Quantitation of Antibiotics and Insecticides in Poultry Feed using Liquid Chromatography Tandem Mass Spectrometry

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Overview

An LC-MS/MS assay has been developed for the analysis of multiclass antibiotics and insecticides in poultry feed.

Introduction

For decades, antibiotics have been added to livestock feeds in low doses to serve as growth promoters. [1] Antibiotics have recently been shown to accumulate in poultry feathers, which is significant because poultry feathers serve as a high protein ingredient in animal feed, such as poultry feed.[1]

The continued use of these antibiotics as feed additives has inadvertently created antibiotic-resistant micro-organisms, which has caused human health concerns.[2] The types and quantities of antibiotics administered to livestock in the U.S. are not reported by the FDA.[1] In 2012, a federal judge ordered to withdraw the approval for the use of common antibiotics in animal feed because overuse could create antibiotic-resistant micro-organisms.[2]

Plant protection products may be introduced into animal feeds through several means, but the most common source of residues is through the legitimate use of pesticides (herbicides, insecticides and fungicides) in the production of crops used in preparation of feeds. Various grains and related glutens are frequently utilized in animal feeds. Animal feeds can in fact contain many nutritional ingredients and additives, including but not limited to proteins, fats, carbohydrates, antimicrobials, emulsifiers, binders, pH control agents, pelleting agents and preservatives.[3, 4] The inherent complexity of the sample matrix demands an efficient extraction and cleanup and a highly sensitive mass spectrometer to accurately quantify low levels of common antibiotics and insecticides in animal feeds in a single method.

In this work, a method has been developed to analyze for nine antibiotics, which included fluoroquinolones, sulfonamides, amphenicols, macrolides and quinolones, and four insecticides in poultry feed.

The preparative method involves a three-part extraction, sample cleanup with Phenomenex® Strata™-XL-CW solid phase extraction (SPE) cartridges and analysis by LC-MS/MS on an Eksigent ekspert™ UltraLC 100-XL with an AB SCIEX QTRAP® 5500 system utilizing Multiple Reaction Monitoring (MRM) with the *Scheduled* MRM™ algorithm and fast polarity switching. For the work presented here, accuracy and reproducibly are demonstrated by evaluating poultry feed samples fortified in triplicate.

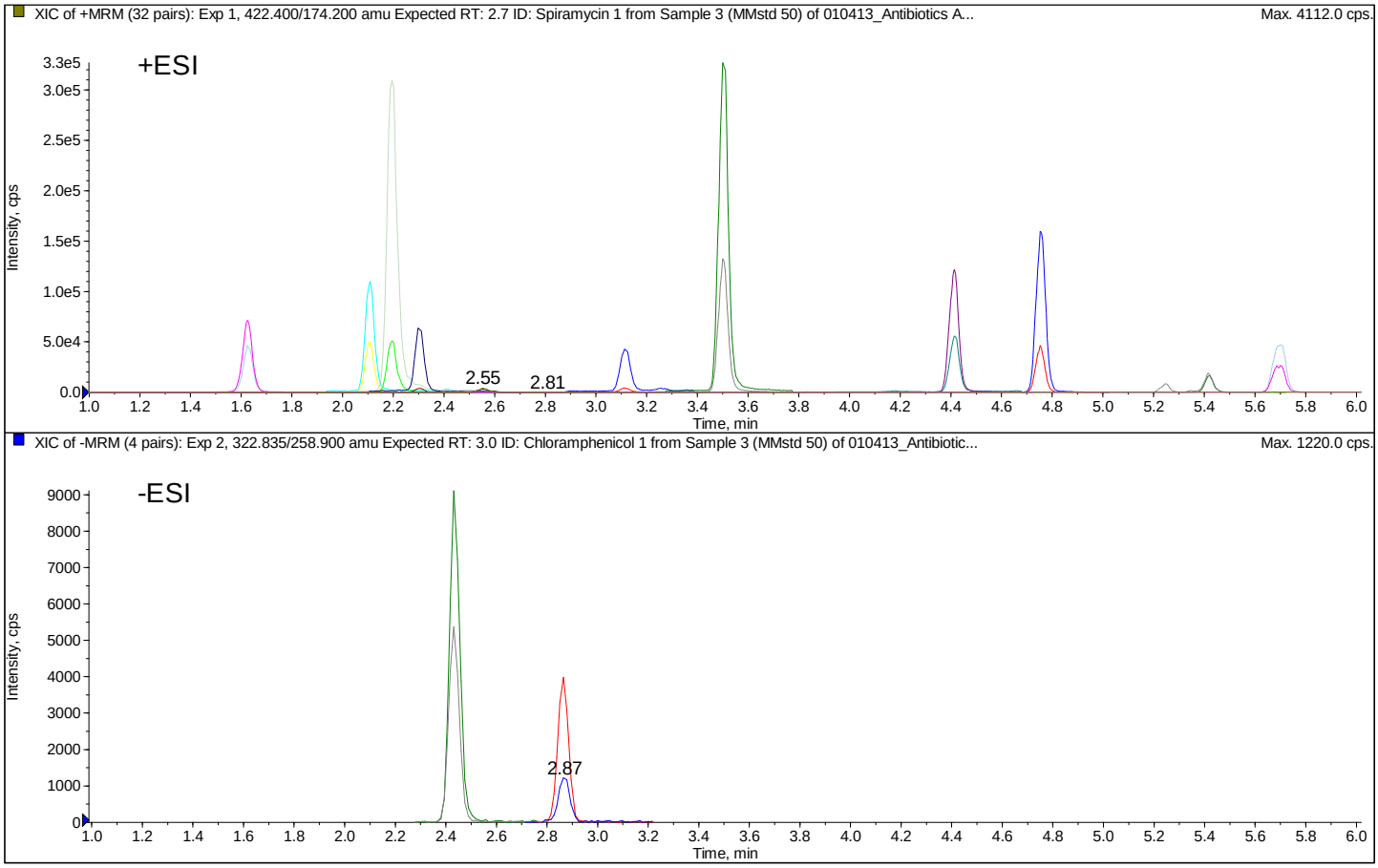

Figure 1. Detection of antibiotics and insecticides in a single run by LC-MS/MS using *Scheduled* MRM™ with polarity switching. Positive mode (+ESI) MRM transitions shown in top pane, and XIC of all negative mode (-ESI) MRM transitions shown at bottom. Peaks are identified by retention time in Table 2.

Experimental

Standards

All targeted analytes were available commercially and were either purchased as pure solid material or as high concentration analytical solutions. To prepare stock solutions of the solid materials, 10 mg of pure material was brought to either 10 or 100 mL with solvent to prepare 1 or 0.1 mg/mL solutions, respectively. The concentration of each stock solution was dependent on it solubility.

Sample Preparation

1) Extraction

Approximately 1.25 g of poultry feed sample were added to a 50 mL polypropylene centrifuge tube. Fortified samples were spiked into the dry sample for an in-sample concentration of 40 µg/kg. The sample was wetted with 10 mL of HPLC water and blended on a horizontal wrist-action shaker for 20 minutes. Three extractions were performed. The first extraction was performed with 5 mL of 1.5 mM EDTA and 5 mL of 1% TCA. The second extraction was performed with 10 mL of 75% methanol in water. The third extraction was performed with HPLC water. Between each extraction step, the sample was vortexed, shaken for 15 minutes on a wrist action shaker, sonicated for 10 minutes and centrifuged at 10,000 rpm for 5 minutes. All extracts were combined and brought to 50 mL with HPLC water.

2) SPE Cleanup

Sample cleanup was performed with Phenomenex® 200 mg Strata™-XL-CW SPE cartridges. This cartridge was selected based on the sorbent's weak cation exchange functionality to extract basic compounds from the poultry feed extract. Moreover, the large particle size of the XL (100 µm) allowed high volume loading and fast flow of the extract through the sorbent without the need to pre-filter the extract.

The final methanol percentage in this combined extraction was 15%, which was optimized for the SPE cleanup by performing a breakthrough study with various methanol percentages ranging

from 0 to 100%. It was determined that at 15% methanol concentration, all the targeted analytes retained on the sorbent during the loading step. At ≥25% methanol, some of the analytes would fail to be retained on the sorbent in the loading step, particularly oxolinic acid, florfenicol and chloramphenicol (data not shown).

The cartridge was conditioned with methanol followed by HPLC water. A 20 mL aliquot of the extract was loaded onto the SPE cartridge and sent to waste. The cartridge was washed with 10 mL of 15% methanol. The cartridge sorbent was dried under a light vacuum after the washing steps and prior to eluting the analytes. A 5 mL aliquot of 5% formic acid in methanol was used to elute the analytes.

3) Concentration/Reconstitution

Samples were evaporated to dryness under a gentle stream of nitrogen on a heating block (≤35°C). It was determined that these conditions resulted in no significant loss of analyte. The samples were reconstituted in 1 mL of 70% methanol in water, which was vortexed and filtered through a 0.22 µm syringe filter into an autosampler vial for analysis. The sample dilution factor was 2x.

LC Separation

The chromatography was performed on an Eksigent ekspert™ UltraLC 100-XL system with a Phenomenex® column configuration that used two Silica SecurityGuard™ cartridges, followed by a Luna® Silica (2) mixer column (30 x 2 mm, 5 µm). A Gemini® 3 µm NX-C18 (50 x 2 mm) served as the analytical column. The column compartment was maintained at 30°C. The gradient is listed in Table 1. Mobile Phase A was HPLC water with 0.1% formic acid and Mobile Phase B was 10 mM ammonium formate in methanol with 0.1% formic acid.

Table 1. LC gradient

Time (min)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.0	0.7	100	0
5.0	0.7	5	95
7.0	0.7	5	95
7.1	0.7	100	0
10.0	0.7	100	0

MS/MS Detection

Analysis was performed on an AB SCIEX QTRAP® 5500 LC/MS/MS system using electrospray ionization (ESI) and *Scheduled* MRM™ in which each analyte's MRM is monitored across a user defined time window around each analyte's expected retention time, maximizing sensitivity. Each analyte's MRM and retention time are listed in Table 2. Most analytes are ionized in positive mode (+ESI) with the exception of florfenicol and chloramphenicol which are ionized in negative mode (-ESI). In order to achieve a single run, polarity switching was used in conjunction with the *Scheduled* MRM™ algorithm. The use of short pause times (2-3 ms) proved to be necessary to achieve optimal peak shapes and sensitivity to quantify the narrow UPLC peaks (FWHM = 3 to 4 s) particularly during polarity switching.

Table 2. Analytes, retention times (RT) and MRM transitions with collision energies (CE)

Analyte	RT (min)	Q1 (amu)	Q3 (amu)
Trimethoprim	1.63	291.2/261.2 (34)	291.2/230.2 (31)
Ciprofloxacin	2.11	332.0/314.0 (27)	332.0/230.9 (51)
Enrofloxacin	2.20	360.1/342.0 (29)	360.1/286.0 (47)
Sarafloxacin	2.30	386.1/368.2 (27)	386.1/348.1 (43)
Florfenicol	2.43	357.9/337.9 (-14)	357.9/184.8 (-46)
Spiramycin	2.55	442.4/174.2 (29)	422.4/101.1 (26)
Chloramphenicol	2.87	332.8/258.9 (-16)	322.8/151.9 (-24)
Oxolinic Acid	3.12	262.0/244.0 (23)	262.0/216.0 (39)
Flumequine	3.50	262.0/243.9 (25)	262.0/201.8 (45)
Diflubenzuron	4.42	311.2/158.1 (18)	311.2/141.1 (42)
Emamectin	4.75	886.7/158.2 (42)	886.7/82.3 (107)
Abamectin	5.42	891.0/305.1 (33)	891.0/568.1 (19)
Ivermectin	5.70	893.3/570.2 (21)	893.3/307.1 (33)

Results and Discussion

Figure 1 shows the extracted ion chromatograms (XIC) of a 10 µL injection of a matrix matched standard at 50 µg/mL.

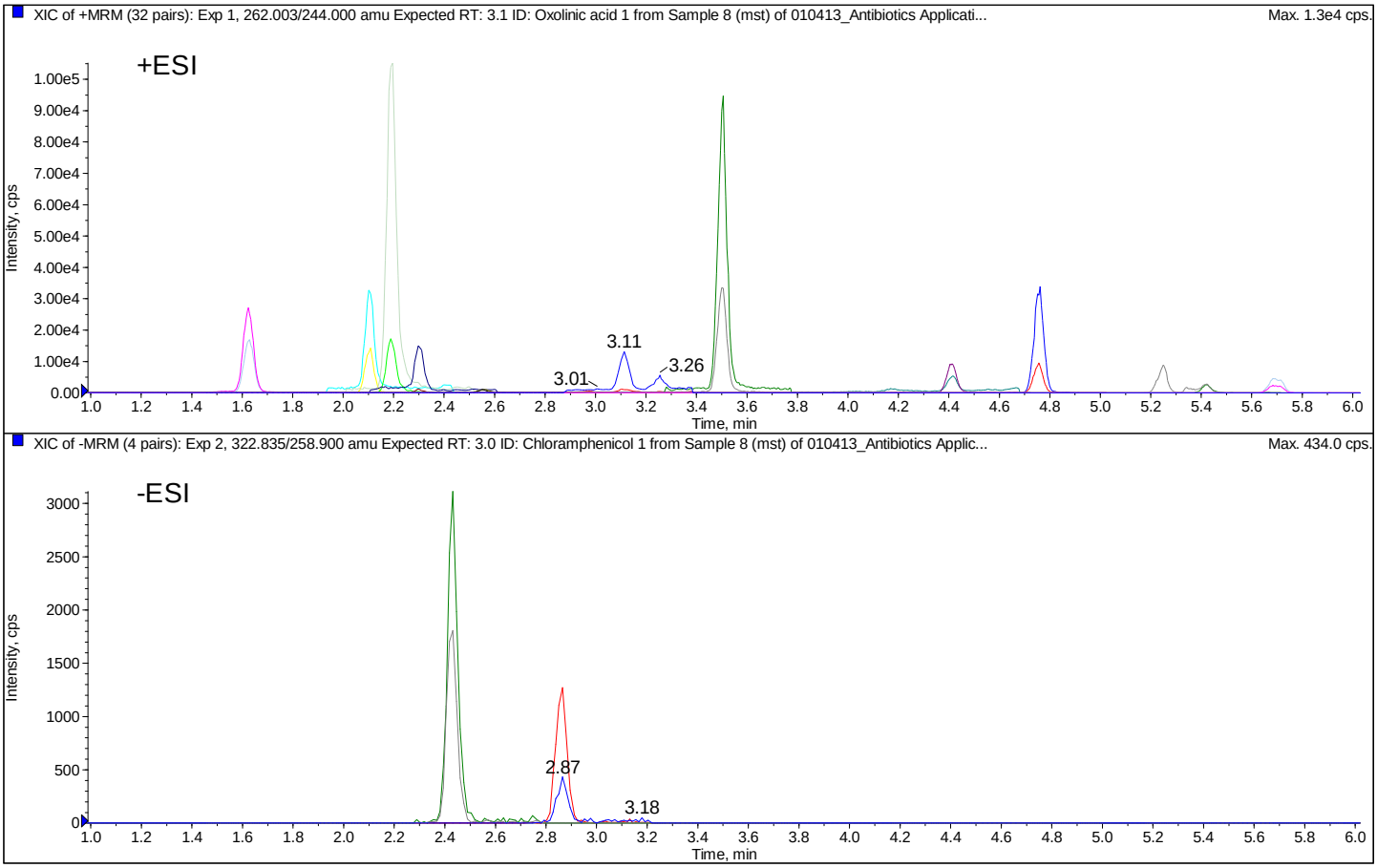

Figure 2. Poultry feed sample fortified at 40 µg/kg in sample (20 µg/mL in extract).

Figure 2 shows the extracted ion chromatograms (XIC) of a 10 µL injection of a poultry feed sample fortified at 40 µg/kg level (20 µg/mL in extract after 2x dilution).

The recoveries for each analyte are shown in Table 3. Given the complexity of the sample matrix and the inherent chemical differences between the target analytes, most analytes were reasonably recovered with the described extraction and cleanup. The method proved to be precise with %RSDs generally less than 5%. Recoveries could potentially be improved with the use of internal standards; however, absolute recoveries are a more accurate approach to assessing the effectiveness of a preparative method.

Table 3. Quantitation and Recovery Data based on MRM 1. Four point calibration using 5, 10, 50 and 100 µg/mL matrix matched standards.

Analyte	r^2	Average recovery (%) ± % RSD
Trimethoprim	0.999	89 ± 4 %
Ciprofloxacin	0.997	60 ± 0 %
Enrofloxacin	0.999	73 ± 4 %
Sarafloxacin	0.996	47 ± 4%
Florfenicol	1.000	85 ± 1 %
Spiramycin	1.000	70 ± 3 %
Chloramphenicol	1.000	77 ± 2 %
Oxolinic Acid	1.000	64 ± 1 %
Flumequine	0.998	64 ± 3 %
Diflubenzuron	1.000	20 ± 5 %
Emamectin	0.999	52 ± 7 %
Abamectin	0.999	40 ± 5 %
Ivermectin	1.000	24 ± 3 %

Summary

A single method has been developed to quantify a wide class of antibiotics and insecticides in poultry feed. The poultry feed extract was cleaned by SPE on a Phenomenex® Strata™-XL-CW prior to analysis utilizing an Eksigent ekspert™ UltraLC 100-XL system with a Phenomenex® Luna® Silica mixer column in series with a Gemini® NX-C18 analytical column with an AB SCIEX QTRAP® 5500 system for detection. *Scheduled* MRM™ in combination with fast polarity switching was used to maximize sensitivity while achieving a single run for all analytes. Analyte recoveries and precision from triplicate fortified poultry feeds were acceptable, given the complexity of the sample matrix and the generic approach to the extraction, and cleanup procedure required to simultaneously test such a variety of analytes.

References

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4 Prince Agri Products: http://www.princeagri.com/Phibro/Products/Catalog/Premixes-Feed-Ingredient-For-Poultry.html

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