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.

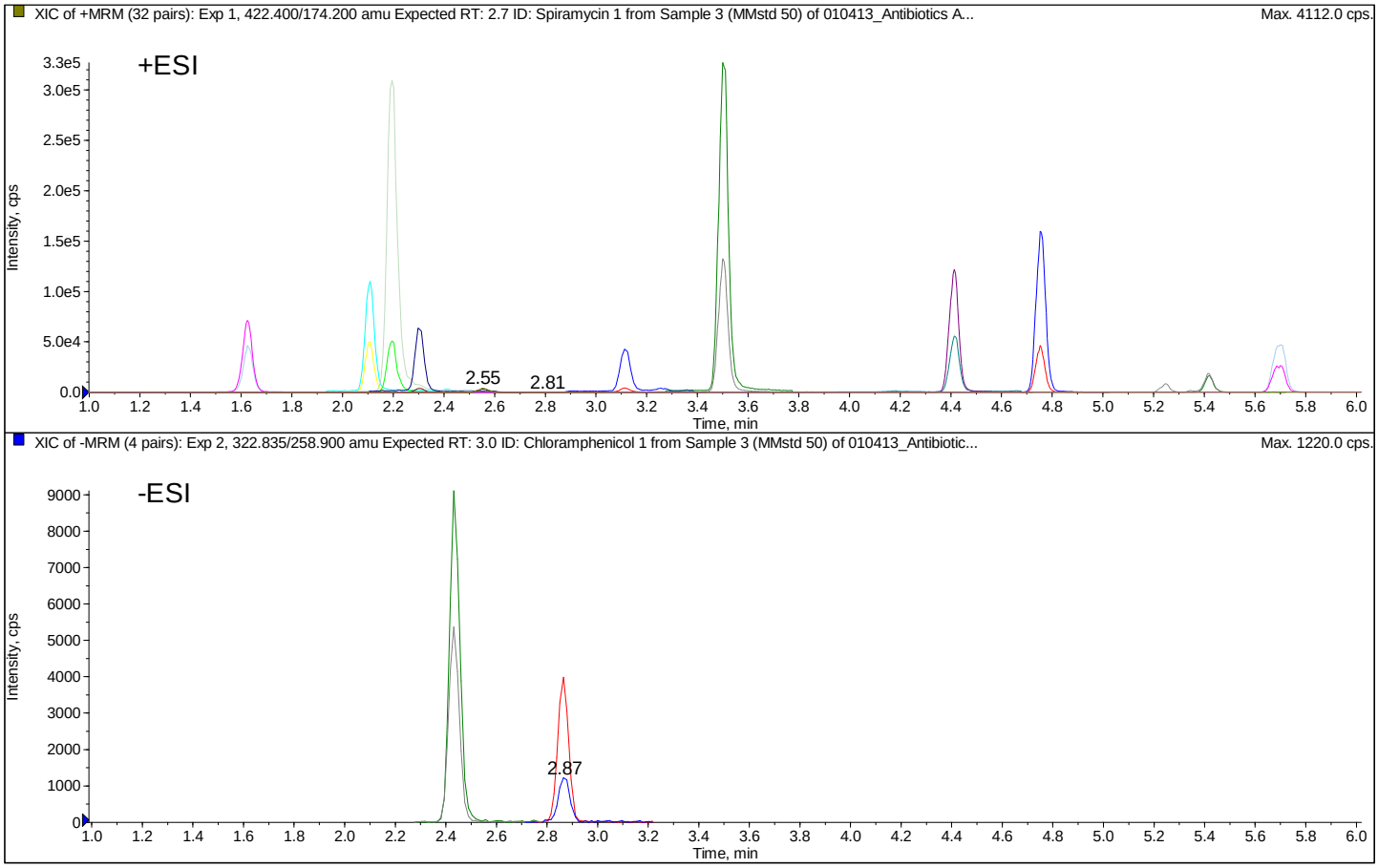

Figure 1. Detection of antibiotics and insecticides in a single run by LC-MS/MS using *Scheduled* MRM™ with polarity switching. Positive mode (+ESI) MRM transitions shown in top pane, and XIC of all negative mode (-ESI) MRM transitions shown at bottom. Peaks are identified by retention time in Table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

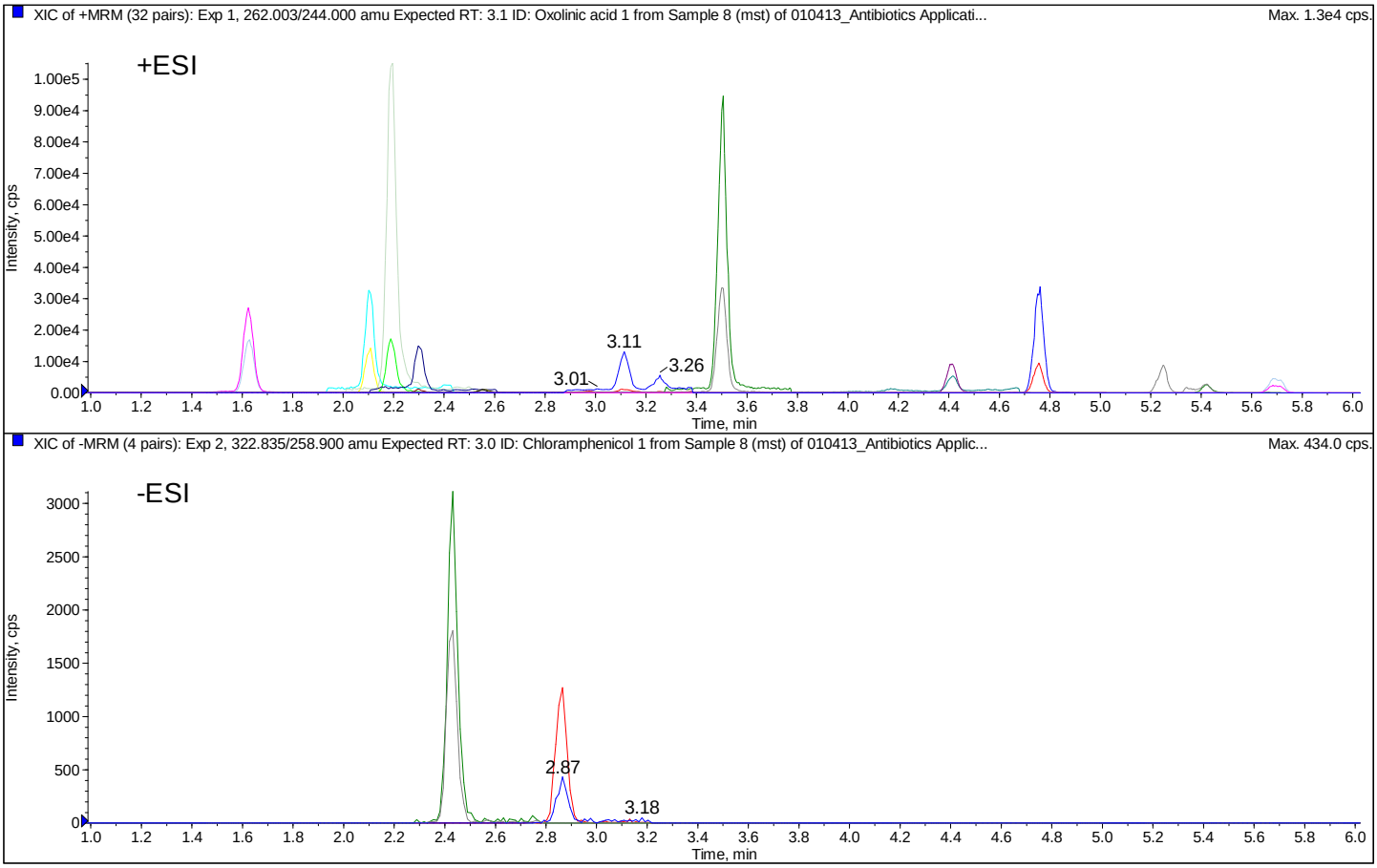

Figure 2. Poultry feed sample fortified at 40 µg/kg in sample (20 µg/mL in extract).

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.

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The Use of Micro Flow LC Coupled to MS/MS in Veterinary Drug Residue Analysis

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Overview

A rapid, robust, sensitive and specific LC-MS/MS method has been developed for the simultaneous detection of veterinary drug residues in milk and meat. The method uses an Eksigent ekspert™ microLC 200 and the AB SCIEX QTRAP® 4500 system utilizing the *Scheduled* MRM™ algorithm with a simplified sample preparation to detect veterinary residues below EU screening requirements.

Introduction

Traditionally, in veterinary drug residue screening of food samples, samples are extracted and analyzed by LC-MS/MS usually at LC flow rates in excess of 500 µL/min and in combination with smaller particle size LC columns result in high UHPLC pressure separations. These conditions result in short chromatographic run times with excellent efficiency and peak shape, but have a drawback in that they require higher volumes of mobile phase. The consumption of organic LC solvents, such as acetonitrile and methanol, is a growing cost of analysts and its disposal has an environmental impact. Therefore, ways to reduce solvent consumption in food residue testing will be beneficial to the environment and reduce running costs of a testing laboratory.

Here we present new data using micro flow LC, running below 40 µL/min, in combination with a LC-MS/MS method developed on an AB SCIEX QTRAP® 4500 system which utilizes the *Scheduled* MRM™ Pro algorithm. Initially this approach has been applied to a screen of veterinary residues including sulfonamides and beta-lactam antibiotics to show its applicability in food analysis. Data presented shows a comparison of micro flow LC-MS/MS with traditional high flow LC-MS/MS and show that low limits of detection (LOD) below legislated levels[1] are easily possible by this approach.

Experimental

Standards and Samples

For this work the target compounds were commercially available and purchased from Sigma Aldrich. Milk and meat samples for spiking experiments were obtained from a local supermarket.

Sample Preparation

The milk samples (2 mL) was simply mixed with acetonitrile (8 mL) and roller mixed for 20 minutes. After mixing the sample extracts was centrifuged for 5 minutes at 2500 rpm. The supernatant (4 mL) was evaporated to dryness (Eppendorf vacuum concentrator at 60°C) and then reconstituted into 0.1% formic acid in water(2 mL). The reconstituted sample was centrifuged for 1 min at 13,000 and the top layer was decanted into plastic HPLC vials ready for LC-MS/MS analysis.

For meat samples the extraction protocol was exactly the same except the initial extraction solvent was acetonitrile/water (87.5/12.5).

LC

All microLC method development and analysis was done using an Eksigent ekspert™ microLC 200 UHPLC system. Final extracted samples (5 µL) were separated over a 3.5 minute gradient (shown in Table 1 where A = water and B = acetonitrile both containing 0.1 % formic acid) on a reversed-phase Triart C18 2.7 µm (50 x 0.5 mm) column (YMC) at 30 µL/min and at a temperature of 60ºC.

For the high flow LC comparison a Shimadzu UFLC$_{XR}$ system was used at a flow rate of 600 µL/min using a Kinetex 2.6 µm XDB-C18 (50 x 2.1 mm) column (Phenomenex). The gradient conditions are shown in Table 2.

Table 1. Gradient conditions used for micro flow LC separation at a flow rate of 30 µL/min

Step	Time	A (%)	B (%)
0	0	98	2
1	0.5	98	2
2	1.7	35	65
3	1.8	0	100
4	2.3	0	100
5	2.4	98	2
6	3.5	98	2

Table 2. Gradient conditions used for traditional high flow LC separation at a flow rate of 600 µL/min

Step	Time	A (%)	B (%)
0	0	98	2
1	2	98	2
2	7	40	60
3	7.2	5	95
4	8	5	95
5	8.1	98	2
6	10	98	2

MS/MS

All analyses were performed on an AB SCIEX 4500 QTRAP® system using the Turbo V™ source in electrospray ionization (ESI) mode. For micro flow LC analysis the electrode was changed to a microLC hybrid electrode (50 µm ID) designed for micro flow rates.[2] In the final micro flow LC method the ion source conditions used were Gas 1, Gas 2 and the Curtain Gas™ interface was set to 30 psi, the temperature (TEM) was set at 350°C and the IS voltage was set to 5500 V.

The veterinary drugs were analyzed using Multiple Reaction Monitoring (MRM) using the *Scheduled* MRM™ algorithm to obtain high selectivity, sensitivity, accuracy and reproducibility. The *Scheduled* MRM™ Pro algorithm in Analyst® software version 1.6.2 allows setting the MRM detection window separately for each compound based on the LC peak width for more efficient scheduling of dwell time (Figure 1).

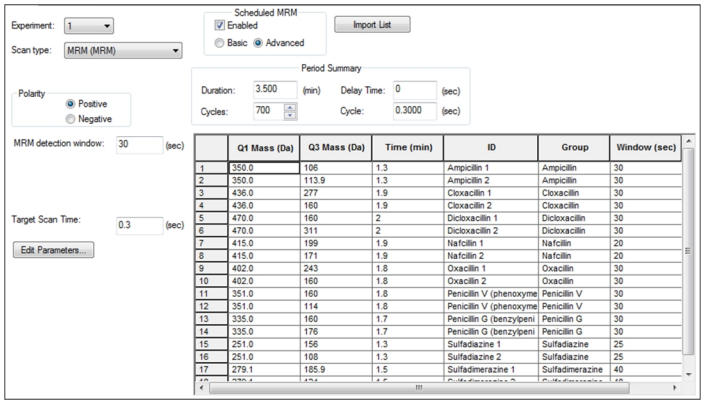

Figure 1. Method editor in Analyst® software version 1.6.2 used to setup the *Scheduled* MRM™ Pro experiment

A total of 32 MRM transitions (Table 3) were monitored to quantify and identify 15 veterinary drug residues and internal standards over a 3.5 minute run time. Only a small set of residues were tested in this project but there is scope to add more compounds to this method. In all the analyses Q1 and Q3 resolution were set to unit.

Table 3. MRM transitions and retention times (RT) of veterinary drug residues investigated in this

Compound	RT (min)	Q1 (amu)	Q3 (amu)	DP (V)	CE (V)
Ampicillin	1.3	350	106, 114	56	23, 41
Cloxacillin	1.9	436	277, 160	51	19, 17
Dicloxacillin	2	470	160, 311	66	19, 21
Nafcillin	1.9	415	199, 171	61	19, 47
Oxacillin	1.8	402	243, 160	46	19, 17
Penicillin V	1.8	351	160, 114	50	19, 45
Penicillin G	1.7	335	160, 176	50	15, 19
Sulfadiazine	1.3	251	156, 108	66	26, 30
Sulfadimerazine	1.5	279	186, 124	80	23, 31
Sulfadimethoxine	1.7	311	156, 92	71	29, 45
Sulfamerazine	1.4	265	108, 92	80	33, 35
Sulfamethaxazole	1.55	254	156, 92	120	21, 35
Sulfamethazine	1.5	279	186, 124	120	23, 31
Sulfaquinoxaline	1.9	301	156, 108	80	27, 37
Sulfathiazole	1.4	256	156, 92	80	19, 33

Results and Discussion

Before the micro LC was used for residue analysis the method was compared against a traditional high flow method that had previously been developed for residue detection in meat and milk. A 1 ng/mL standard of a mixture of different veterinary residues was prepared and analyzed (Figure 2).

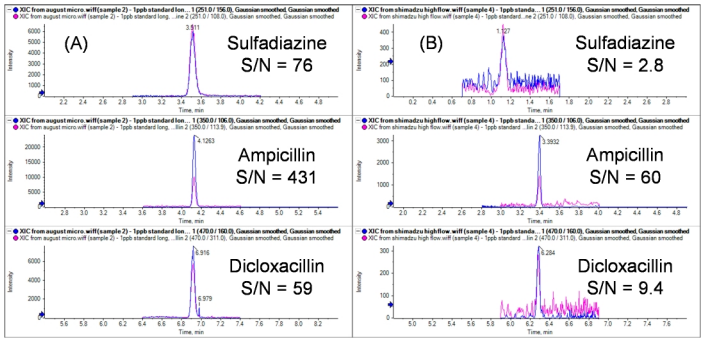

Figure 2. Comparison of microLC (A) with traditional high flow LC (B) using a 1 ng/mL standard.

For the high flow separation a Kinetex 2.6 µm XDB-C18 column at a flow rate of 600 µL/min was used and a Triart C18 column was used for microLC at 25 µL/min. The gradient conditions (Table 2) were kept the same as was the injection volume and column temperature. The results showed sensitivity increases of factors greater than 4 fold to over 10 fold for the veterinary drugs tested with none of the compounds showing a sensitivity loss.

The gradient on the microLC was then adjusted and the flow rate increased to 30 µL/min, to shorten the run time down to 3.5 minutes (Figure 3).

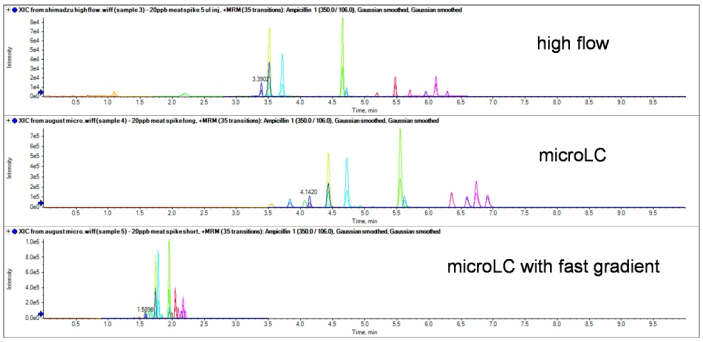

Figure 3. Comparison of meat sample spiked at 20 µg/kg and analyzed by traditional high flow LC and micro flow LC-MS/MS,. In this example analysis time was decreased from 10 min to 3.5 min using micro flow LC and by speeding up the gradient. In all methods peak widths at the base were 3 seconds or less.

Table 4. Results for the calibration lines for a selection of veterinary drug residues and the repeat analysis of spiked milk and meat samples. Displayed are the coefficient of regression (r), coefficient of variation (CV), and signal-to-noise (S/N) obtained. Linearity ranged from 0.1 to 100 ng/mL with linear fit and no weighting used except for sulfamerazine where linear fit and 1/x weighting was used.

Compound	r	CV (%) at 20 µg/kg spiked into milk (n=20)	S/N at 2 µg/kg spiked into milk	S/N at 20 µg/kg spiked into milk	CV (%) at 20 µg/kg spiked into meat (n=20)	S/N at 20 µg/kg spiked into meat
Ampicillin	0.999	5.8	67	712	3.6	285
Cloxacillin	0.999	4.7	94	934	9.1	591
Dicloxacillin	1.000	5.7	50	389	9.0	508
Nafcillin	0.999	2.7	39	379	10.2	800
Oxacillin	0.999	5.6	39	337	8.4	299
Penicillin V	0.999	4.3	101	1162	5.5	272
Penicillin G	0.991	5.8	19	150	14.0	175
Sulfadiazine	0.997	11.1	24	208	6.9	196
Sulfadimerazine	0.995	6.1	30	2131	8.3	1119
Sulfadimethoxine	0.999	4.2	152	1549	1.4	539
Sulfamerazine	0.996	3.5	44	366	3.0	333
Sulfamethaxazole	0.993	7.2	40	356	5.7	189
Sulfamethazine	0.997	10.4	55	662	2.8	357
Sulfaquinoxaline	0.998	4.8	25	275	3.7	705
Sulfathiazole	0.998	3.4	25	290	5.2	131

The results showed that for the late eluting compounds there was some sensitivity loss due to peak broadening but again sensitivity gains were also observed for early eluting compounds. Generally speaking increasing the speed of analysis three fold did not have a negative effect on the response observed for these veterinary residues.

Calibration standards were analyzed for all compounds using the shortened microLC method and three examples of calibration lines for different compounds are shown in Figures 4a to 4c. In each figure the calibration lines were linear and the residues could be detected at a level of 0.1 ng/mL or below (see peak review in each figure).

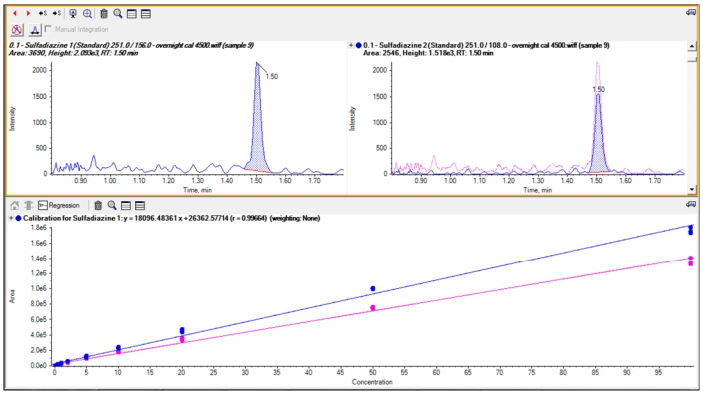

Figure 4a. Quantifier, qualifier MRM transition at 0.1 ng/mL (top), and calibration line of sulfadiazine from 0.1 to 100 ng/mL (bottom), the linearity is provided without the use of any internal standards

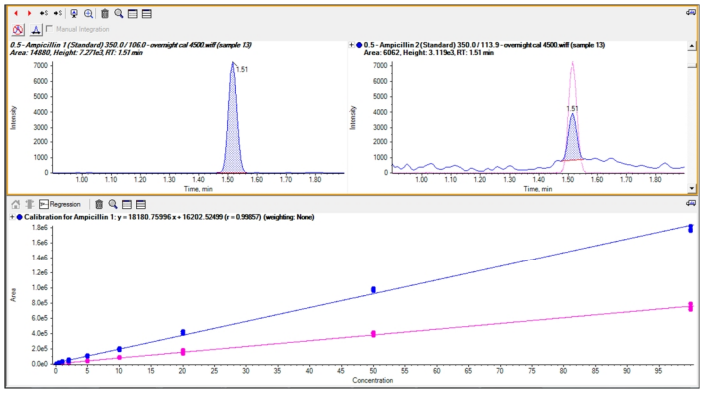

Figure 4b. Quantifier, qualifier MRM transition at 0.5 ng/mL (top), and calibration line of ampicillin from 0.1 to 100 ng/mL (bottom), the linearity is provided without the use of any internal standards

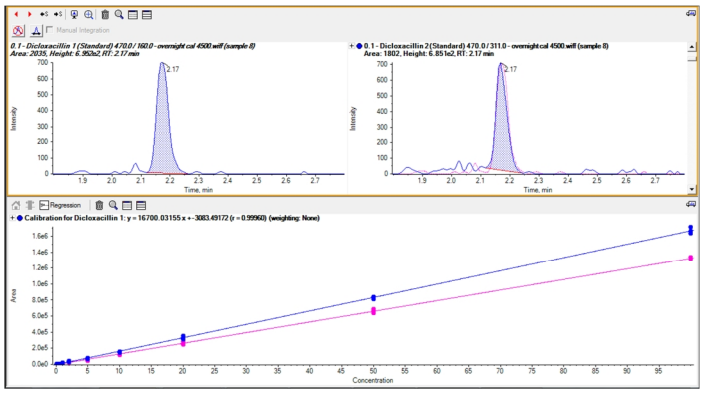

Figure 4c. Quantifier, qualifier MRM transition at 0.1 ng/mL (top), and calibration line of dicloxacillin from 0.1 to 100 ng/mL (bottom), the linearity is provided without the use of any internal standards

The calibration data for each compound is shown in Table 4. Following on from the assessment of linearity milk, meat samples were spiked and extracted and repeatedly analyzed to assess reproducibility with the results displayed in Table 4. For both the calibration lines and the spiking experiments no internal standards were used.

From the results displayed in Table 4 it can be seen that the method can easily provide detection limits which comply with current EU legislation. Linearity was excellent from 0.1 to 100 ng/mL with coefficients of regression greater than 0.99. The repeatability observed and signal-to-noise (S/N) measured varied with the matrix showing the need of internal standards to counter matrix effects from the simplified sample extraction protocol used. However, no coefficient of variation (CV) was over 15% which mirrored a previous study of pesticide residue analysis using microLC[3] with most generally below 10%. All S/N (calculated using 3x standard deviation algorithm in Analyst® software) were greater than 15/1 even in the 2 µg/kg spike into milk.

Summary

This study has clearly demonstrated that using microLC is a valid approach in veterinary residue analysis. The method developed using Eksigent ekspert™ microLC 200 and the AB SCIEX QTRAP® 4500 system was rapid, sensitive, reproducible, and easily reached the requirements of current EU legislation. Micro flow LC offers the opportunity to cut the analysis time by over half without a loss in performance and in the majority of cases a gain in signal by over a factor of 5 was observed.

Micro LC also provides huge cost saving to laboratories. With LC grade acetonitrile running at a cost of £100/L this 3 day study could have cost about £ 100 with conventional chromatography (0.6 mL/min running for 24hrs a day) and less than £10 with microLC. Over a year this amounts to savings of over £4000 (£90 x 50 weeks) in solvent consumption alone.

Although this method is still under development, with plans to expand the number of compounds in this screen, this work has shown the clear potential of Micro LC in this application area.

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