APPLICATION NOTE

Analysis of Tetracycline in Animal

Authors:

Saba Hariri
Abir Khaled
Avinash Dalmia
Feng Qin

PerkinElmer, Inc.
Toronto
Canada

Shelton, CT
USA

High Throughput Analysis of Tetracycline in Animal Tissue Using UHPLC/MS/MS System equipped with High Capacity QS-works Autosampler

Introduction

Tetracyclines (TCs) are one of the most frequently used antibiotics in animal production to treat diseases, prevent infection and protect growth of animal. Excessive usage of antibiotics might result in residue violations in animal originated food such as meat, milk and others, and this can pose a risk to health of humans. The presence of tetracycline residues in food can cause toxicity and side effects such as allergic reaction as well as bacterial resistance which may transfer to human beings. Therefore, in order to protect human health, regulatory agencies around the world have established maximum residue levels (MRLs) or tolerances of veterinary drugs in foods (for example, the European Union, Canada, China and many other countries set "maximum residue levels of drug residues in foods", while in the US, these are called "tolerances")[1-2]. The use of highly sensitive and selective techniques for accurate monitoring of tetracyclines based on regulatory requirements is essential to protect human health. This work presents a fast and sensitive UHPLC/MS/MS method for the quantitative analysis of tetracyclines in meat. To increase lab productivity, QS-Works high throughput autosampler was used due to large capacity, temperature-controlled trays and robotic arms which allows unattended operation for long time. QS works also allows labs to run up to 600 samples in a sequence which improves testing labs efficiencies. The developed method showed good sensitivity, linearity, recovery, precision and selectivity required for analysis of tetracyclines in meat at low tolerance levels set by different regulatory bodies in the world.

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Experimental

Hardware/Software

Chromatographic separation was conducted on a PerkinElmer QSight® LX50 UHPLC system, while detection was achieved using a PerkinElmer QSight® 420 MS/MS detector. High capacity QS Works autosampler was used to enhance lab productivity. All instrument control, data acquisition and data processing were performed using the Simplicity 3Q™ software platform.

LC Method and MS Source Conditions

The LC method and MS source parameters are shown in Table 1 and multiple reaction monitoring mode (MRM) transitions of the tetracyclines are shown in Table 2. At least two MRM transitions were monitored for each analyte to reduce the number of false positive and negative in the method. Optimization of MS/MS parameters, including choice of parent ions and product ions, collision energies (CE), entrance voltages (EV), (CCL2) and so on, was done by infusion of standards. Source conditions were optimized by t-infusion of neat standards with LC flow. The acquisition MS method was created based on those optimized conditions.

Table 1. LC Method and MS Source Parameters.

LC and MS Condition	
Mobile phase A	Water (contains 0.1% Oxalic acid)
Mobile phase B	Acetonitrile (contains 0.1% formic acid)
Column	PerkinElmer Quasar SPP Pesticides, 100 × 4.6 mm; 2.7 µm, (N9306880) mm
Column oven temperature	20 °C
Autosampler temperature	20 °C
Injection volume	5 µL
ESI voltage (positive)	5100 V
Drying gas	120
Nebulizer gas	250
Source temperature	350 °C
HSID temperature	250 °C

Table 2. MRM Transitions.

Compound name	Precursor ion/Da	Product ion/Da	CE/V	EV/V	CCL2/V
Oxytetracyline	461.3	426.3 201.2	-21 -49	31 25	-136 -180
Tetracycline	445.3	410.3 427.3	-22 -15	29 27	-144 -124
Chlortetracycline	479.2	154.0 444.2	-34 -25	26 29	-136 -164
Doxytetracycline	445.3	154.1 154.0	-35 -34	29 26	-140 -136

Standards and Sample Preparation

All solvents, reagents and diluents used were LC/MS grade. All TC standards were obtained from Sigma-Aldrich® Inc. and stored at -20 °C in freezer to prevent their degradation. Stock and mixed drug solutions for spiking and calibration were prepared in methanol for all TCs. To prevent degradation of standards, all stock and working standards were stored in freezer until used.

To make a homogenized sample, a representative meat sample was cut into small pieces and blended. A one-gram aliquot of the homogenized meat sample, along with 8 mL of 0.1 M aqueous EDTA solution were added into a 50 mL centrifuge tube and vortexed. Next, 17 mL of acetonitrile was added into the same tube and vortexed. The tube was then placed into freezer maintained at -20 °C for 30 min followed by centrifuging for 10 minutes at 4000 rpm. Next, 8 mL of supernatant was transferred into a 50 mL clean centrifuge tube, and 5 mL of hexane was added for defatting of sample, and the tube was vortexed for 3 min. The solution was then centrifuged for 1 min at 4000 rpm. The 3 mL of lower phase was transferred into a new 15 mL centrifuge tube and evaporated to dryness by blowing nitrogen over it at a temperature of 50 °C. The dried extract was reconstituted by dissolving it in a 1 mL of methanol/water (30/70, v/v) solution containing 0.01 M oxalic acid. Before injection onto the LC/MS/MS system, sample extracts were vortexed and filtered by means of a 0.2 µm PTFE syringe filters[3].

Results and Discussion

The objective of this work was to assess the performance of high capacity QS-Works autosampler with a validated sample preparation method for the determination of tetracyclines in meat. Using high throughput autosampler like QS-Works improves testing lab efficiencies and productivity. For food testing labs with large numbers of samples, QS-Works provides processing of more sample rapidly, while improving data quality and consistency due to robotic sampling unit (Figure 1).

The maximum residue levels (MRLs) established for tetracyclines in foods by the Health Canada or tolerances by US FDA were used as the reference target levels (X in Table 3) and the detection threshold ('yes/no' screening level) should be at or below 0.5X. Figure 2 shows the chromatograms (XIC) of 4 tetracyclines, with good peak resolution. Two ion transitions were monitored for identification and confirmation purposes. This results higher selectivity and specificity in identification. All ion ratios were computed by calculating peak area for less intense ion to peak area for more intense ion to generate ion ratios.

Sample matrix effects (MEs) are the main concerns for LC/MS/MS method development, especially for food analysis due to the diversity and complexity of food sample matrices. ESI is susceptible to ionization suppression of analytes in the presence of charge-competing matrix components. Matrix-induced enhancement effects are also known to occur in ESI, which can also introduce a large bias in quantification. To overcome sample MEs, several approaches have been used, such as sample dilution, use of stable isotope internal standards, matrix-matched (MM) calibration, standard addition, sample clean-up and use of high efficiency columns for improved separation. Sample matrix effects (MEs) were evaluated by comparing the slopes of calibration curves obtained from meat sample matrix to slopes obtained from reagent-only (RO). Sample ME (%) for each analyte was calculated by the percentage difference between the slopes. As shown in Table 3, the MEs for most of the compounds were less than 20%. To overcome matrix effects and reduce variations in analytical results, matrix-matched calibrations were used in this study for quantification of all analytes. Calibration was performed using

both matrix-matched (MM) and reagents-only (RO) standards. All calibration curves built from both RO and meat sample matrix showed good linearity with correlation coefficient (R^2) larger than 0.99 (see Figures 3 and 4 for typical examples of calibration curves). Carryover was assessed by injecting the reagent blank after a high concentration standard and no carry-over was observed in any of the experiments. To avoid chelation of tetracyclines by metal ions of LC parts or column body and maintain good peak shape and reproducibility over the run, oxalic acid was added into aqueous mobile phase[3]. As a result, the method demonstrated good precision with RSD less than 5% for five replicates. Table 3 also lists the effect of using oxalic acid in mobile phase and reconstitute solvent on the reproducibility. As can be seen, the reproducibility and peak shape of all analytes was improved by adding the oxalic acid into mobile phase and reconstitute solvent. The LOQ for each analyzed tetracycline was lower than their allowed tolerance level in meat samples. This demonstrates that the method is more than adequately sensitive for tetracycline analysis in meat at tolerance level. The limits of quantification (LOQs) for the TCs were estimated based on signal/noise (S/N) ratio of 10 and all the LOQs for the drugs studied are 1.5 ± 1.0 µg/L. The results demonstrated that the developed method can be applied for the fast screening and quantification of tetracyclines in meat samples. The absolute recoveries of tetracyclines were in range of 70-120% (see Table 3). In future, the use of a deuterated tetracycline can be used to compensate for losses of tetracyclines with this extraction process.

Figure 1. QS-Works autosampler.

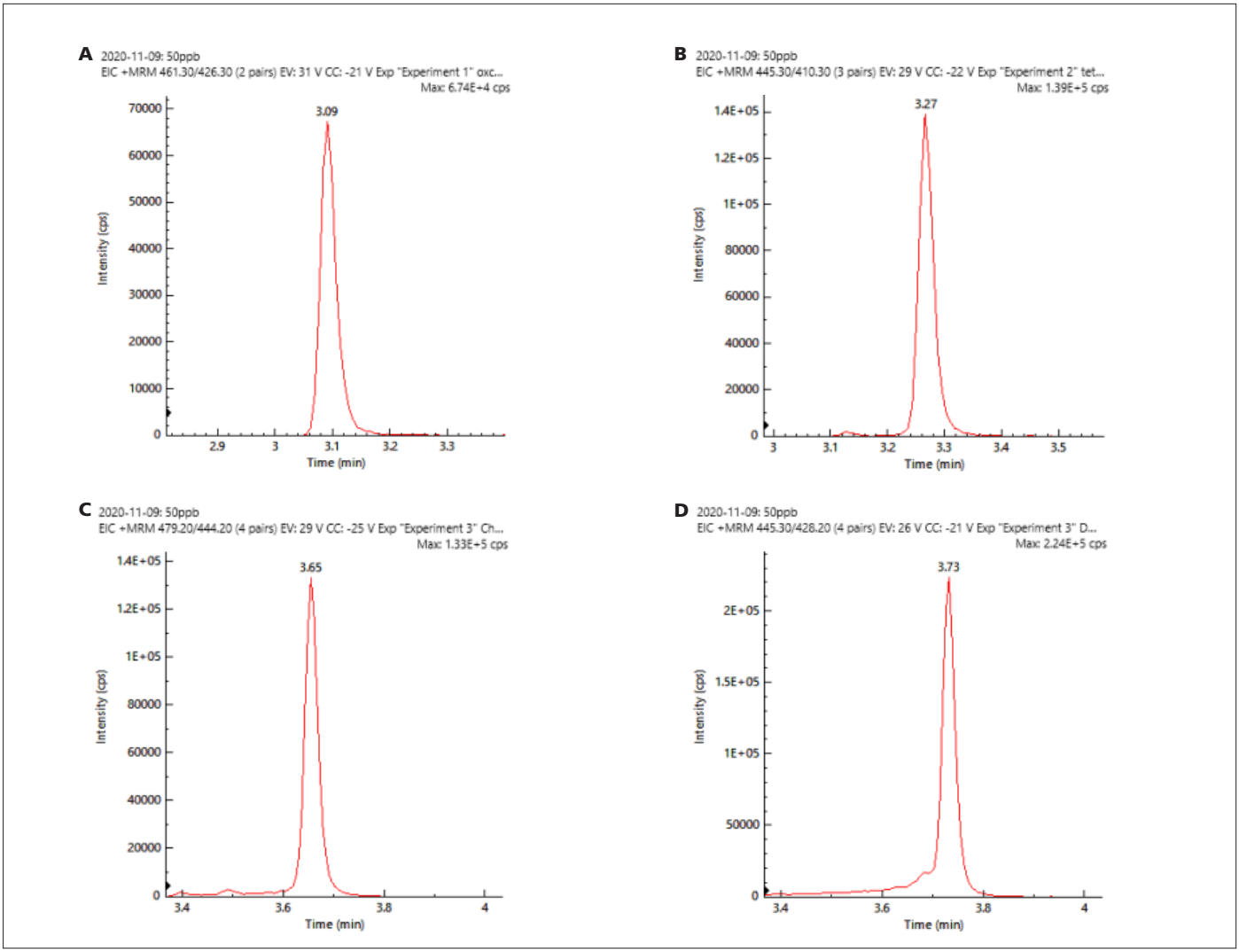

Figure 2. XIC chromatograms of a) oxytetracycline, b) tetracycline, c) chlortetracycline, d) doxytetracycline.

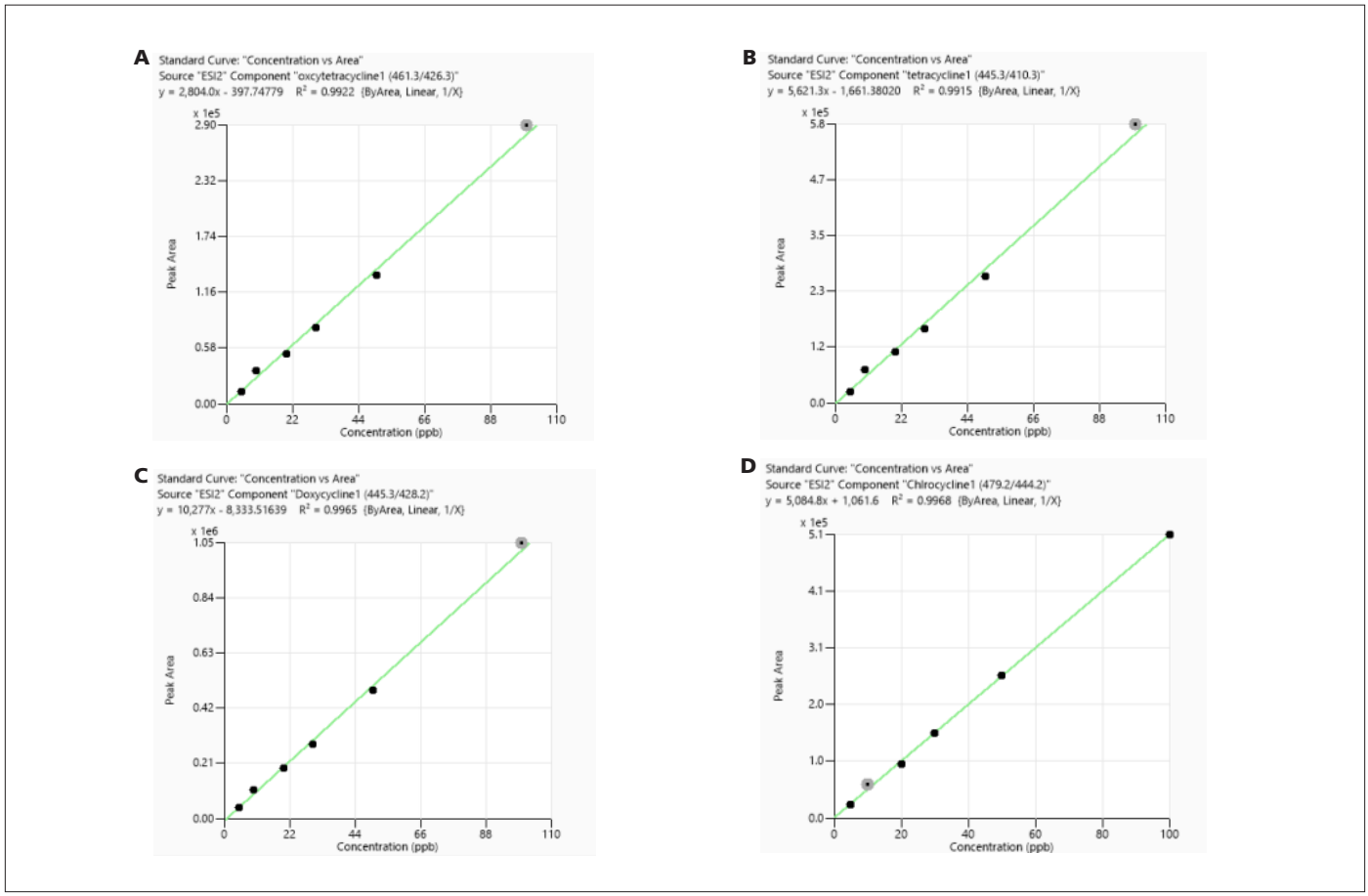

Figure 3. Calibration curves of tetracyclines obtained from standards prepared in reagent only.

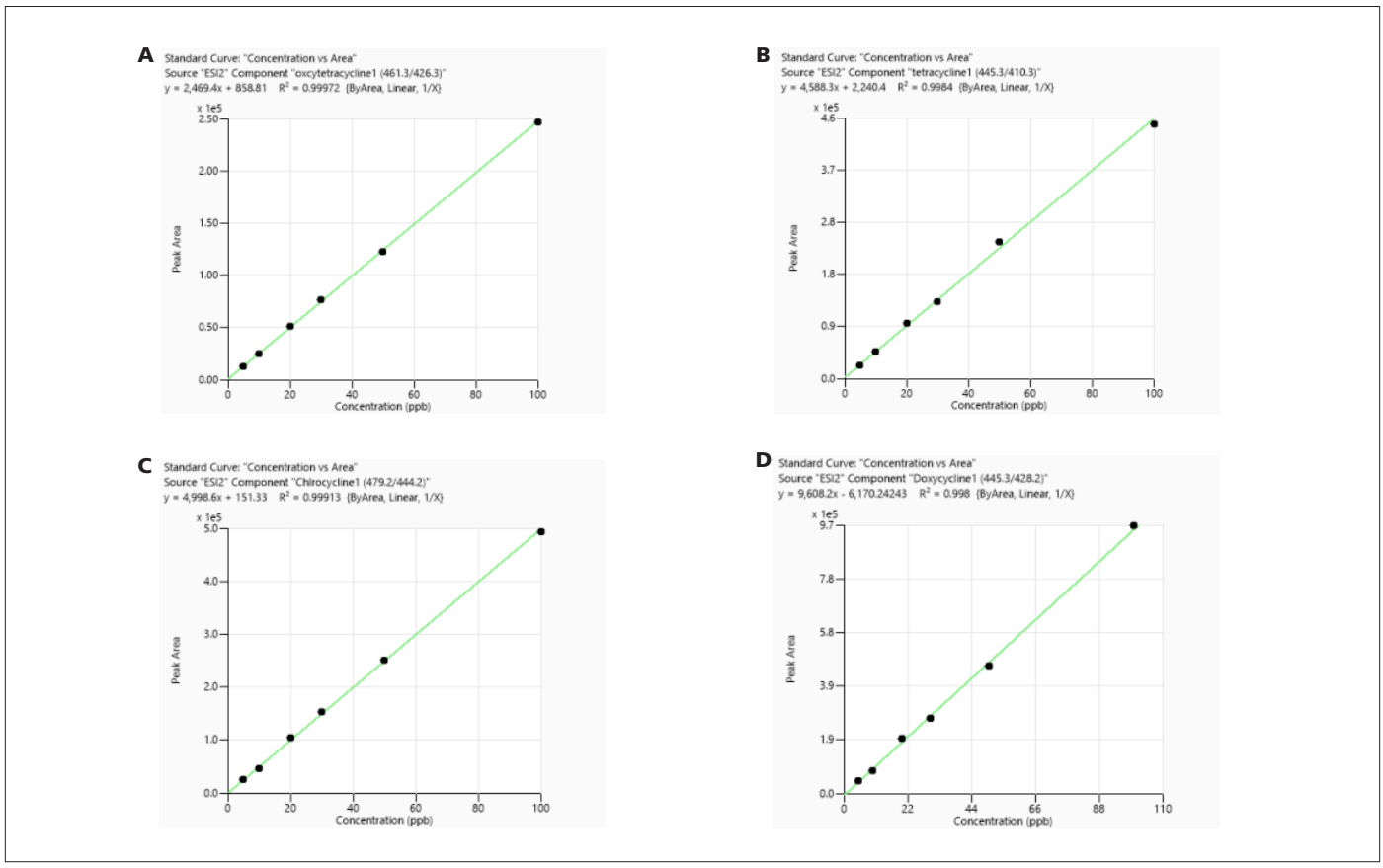

Figure 4. Calibration curves of tetracyclines obtained from standards prepared in meat sample matrix.

Table 3. Results of retention time, matrix effect (ME), linearity, recovery, and reproducibility (%RSD).

Compound name	Level (ng/g) Tolerance (x)	RT (min)	ME (%)	Linearity (R^2)	Recovery (%)	RSD with oxalic acid (%) (n=5)	RSD without oxalic acid (%) (n=5)
Oxytetracyline	200	3.09	86	0.9984	70.0	4.6	11.0
Tetracycline	200	3.26	80	0.9997	116.0	4.9	16.0
Chlortetracycline	200	3.65	96	0.9991	87.3	1.9	9.5
Doxytetracycline	200	3.73	94	0.9980	71.6	1.5	8.0

Conclusion

An UHPLC/MS/MS method utilizing a PerkinElmer high throughput QS-Works autosampler and LX 50 UHPLC coupled to a QSight 420 MS/MS system has been developed which demonstrates good sensitivity for the identification and quantification of tetracyclines in a homogenized meat sample. The low detection levels achieved in the method can aid in the support of low regulatory limits for routine screening and quantitation analysis.

References

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2. Standard Method Performance Requirements (SMPRs®) for Screening and Identification Method for Regulated Veterinary Drug Residues in Food. SMPR2018_010.pdf (aoac.org). Accessed November, 2020.
3. Aurélien Desmarchelier, Sébastien Anizan, Mai Minh Tien, Marie-Claude Savoy & Cindy Bion (2018) Determination of five tetracyclines and their epimers by LC-MS/ MS based on a liquid-liquid extraction with low temperature partitioning, Food Additives & Contaminants: Part A, 35:4, 687-695, DOI: 10.1080/19440049.2018.1427894

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

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