comparability and reliability (Goessens et al., 2020; Hoff et al., 2020; ÖNORM EN 17299, 2019). Cone voltages were adapted from ÖNORM EN 17299 and Goessens et al. (2020) as the latter authors used the same mass spectrometry settings and further adjusted based on the measurement results.

Collision energies were determined using untargeted experiments and Skyline (version 24.1) as an evaluation tool and were increased where insufficient fragmentation was observed. Final collision energies were experimentally confirmed for each compound. The application of the QTOF mass spectrometer offered methodological advantages such as the use of target enhancement to increase the duty cycle, which led to improved signal intensity. The target enhancement was set to enhance the most relevant ions for analysis. The effects of the target enhancement varied by analyte: for example, the average peak area for robenidine approximately doubled, while for narasin and halofuginone it increased by factor of 14 and 19, respectively. This is in line with the recommendations provided by Waters in their “Hints and

Tips” guide on setting up and optimizing target enhancement. According to their documentation, an increase in signal response by a factor of 5 to 15 can be achieved, depending on the m/z ratio of the ions of interest. The most significant improvements are observed for low m/z values, typically below m/z 300. (Waters Corporation, 2021) This effect was particularly evident for coccidiostats such as halofuginone, robenidine, and decoquinate, all of which fall into this low m/z range. However, the implementation of target enhancement resulted in consistently strong signal improvements across all analytes improving signal to noise ratios and limit of detections. Moreover, this optimisation enables the reliable quantification of carry-over levels of coccidiostats in animal feed of 1 % and 3 % which was particularly relevant for this master thesis.

The mass spectrometric parameters (e.g., capillary voltage, desolvation temperature, sampling cone voltage) were adopted from the study by Goessens et al. (2020) which employed the Xevo TQ-XS. As both the Xevo TQ-XS and the Xevo G2-XS QTOF are equipped with the same Waters (Milford, USA) ESI source design, these settings were directly transferable. Their suitability for the Xevo G2-XS QTOF was confirmed experimentally by assessing signal stability and peak quality of the analytes, as well as by calibration with a 5 mM sodium formate solution in 2-propanol/water (90/10, v/v).

## 3.4 Method validation

Method validation with reference to the principles of *Eurachem Guide* (Cantwell, 2025) included the assessment of LOD, LOQ, linearity, recovery, and a proficiency testing for narasin. However, due to limited time and resources, validation was only performed for the positive and negative coccidiostats. The antibiotics and their corresponding internal standard were not included in the validation process. In the case of semduramicin validation was also not possible as the analytical standard was not commercially available at a reasonable price and international shipping costs were very high. The results of the validation process are presented below.

### 3.4.1 Linearity

The linearity of the method was evaluated by using matrix mixed calibration curves of each analyte. To simulate a 10 % matrix load, an extract from a blank feed matrix composed of soybean and maize was fortified with standard solutions of coccidiostats at concentrations of 0.001 µg/mL and 0.01 µg/mL. To obtain different levels for the calibration the mixture was injected at varying injection volumes (0.5-30 µL), resulting in total of 13 calibration points. This high number of calibration levels was chosen to establish a common linear range for all included coccidiostats, as they all exhibited different linearity ranges. Calibration curves were obtained by plotting the peak area against concentration in pg on column. A representative calibration curve for narasin is shown in *figure 13*, while the correlation coefficients and linear ranges of all the remaining 12 compounds are summarized in *table 5.* All the remaining calibration curve are shown in Appendix.

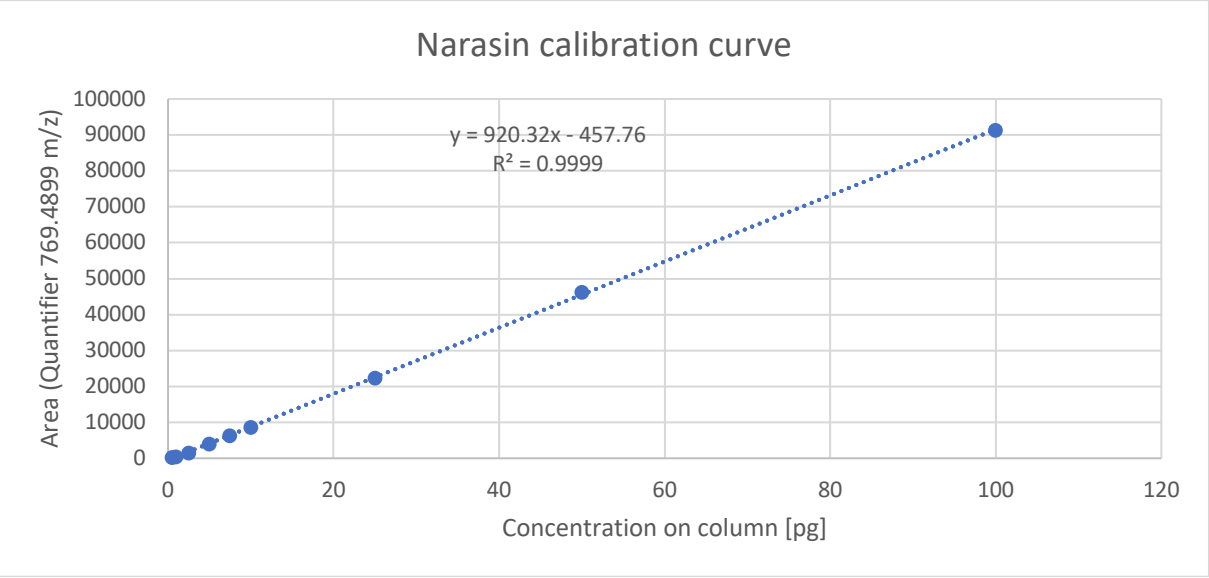



***Figure 13:*** *Calibration curve of narasin with nine concentration levels between 0.5 and 100 pg on column ($R^2$ = 0.999).*

In accordance with Eurachem Guide (Cantwell, 2025), at least five calibration points were selected within the linear range for each analyte. The resulting on column concentrations ranged from 1-500 pg depending on analyte. All analytes demonstrated excellent linearity across the tested range with correlation coefficients (R2) equal to or greater than 0.99. Calibration points were calculated as the mean of triplicate injections. The R2 values of equal to or above 0.99 are in line with findings from other publications. For example, Valesea at al. (2017) reported R2 values between 0.966 and 0.990 for coccidiostats in animal feed. Similar results were achieved by Goessens et al. (2020) with R2 values > 0.994.

***Table 5:*** *Summary of linearity parameters for coccidiostats spiked into 10 % feed matrix, including linear range, number of calibration levels and coefficient of determination (R2).*

| | **Linear range [pg on column]** | **Calibration points** | **$R^2$** |
|---|---|---|---|
| ***Coccidiostats ESI +*** | | | |
| Halofuginone | 1-100 | 6 | 0.9994 |
| Dimetridazole | 1-500 | 6 | 0.9876 |
| Robenidine | 1-100 | 6 | 0.9947 |
| Ethopabate | 2.5-200 | 5 | 0.9955 |
| Narasin | 0.5-100 | 9 | 0.9999 |
| Lasalocid A | 1.5-300 | 7 | 0.9938 |
| Salinomycin | 0.5-10 | 5 | 0.9918 |
| Monensin | 0.5-10 | 6 | 0.9982 |
| Decoquinate | 0.5-10 | 6 | 0.9974 |
| Maduramicin | 1-7.5 | 4 | 0.9989 |
| ***Coccidiostats ESI -*** | | | |
| Diclazuril | 0.5-10 | 7 | 0.9983 |
| Dinitrocarbanilide (Nicarbazin) | 0.05-0.5 | 6 | 0.9969 |

Dimetridazole exhibited the widest linear range, whereas salinomycin, monensin, decoquinate, and maduramicin showed narrower ranges between 0.5 and 10 pg on-column. Differences in linear range between analytes was also observed in the study by Goessens et al. (2020). In their study halofuginone for example showed a linear range of 2.5-200 ng L-1 while other compounds such as robenidine and salinomycin exhibited broader ranges of 25-20000 ng L-1. The observed variation in linear range among the analytes can likely be attributed to differences in ionization efficiency, matrix interaction and detector response. Dinitrocarbanilide had the smallest linear range from 0.05 to 0.5 pg. This limitation is likely due to signal saturation and detector overload, which affected the linearity. Narasin had the highest number of valid calibration points, with nine included in the regression, while maduramicin only met the criteria for four calibration levels which can be adopted by further experiments. While *Eurachem Guide* (Cantwell, 2025) does not set strict numerical thresholds for linearity, its fit-for-purpose principle is fulfilled in this method

through the broad linear ranges, sufficient number of calibration points, and strong alignment with other published data providing evidence of adequate linearity.

### 3.4.2 Limit of detection and limit of quantification

LOD and LOQ were calculated based on matrix matched calibration results using the analytical software Valoo (Analytic-Software, 1999–2024). The calculation was based on linear regression analysis and the residual variance of the calibration function, in accordance with ÖNORM DIN 32645. The results for LOD and LOQ are presented in *table 6.* For better comparability, the LOQ values expressed as pg on column were also converted to mg/kg, corresponding to the respective sample preparation. Values for the permitted 1 % and 3 % carry-over are listed in *table 1.*

***Table 6:*** *LOD and LOQ values of coccidiostats expressed in pg on column, with additional conversion to ng/kg feed for comparability.*

| | **LOD [pg on column]** | **LOQ [pg on column]** | **LOQ [ng/kg]** |
|---|---|---|---|
| ***Coccidiostats ESI +*** | | | |
| Halofuginone | 3.068 | 10.331 | 82.65 |
| Dimetridazole | 56.762 | 190.659 | 1525.27 |
| Robenidine | 9.455 | 31.845 | 254.76 |
| Ethopabate | 6.507 | 21.876 | 175.01 |
| Narasin | 1.207 | 4.051 | 32.41 |
| Lasalocid A | 1.027 | 3.365 | 26.92 |
| Salinomycin | 0.458 | 1.522 | 12.18 |
| Monensin | 0.469 | 1.556 | 12.44 |
| Decoquinate | 0.861 | 2.825 | 22.60 |
| Maduramicin | 0.094 | 0.318 | 2.54 |
| ***Coccidiostats ESI -*** | | | |
| Diclazuril | 0.430 | 1.421 | 11.44 |
| Dinitrocarbanilide (Nicarbazin) | 0.046 | 0.147 | 1.18 |

Given the very low LOD and LOQ values, also calculated in ng/kg, carry-over levels of 1 % and 3 % could easily be detected. This enables reliable identification even at very low concentrations and is particularly relevant for the two non-approved coccidiostats ethopabate and dimetridazole which can thus be detected even at trace levels. The LOQ values achieved in this study, ranging from 1.18 ng/kg to 1525.27 ng/kg, demonstrate a high sensitivity compared to previously published studies. These values are theoretically derived, but their plausibility was supported by recoveries performed at concentration levels close to the calculated LOD and LOQ. For example, Valesea at al. (2017) reported LOQ values between 25 and 50 µg/kg while Hoff et al. (2020) described values of LOQ of 70 µg/kg. A third study by Pietruk et al. (2015) expressed LOQ in the range of 0.004 to 0.22 mg/kg which is far higher than the calculated amounts in this

study. These results underline the method's suitability for detecting even trace level concentrations of coccidiostats including also non authorized substances. The application of target enhancement and the resulting selective boosting of specific m/z values has likely contributed to the low LOD and LOQ values, thereby improving the overall performance of the method and making it suitable for the determination of 1 % and 3 % carry-over levels in animal feed.

### 3.4.3 Recovery

To verify the method's performance not only in terms of sensitivity but also in terms of accuracy, recovery was determined at two spiking levels. One level represented 3 % carry-over from non-target feed and was individually calculated for each analyte (*see table 1*), while the second level corresponded to concentrations close to the respective LOD. The results of the recovery experiments are shown in *table 7*. Due to technical issues during the analysis period recovery for ethopabate and dimetridazole could not be completely determined. However, this should not be interpreted as a limitation of the method itself because recovery for these compounds can be evaluated in further analysis.

***Table 7**: Recovery data for coccidiostats spiked at two concentration levels (low and high) in feed matrix (maize and soy).*

| | **Results recovery experiments** | | | |
|---|---|---|---|---|
| | **concentration high-level (3 % carry-over) [µg/mL]** | **concentration low-level [µg/mL]** | **recovery high-level [%], mean** | **recovery low-level [%], mean** |
| **Coccidiostats ESI +** | | | | |
| Ethopabate | 1.5000 | 0.015 | - | - |
| Dimetridazole | 1.5000 | 0.015 | 0.04 | - |
| Halofuginone | 22.500 | 0.0113 | 4.39 | 9.15 |
| Robenidine | 0.263 | 0.0263 | 17.39 | 3.54 |
| Decoquinate | 0.150 | 0.0150 | 41.06 | 15.62 |
| Monensin | 0.469 | 0.0047 | 131.93 | 142.43 |
| Salinomycin | 0.263 | 0.0026 | 171.49 | **78.48** |
| Maduramicin | 0.019 | 0.0002 | 168.80 | 16.05 |
| Narasin | 0.263 | 0.0026 | **106.39** | **71.60** |
| Lasalocid A | 0.469 | 0.0047 | 287.67 | **83.98** |
| **Coccidiostats ESI -** | | | | |
| Diclazuril | 0.00375 | 0.000375 | 7.38 | 4.34 |
| Dinitrocarbanilide (Nicarbazin) | 0.1875 | 0.001875 | 0.08 | 0.14 |

Recovery was calculated by comparing the measured concentrations in spiked matrix samples with the known amount added and analyzed in triplicate. The observed recoveries varied between analytes and concentration levels. At the higher spiking level, the recovery values

ranged from 0.04 % to 287.67 %, while at the lower-level values ranged from 3.54 % to 142.43 %. Acceptable results were obtained for salinomycin (78.48 %) and lasalocid (83.98 %) at low-level concentrations. Among all the analytes only narasin showed acceptable recovery levels across both spiking levels with 106.39 % at high-level and 71.60 % at low- level. According to acceptance criteria based on the ÖNORM EN 17299 guidelines, recovery rates are expected to fall within the range of 70–130 %. This requirement was not met by the majority of analytes in the present study, indicating potential losses during extraction or matrix-related interferences. According to Eurachem Guide (Cantwell, 2025), recovery is assessed based on the fit-for-purpose principle, rather than strict numerical thresholds. With additional repetitions of the recovery experiments, consistency could have been evaluated and used to support the reliability of the method. However, due to time constraints, such verification was not possible within the current study. Due to technical difficulties with the negative ionization mode of the TOF instrument at the time of analysis, the corresponding recovery values should be regarded as approximate estimates. While they provide useful orientation, they may not fully reflect the true recovery performance under optimal conditions. When comparing the recovery values obtained in this study with those reported in the literature, a similar trend can be observed as recoveries vary considerably across analytes but generally remain within acceptable limits. Goessens et al. (2020) however also reported a notably low recovery for robenidine, with a value of 29.0 %, while all other analytes showed recoveries ranging from 99.5 % to 115.5 %. In the present study, robenidine also performed poorly, with recovery rates of 17.39 and 3.54 % at the two spiking levels. The study by Kenjeric et al. (2024) further demonstrated that recovery is highly dependent on the extraction conditions. They showed that under acidic extraction conditions, only 35 % of the analytes fell within the acceptable recovery range of 70–120 %, whereas under neutral extraction conditions, approximately 50 % of the analytes met the criteria. This clearly highlights the influence of the extraction procedure and solvent composition on recovery.

In this study compounds such as ethopabate, dimetridazole, halofuginone, robenidine, and decoquinate exhibited notably lower recoveries compared to compounds like monensin, salinomycin, maduramicin, and lasalocid. This discrepancy may be attributed to the extraction solvent used (80 % acetonitrile, 10 % methanol, 10 % water), which is likely not optimal for all analytes. In particular, the former compounds show only low to moderate solubility in acetonitrile due to their higher polarity, whereas the latter group consists of highly lipophilic ionophores that are soluble in organic solvents like ACN. More experiments with adjusted acetonitrile content in the extraction solvent would have been necessary to improve recoveries,

particularly for the more polar compounds. This highlights a key challenge in multiclass methods as the chemical diversity and varying polarity of the target analytes require compromises in the extraction conditions since no single solvent system can ensure optimal recovery for all compounds. As a result, compromises in analyte recovery are often unavoidable. Nevertheless, recovery can be mathematically accounted for to correct systematic losses, thereby ensuring that the reported concentrations remain accurate and analytically meaningful.

### 3.4.4 Proficiency test sample (narasin)

The final step of the validation involved the comparison of narasin concentrations in an interlaboratory test sample (IAG Interlaboratory Test 2024). The proficiency test sample (mixed feed) was processed according to the described sample preparation protocol and subsequently analyzed. Quantification was performed using the internal standard nigericin by calculating the response factor. The determination was carried out in triplicate and the internal standard was spiked as standard solution prior to extraction. The measurement and calculation resulted in a narasin concentration of 49.8 mg/kg ± 18.2 mg/kg (n=3). The mean value of the proficiency test was 51.4 mg/kg, with a tolerance range of [36.9-65.8] mg/kg. The calculated z-score of –0.09 indicates that the deviation from the assigned mean value is small and within the acceptable range, showing that the method gives accurate and reliable results. This confirms that the measured values match the proficiency testing results and supports the validity of the method.

### 3.4.5 Summary of validation results

Validation results demonstrated that the developed LC-MS/MS method is suitable for determining positive and negative coccidiostats in animal feed. The method showed a broad linear range, high coefficients of determination ($R^2$), and low detection limits, sufficient for reliable identification of 1 % and 3 % carry-over levels. Although the LOD and LOQ values are based on calculations and are expected to be higher in real feed samples, they remain fully adequate for the intended application. These performance characteristics underline the relevance of the method in the context of feed cross-contamination and regulatory compliance. Acceptable recoveries were obtained for three analytes (salinomycin, narasin, and lasalocid), while lower recoveries for the others currently limit broader applicability. Further recovery experiments and optimisation of the extraction solvent may improve performance but were beyond the scope of this thesis. The methods robustness was further confirmed by successful participation in a proficiency test for narasin, demonstrating applicability under realistic feed conditions.

## 3.5 Calculation of response factors

In order to demonstrate the suitability of the newly developed LC-MS/MS method rabbit feed samples collected in Austria were analysed. A list of the tested samples is shown in *table 4*. Quantification was performed using the internal standard nigericin and by calculating the corresponding response factor for each analyte. Due to instrumental limitations in negative ionization mode, response factors could only be determined for the coccidiostats detectable in positive mode. Due to analytical limitations, it was also not possible to determine a response factor for decoquinate. *Table 8* presents the response factors for each analyte, calculated as the mean of triplicate measurements across different analyte concentrations, with a fixed concentration of the internal standard nigericin.

***Table 8:*** *Calculated response factors for the quantification of positive coccidiostats calculated as mean of triplicate measurements across different analyte concentrations.*

| | **Response factor (RF)** | |
|---|---|---|
| | Mean ± SD | % RSD |
| **Dimetridazole** | 0.028 ± 0.004 | 12.94 |
| **Robenidine** | 0.337 ± 0.016 | 4.80 |
| **Halofuginone** | 0.069 ± 0.011 | 16.63 |
| **Narasin** | 0.474 ± 0.055 | 11.54 |
| **Monensin** | 0.917 ± 0.071 | 7.78 |
| **Salinomycin** | 0.026 ± 0.005 | 18.99 |
| **Lasalocid** | 0.042 ± 0.013 | 37.70 |
| **Ethopabate** | 1.425 ± 0.442 | 31.01 |
| **Maduramicin** | 0.020 ± 0.010 | 52.74 |

The calculated response factors (RF) for the analysed coccidiostats exhibited varying degrees of repeatability, with % RSD values ranging from 4.8 % (robenidine) to 52.7 % (maduramicin). While several analytes such as robenidine, halofuginone, narasin, monensin, and salinomycin showed acceptable reproducibility (%RSD < 20%), others such as lasalocid, ethopabate, and maduramicin displayed considerable variability. Narasin demonstrated reliable quantification, which is supported by its consistent performance in an interlaboratory proficiency test. Unfortunately, no comparable response factors could be found in the literature for direct comparison likely due to differences in instrumentation, internal standards, and analytical conditions.

Nigericin was used as a universal internal standard however, the wide range of % RSDs suggests that it may not be equally suitable for all analytes. Nevertheless, nigericin is generally considered a reliable internal standard and has been successfully applied in various LC-MS/MS methods for the quantification (Goessens et al., 2020; Pietruk et al., 2015; Vincent et al., 2011). The Austrian

standard ÖNORM EN 17299 recommends the use of additional standards such as robenidine-d8, bis-diclazuril, decoquinate-d5, dimetridazole-d3 and ethopabate-d5 to improve accuracy and precision, but these could not be implemented in this study due to budgetary and availability constraints. Similar challenges have been described by Pietruk et al. (2015), who highlighted the difficulty of identifying suitable internal standards for coccidiostats, particularly due to the limited availability of isotope-labelled internal standards.

## 3.6 Analysis of animal feed samples

The rabbit feed samples selected for LC-MS/MS analysis covered a broad range, as both breeding-specific and conventional rabbit feed were analysed to demonstrate the applicability of the method. The samples were collected in Austria and sent to the Austrian Agency for Health and Food Safety. There they were grounded, processed, and analysed using the newly developed LC-MS/MS method. *Table 9* displays the obtained results. No coccidiostats were detected in the breeding rabbit feed, Alpenhorn Kanin, or K-75 rabbit feed.

***Table 9:*** *Results of coccidiostats analysis in rabbit feed samples with additional comparison to the 3 % carry-over.*

| **Results of coccidiostats analysis in rabbit feed samples** | | | |
|---|---|---|---|
| | **Monensin**<br>mg/kg (MW ± SD)<br>n =2 | **Salinomycin**<br>mg/kg (MW ± SD)<br>n =2 | **Narasin**<br>mg/kg (MW ± SD)<br>n =2 |
| Kaninkorn Classic (24134081-001) | 0.36 ± 0.03 | 9.3 ± 0.60 | - |
| Kaninkorn Classic (24153645-001) | 0.14 ± 0.05 | 0.2 ± 0.02 | - |
| Kaninkorn Classic (24065374-001) | 3.65 ± 0.37 | not in linear range | 0.61 ± 0.07 |
| 3 % carry-over rabbit feed | 3.75 | 2.1 | 2.1 |

The detected coccidiostats were monensin, salinomycin, and narasin. All three compounds were identified and quantified in the three analysed “Kaninkorn Classic” samples. For monensin concentrations ranged from 0.14 to 3.65 mg/kg remaining below the 3 % carry-over threshold of 3.75 mg/kg. In contrast, one sample exceeded the 3 % carry-over limit for salinomycin (2.1 mg/kg), with a measured concentration of 9.3 mg/kg. Narasin was also detected in one sample at a level of 0.61 mg/kg, which remained below its respective 3 % carry-over threshold. The coccidiostat robenidine which is one of the two currently authorized coccidiostats in rabbit feed, was not detected in any of the samples. Diclazuril, which is also authorized but must be analysed

in negative ionization mode, could not be evaluated due to technical limitations in the negative mode. One sample containing salinomycin could not be quantified, as the signal exceeded the linear range and required additional dilution. However, due to time constraints, this measurement could not be repeated.

The detected substances monensin, salinomycin, and narasin are among the most used coccidiostats in animal feed. This observation aligns with findings from Pietruk et al. (2015) who also detected these compounds in a total of 26 animal feed samples. In their study, salinomycin was the most frequently found coccidiostat (15 samples), followed by narasin (6 samples) and monensin (5 samples). Monensin, narasin and salinomycin were also among the most frequently detected coccidiostats in pig, cattle, and poultry feed in the study by Borràs et al. (2013). Cronly et al. (2010) detected salinomycin concentrations of 2.24 mg/kg and monensin concentrations of 1.61 mg/kg in pig and poultry feed.

According to current EU regulations (Commission Directive 2009/8/EC), only robenidine and diclazuril are approved for use in rabbits. All other coccidiostats are not permitted and may only be present as carry-over from the feed production process. Despite this regulatory restriction, monensin, narasin, and salinomycin which are three of the most frequently detected coccidiostats in pig, cattle, and poultry feed, were also found in the rabbit feed samples analysed in this study. Their detection at trace levels is consistent with carry-over contamination and suggests cross-contamination during feed manufacturing. This study is the first to examine coccidiostat residues in rabbit feed in more detail, but due to the lack of comparable data in the literature, a direct assessment of the contamination levels in relation to other studies is not possible. This highlights a clear data gap and demonstrates the need for further research and monitoring in this area.

# 4 Conclusion

A new LC-MS/MS method was developed and validated for the simultaneous detection and quantification of eleven authorized and two unauthorized coccidiostats in rabbit and poultry feed. In addition, selected tetracyclines were included in the method setup, although they were not fully validated. The method was developed to remain flexible and allow for the integration of further antibiotics and non-authorised coccidiostats in future applications. While coccidiostat monitoring is well established in poultry feed, data on rabbit feed remains limited. This study therefore aimed to provide a practical approach to address this gap and support future monitoring of rabbit feed, contributing to both animal and human safety.

Initially, different extraction solvents were compared to evaluate their impact on ion suppression or enhancement in the electrospray ionisation process. The mixture of 80 % acetonitrile, 10 % methanol and 10 % water showed the best performance and was selected for the final method. In addition, different sample preparation techniques including dilute and shoot, QuEChERS and SPE were compared. In this study, the dilute and shoot approach proved to be the most suitable for five out of ten tested analytes, offering the highest peak areas while reducing both time and resource consumption which is particularly important in routine analysis.

Method development focused on optimizing both chromatographic separation and MS/MS parameters. The final method consisted of two separate LC-MS/MS runs in positive ionisation mode (45 min) and negative mode (25 min). Different gradient elution programs were used including water with 0.1 % formic acid, acetonitrile with 0.1 % formic acid, as well as a 50 mM ammonium formate solution in methanol/water (80:20, v/v) with 0.1 % formic acid and acetonitrile. For MS-based identification and quantification at least the two most intense product ions were selected for each analyte and a targeted method was created. Additionally target enhancement was applied to increase the signal of selected m/z values and improve overall sensitivity. This proved particularly advantageous for the detection of low-level residues resulting from 1 % or 3 % carry-over in non-target feed.

The results of method validation are in line with current Eurachem guidelines (Cantwell, 2025). Linearity was demonstrated across a broad concentration range, with correlation coefficients equal to or greater than 0.99 for all analytes. Limits of detection were below relevant regulatory thresholds, while limits of quantification ranged between 1.18 ng/kg and 1525.27 ng/kg.

Recovery rates varied considerably, ranging from 0.04 % to 287.67 %, which represents a notable limitation of this study. According to the acceptance criteria defined in the ÖNORM EN 17299 guidelines, recoveries should fall within the range of 70–130 %, which was not achieved in several cases. Nevertheless, acceptable values were obtained for key analytes such as salinomycin, lasalocid, and narasin. This reflects a central challenge of multiclass methods as ionophoric and chemical coccidiostats differ greatly in their physicochemical properties but frequently occur simultaneously, making it difficult to establish extraction conditions that ensure consistent recoveries for all compounds within a single multi-analyte method. To ensure reliability of quantification despite low or variable recoveries, recovery correction can be applied during data evaluation. The suitability of the method for practical application was further confirmed by the successful detection of narasin in the IAG proficiency test for mixed feed, where the measured concentration fell within the assigned tolerance range.

The method's applicability was further evaluated by analysis of six different rabbit feed samples. Salinomycin, narasin, and monensin were detected at concentrations consistent with a 3 % carry-over, with one sample exceeding the expected threshold for salinomycin. This highlights the importance of monitoring carry-over effects in non-target feed. In contrast, the authorised coccidiostats for rabbit feed, robenidine and diclazuril, were not detected in any of the samples. Together, the results of the proficiency test and the analysis of real rabbit feed samples confirm the suitability and robustness of the developed method, particularly for the reliable detection of residues at levels consistent with carry-over in feed control.

Although the method performed well for the intended application, some steps offer potential for improvement. For quantification nigericin was used as internal standard for the eleven coccidiostats measured in positive ESI mode. Although this enabled the estimation of analyte concentrations, the use of individual isotope-labelled standards for each analyte (deuterated analogues) would further improve accuracy because they compensate for matrix effects and allow more reliable quantification through ion ratios. At least one internal standard for the chemical coccidiostats and one for the ionophoric compounds should be included to account for their different properties.

Moreover, due to technical issues during negative electrospray ionisation no response factors or ion ratios were determined in negative mode. As a result, only qualitative detection was possible for these compounds within the current study. However, the internal standard required for

quantification in the negative mode has already been included in the method setup and only additional experiments are needed to fully establish its quantitative performance. Furthermore, to strengthen the method's broader validation, additional proficiency tests involving a wider range of coccidiostats should be conducted. This would allow for a more comprehensive confirmation of the method’s reliability across all target analytes.

In conclusion, the newly developed LC-MS/MS method enables the detection of coccidiostats in feed including low-level residues resulting from 1 % and 3 % carry-over. This represents a major advantage in regulatory monitoring, as such contamination levels are critical but often analytically challenging to verify. With some modification in sample preparation, the method could be suitable not only for use in animal feed but could also be adopted for routine screening of various matrices like meat, eggs and drinking water. Furthermore, the study provides new data on coccidiostats residues in rabbit feed which is a matrix that has received limited attention in the past. The method therefore not only closes a relevant data gap but also sets the basis for reliable routine monitoring. This work contributes to the protection of animal health as well as consumer safety and supports the fight against antimicrobial resistance.

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# 5 Appendix

## 5.1 Calibration curves

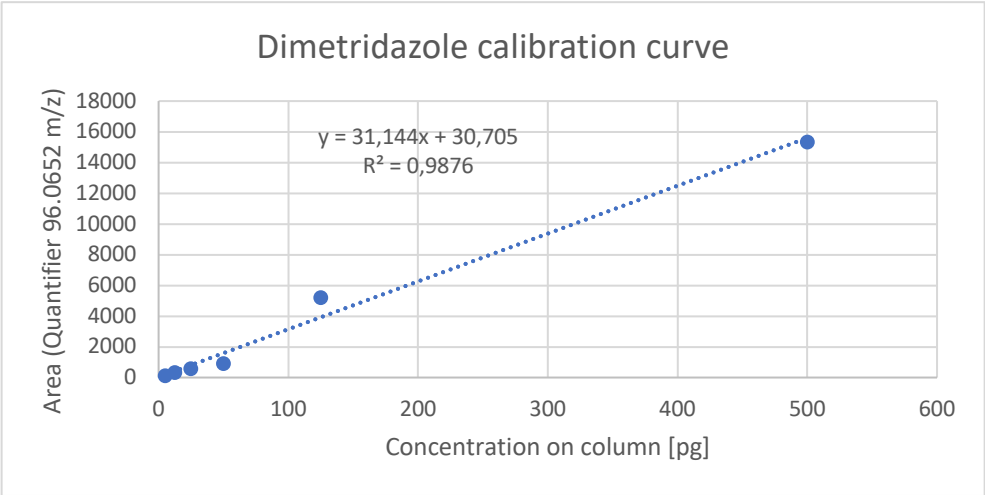


***Figure 14:*** *Calibration curve of dimetridazole with six concentration levels between 5 and 500 pg on column ($R^2$ = 0.9876).*

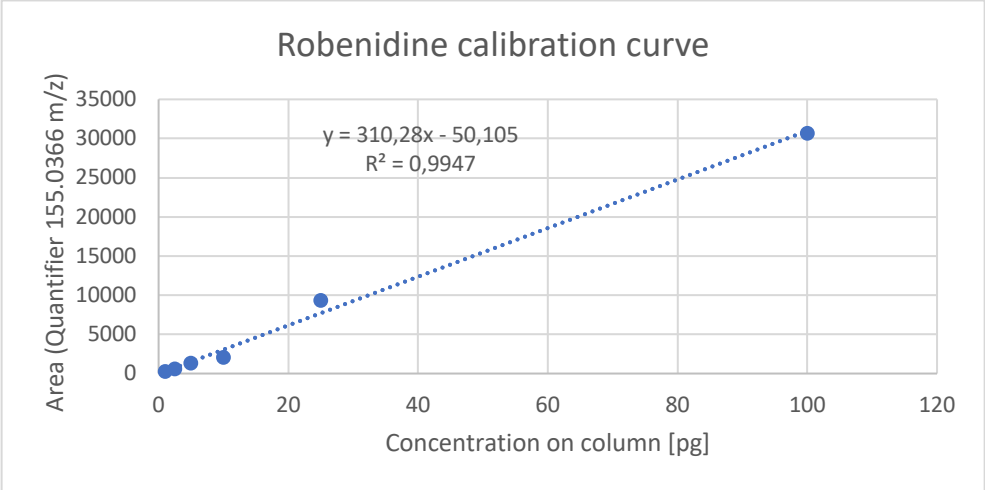


***Figure 15:*** *Calibration curve of robenidine with six concentration levels between 1 and 100 pg on column ($R^2$ = 0.9947).*

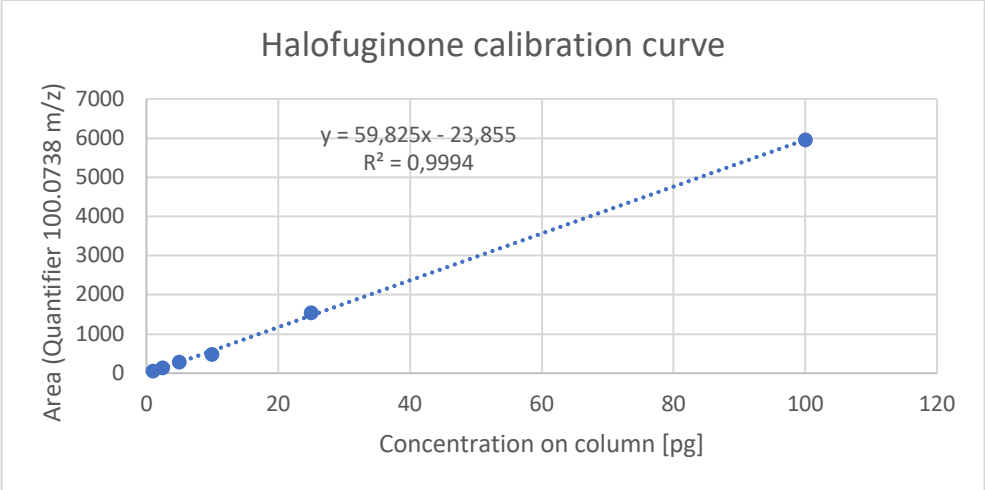


***Figure 16:*** *Calibration curve of halofuginone with six concentration levels between 1 and 100 pg on column ($R^2$ = 0.9994).*

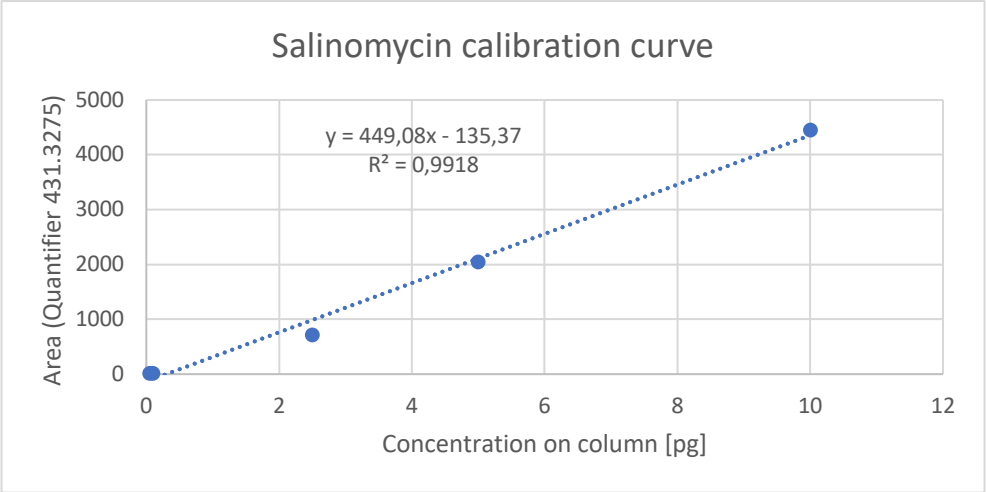


***Figure 17:*** *Calibration curve of salinomycin with five concentration levels between 0.5 and 10 pg on column ($R^2$ = 0.9918).*

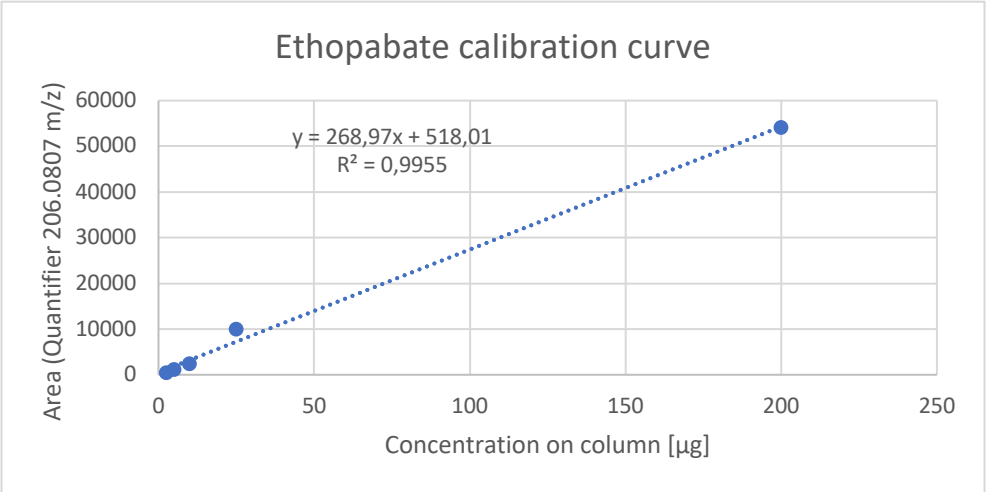


***Figure 18:*** *Calibration curve of ethopabate with five concentration levels between 2.5 and 200 pg on column ($R^2$ = 0.9955).*

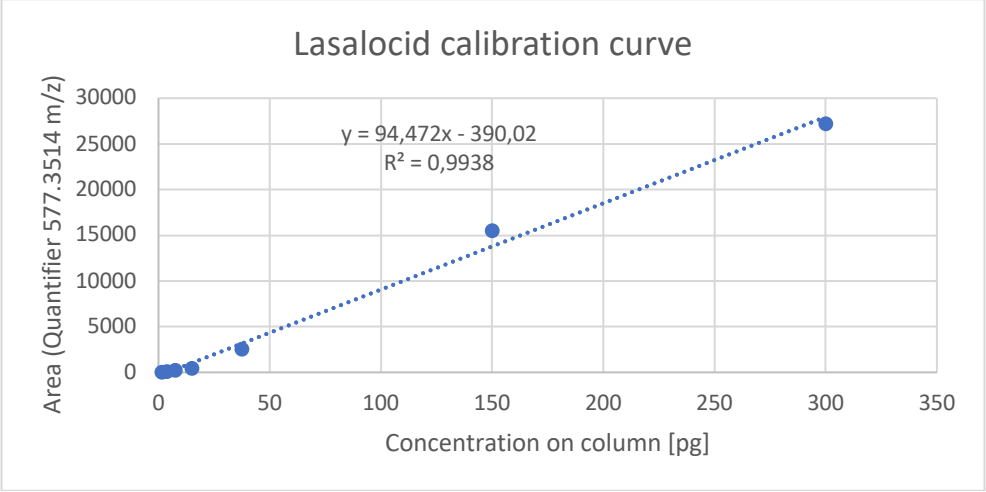


***Figure 19:*** *Calibration curve of lasalocid with seven concentration levels between 1.5 and 300 pg on column ($R^2$ = 0.9938).*

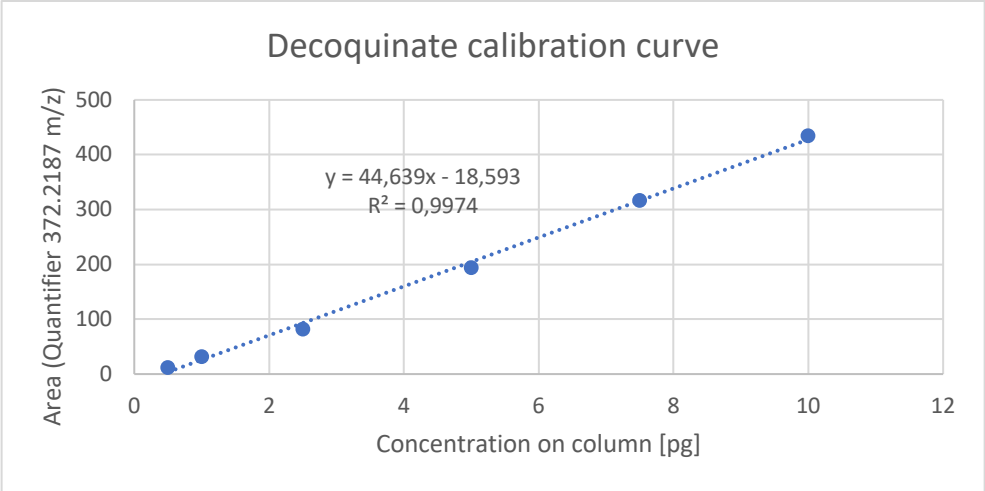


***Figure 20:*** *Calibration curve of decoquinate with six concentration levels between 0.5 and 10 pg on column ($R^2$ = 0.9974).*

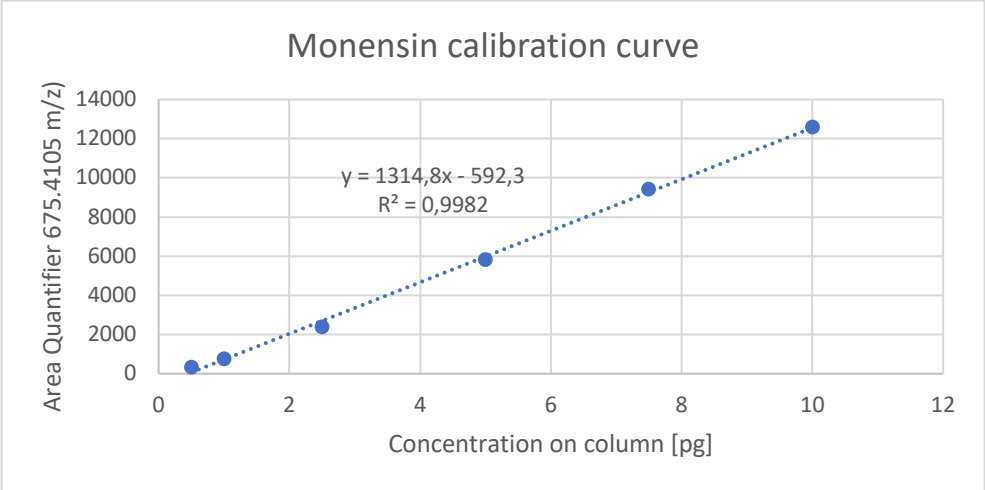


***Figure 21:*** *Calibration curve of monensin with six concentration levels between 0.5 and 10 pg on column ($R^2$ = 0.9982).*

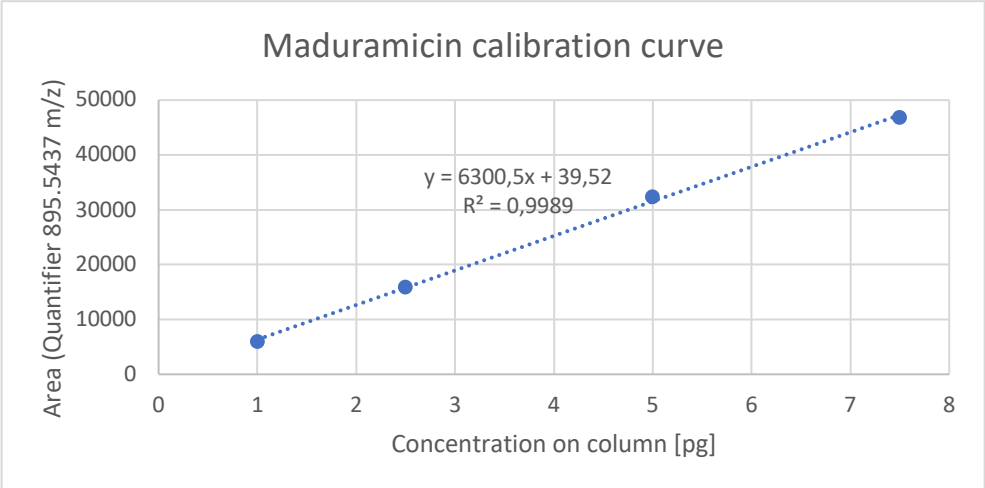


***Figure 22****: Calibration curve of maduramicin with four concentration levels between 1 and 7.5 pg on column ($R^2$ = 0.9989).*

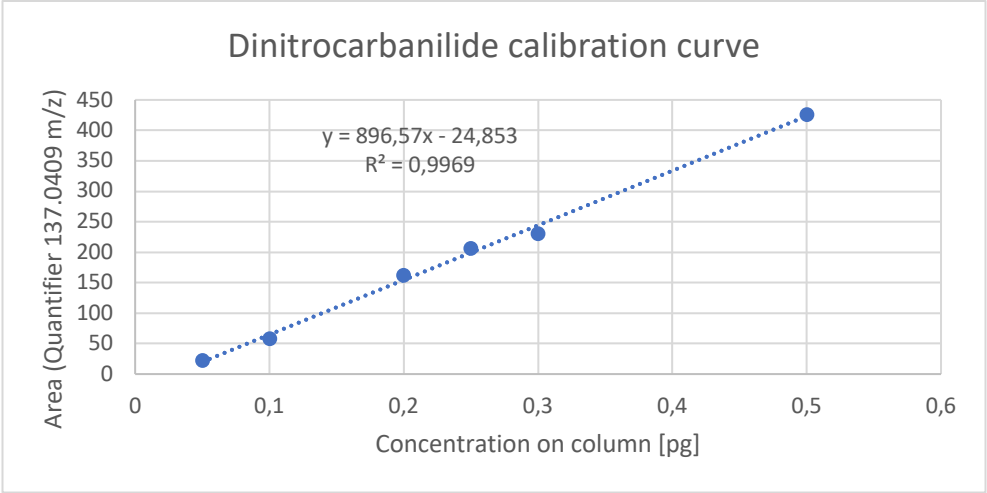


***Figure 23:*** *Calibration curve of dinitrocarbanilide with six concentration levels between 0.05 and 0.5 pg on column ($R^2$ = 0.9969).*

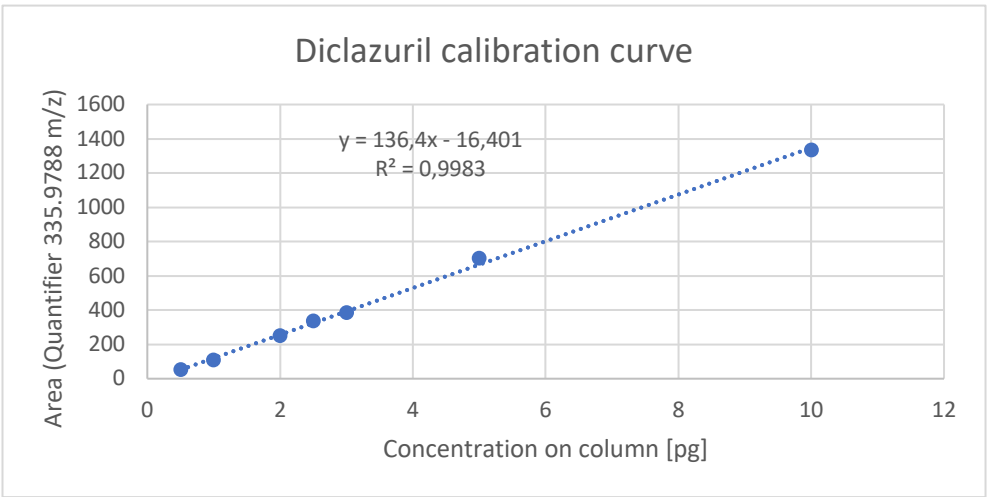


***Figure 24:*** *Calibration curve of diclazuril with seven concentration levels between 0.5 and 10 pg on column ($R^2$ = 0.9983).*