Enhancements to LIB 4560 to include method modifications, and additional drug residues in honey using LC-MS/MS

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Abstract

The liquid chromatography tandem mass spectrometry method, LIB 4560, was developed in 2014 by the United States Food and Drug Administration, Arkansas Laboratory. Although the method has been effective for regulatory use, substantial modifications to the method were needed to meet the changing regulatory needs for analysis of veterinary drugs in honey. These modifications were all implemented in the method as described herein and did not adversely affect quantitation, robustness, or confirmation abilities. Validation of the drug analytes demonstrated acceptable accuracies and detection levels.

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Introduction

The economic impact of honey bees is estimated to be more than $15 billion annually (1-4). Yet, studies have shown that honey bee numbers have substantially decreased and not recovered over the last several years (5). Much of the decline has been attributed to diseases, such as American Foulbrood, European Foulbrood, and Nosema disease (6-8). In attempts to treat and prevent such illnesses in honey bees, antibiotics and/or veterinary drugs are often administered to bees through feeding processes. Although some veterinary drugs are approved to treat and prevent bee disease, the drug residues are not allowed in foodstuff.

LIB 4560 is a multi-residue method for the determination of numerous veterinary drug residues with vastly different chemical properties in honey using liquid chromatography coupled to a triple quadrupole mass spectrometer (LC-MS/MS) (3). In order to address all of these chemical properties, the method is divided into two or more separate extractions and four different LC-MS/MS acquisitions. This method has been used since 2014 for regulatory analysis by the U.S. Food and Drug Administration.

Since LIB 4560's initial development, changes in the testing program have occurred. These changes include adjustments to the target testing levels (TTL) for several drug residues in honey, expanding the number of targeted residues (sulfadoxine, sulfapyridine, sulfamethoxypyridazine, sulfamethoxazole and sulfaethoxypyridazine), and changes in technology to provide increased throughput. The method modifications described herein pertain exclusively to the multi-residue and primary extraction referenced in LIB 4560 (3).

This study encompasses the extraction and determination of 25 different drug residues in honey. All of the analytes in this method can be extracted and determined in a single procedure.

Experimental

(Equipment and reagents have been provided for guidance. Equivalent products may be substituted as appropriate)

Equipment:

a) Mass Spectrometer: Sciex QTRAP 5500 Mass Spectrometer with Turbo V source and electrospray ionization
b) Liquid Chromatograph: Agilent 1260 Liquid Chromatograph.
c) Chromatographic Column: Agilent Poreshell EC-C18 column (2.7 µm, 4.6 X 50 mm)
d) Centrifuge: Must be capable of holding 15 mL and 50 mL centrifuge tubes, at 4°C, and approximately 3950 g
e) Turbo-Vap nitrogen evaporator (Biotage, Uppsala, Sweden)
f) 15 mL disposable polypropylene centrifuge tubes with screw cap lids, (Sarstedt, Newton, NC)
g) 50 mL disposable polypropylene centrifuge tubes with screw cap lids (Sarstedt, Newton, NC)
h) Vortex Mixer
i) Multi-Tube vortex shaker that is capable of holding 50 mL centrifuge

tubes or Geno/Grinder

j) Ultrasonic bath: water filled
k) Auto-Sampler vials (#5182-0716, Agilent Technologies, Santa Clara, CA)
l) Auto-Sampler screw caps (#5185-5861, Agilent Technologies, Santa Clara, CA)

Reagents and Standards:

a. Acetonitrile: LC/MS grade – (Fisher Scientific, Houston, TX)
b. Acetonitrile: HPLC grade – (Fisher Scientific, Houston, TX)
c. Water: LC/MS grade – (Fisher Scientific, Houston, TX)
d. Water: 18 MΩ-cm or equivalent for extraction use only
e. Formic Acid: LC/MS grade – (Fisher Scientific, Houston, TX)
f. Glacial Acetic Acid: Reagent grade – (Fisher Scientific, Houston, TX)
g. Methanol: HPLC grade – (Fisher Scientific, Houston, TX)
h. Sodium Chloride: Reagent grade – (Fisher Scientific, Houston, TX)
i. Enrofloxacin (ENRO) – (Sigma Aldrich, St. Louis, MO)
j. Sarafloxacin (SARA) – (Sigma Aldrich, St. Louis, MO)
k. Ciprofloxacin (CIPRO) – (Sigma Aldrich, St. Louis, MO)
l. Danofloxacin (DANO) – (Sigma Aldrich, St. Louis, MO)
m. Difloxacin (DFLX) – (Sigma Aldrich, St. Louis, MO)
n. Norfloxacin (NOR) – (Sigma Aldrich, St. Louis, MO)
o. Lincomycin (LIN) – (Sigma Aldrich, St. Louis, MO)
p. Doxycycline (DC) – (Sigma Aldrich, St. Louis, MO)
q. Tetracycline (TC) – (Sigma Aldrich, St. Louis, MO)
r. Oxytetracycline (OTC) – (Sigma Aldrich, St. Louis, MO)
s. Chlortetracycline (CTC) – (Sigma Aldrich, St. Louis, MO)
t. Sulfamethazine (SMZ) – (Sigma Aldrich, St. Louis, MO)
u. Sulfamerazine (SMR) – (US Pharmacopeia, Rockville, MD)
v. Sulfadimethoxine (SDM) – (US Pharmacopeia, Rockville, MD)
w. Sulfadiazine (SDZ) – (US Pharmacopeia, Rockville, MD)
x. Sulfachloropyridazine (SCP) – (Sigma Aldrich, St. Louis, MO)
y. Sulfaquinoxaline (SQX) – (US Pharmacopeia, Rockville, MD
z. Sulfathiazole (STZ) – (US Pharmacopeia, Rockville, MD
aa. Sulfacetamide (SAA) – (US Pharmacopeia, Rockville, MD
bb. Sulfadoxine (SDX) – (Sigma Aldrich, St. Louis, MO)
cc. Sulfamethoxypyridazine (SMP) – (Sigma Aldrich, St. Louis, MO)
dd. Sulfamethoxazole (SMX) – (Sigma Aldrich, St. Louis, MO)
ee. Sulfaethoxypyridazine (SEP) – (Sigma Aldrich, St. Louis, MO)
ff. Sulfapyridine (SPD) – (Sigma Aldrich, St. Louis, MO)
gg. Monensin (MON) – (Sigma Aldrich, St. Louis, MO)
hh. Demeclocycline (DEME) – (Sigma Aldrich, St. Louis, MO)
ii. Sulfamethazine $^{13}C_6$ – (Sigma Aldrich, St. Louis, MO)
jj. Ciprofloxacin $^{13}C_3$ – (Cambridge Isotope Laboratories, Andover, MA)
kk. Lincomycin d_3 – (Sigma Aldrich, St. Louis, MO)

Standard Preparation:

1. Stock Internal Standard (ISTD) – These solutions should be stored in a freezer at ≤0 °C:

    a) Sulfamethazine $^{13}C_6$ labeled (100 µg/mL): 10.0 mg (after correcting for impurities) diluted to 100 mL in methanol. This solution has a shelf life of six months.

    b) Demeclocycline (200 µg/mL): 20.0 mg (after correcting for impurities) diluted to 100 mL in methanol. This solution has a shelf life of six months.

    c) Ciprofloxacin $^{13}C_3$ (100 µg/mL): 10.0 mg (after correcting for impurities) diluted to 100 mL in methanol, a few drops of ~0.1N HCL maybe needed to ensure Ciprofloxacin properly dissolves. This solution has a shelf life of six (6) months.

    d) Lincomycin d_3 (200 µg/mL): 20.0 mg (after correcting for impurities) diluted to 100 mL in methanol. This solution has a shelf life of six (6) months.

2. Mixed Intermediate ISTD: This solution has a shelf life of six (6) months if stored in a freezer (≤-70°C).

Table 1:

Analyte	Conc. of Stock Solution	Volume Used	Final Volume	Final Concentration
Sulfamethazine $^{13}C_6$	100 µg/mL	500 µL	5.00 mL	10.0 µg/mL
Demeclocycline	200 µg/mL	200 µL	5.00 mL	8.00 µg/mL
Ciprofloxacin $^{13}C_3$	100 µg/mL	20.0 µL	5.00 mL	0.400 µg/mL
Lincomycin d_3	200 µg/mL	200 µL	5.00 mL	8.00 µg/mL

3. Stock Analytical Standards–These solutions should be stored at ≤ 0 ˚C:

***** *Indicates two independent stock solutions should be prepared. One of the stock solutions will serve as a calibration standard and the other will serve as the matrix spike/initial calibration verification (ICV) fortification standard.*

    a) Ciprofloxacin (200 µg/mL): 10.0 mg (after correcting for impurities) diluted to 50.0 mL in methanol, a few drops of ~0.1N HCL may be needed to ensure Ciprofloxacin properly dissolves. This solution has a shelf life of six months.

    b) Enrofloxacin (200 µg/mL): 10.0 mg (after correcting for impurities) diluted to 50.0 mL in methanol. This solution has a shelf life of six months.

    c) *Norfloxacin (200 µg/mL): 10.0 mg (after correcting for impurities) diluted to 50.0 mL in ethanol, DMSO, or methanol, solution should be sonicated at 50°C to ensure Norfloxacin properly dissolves. This solution has a shelf life of six months.

    d) Difloxacin (200 µg/mL): 10.0 mg (after correcting for impurities) diluted to 50.0 mL in methanol. This solution has a shelf life of six months.

    e) Sarafloxacin (200 µg/mL): 10.0 mg (after correcting for impurities) diluted to 50.0 mL in methanol. This solution has a shelf life of six months.

f) Danofloxacin (200 µg/mL): 10.0 mg (after correcting for impurities) diluted to 50.0 mL in methanol. This solution has a shelf life of six months.

g) Oxytetracycline (2.00 mg/mL): 100 mg (after correcting for impurities) diluted to 50.0 mL in methanol. This solution has a shelf life of six months.

h) Chlortetracycline (2.00 mg/mL): 100 mg (after correcting for impurities) diluted to 50.0 mL in methanol. This solution has a shelf life of six months.

i) *Tetracycline (2.00 mg/mL): 100 mg (after correcting for impurities) diluted to 50.0 mL in methanol. This solution has a shelf life of six months.

j) Doxycycline (2.00 mg/mL): 100 mg (after correcting for impurities) diluted to 50.0 mL in methanol. This solution has a shelf life of six months.

k) Monensin (100 µg/mL): 10.0 mg (after correcting for impurities) diluted to 100 mL in methanol. This solution has a shelf life of six months.

l) * Lincomycin (2.00 mg/mL): 100 mg (after correcting for impurities) diluted to 50.0 mL in methanol. This solution has a shelf life of six months.

m) Sulfacetamide (500 µg/mL): 50.0 mg (after correcting for impurities) diluted to 100 mL in methanol. This solution has a shelf life of six months.

n) Sulfamerazine (500 µg/mL): 50.0 mg (after correcting for impurities) diluted to 100 mL in methanol. The standard should be stored in freezer (0–10°C). This solution has a shelf life of six months.

o) Sulfadiazine (500 µg/mL): 50.0 mg (after correcting for impurities) diluted to 100 mL in methanol. This solution has a shelf life of six months.

p) Sulfachloropyridazine (500 µg/mL): 50.0 mg (after correcting for impurities) diluted to 100 mL in methanol. This solution has a shelf life of six months.

q) Sulfathiazole (500 µg/mL): 50.0 mg (after correcting for impurities) diluted to 100 mL in methanol. This solution has a shelf life of six months.

r) Sulfaquinoxaline (500 µg/mL): 50.0 mg (after correcting for impurities) diluted to 100 mL in methanol. This solution has a shelf life of six months.

s) *Sulfamethazine (500 µg/mL): 50.0 mg (after correcting for impurities) diluted to 100 mL in methanol. This solution has a shelf life of six months.

t) Sulfadimethoxine (500 µg/mL): 50.0 mg (after correcting for impurities) diluted to 100 mL in methanol. This solution has a shelf life of six months.

u) Sulfamethoxazole (500 µg/mL): 50.0 mg (after correcting for impurities) diluted to 100 mL in methanol. This solution has a shelf life of six months.

v) Sulfaethoxypyridazine (500 µg/mL): 50.0 mg (after correcting for impurities) diluted to 100 mL in methanol. This solution has a shelf life of six months.

w) Sulfamethoxypyridazine (500 µg/mL): 50.0 mg (after correcting for impurities) diluted to 100 mL in methanol. This solution has a shelf life of six months.

x) Sulfadoxine (500 µg/mL): 50.0 mg (after correcting for impurities) diluted to 100 mL in methanol. This solution has a shelf life of six months.

y) Sulfapyridine (500 µg/mL): 50.0 mg (after correcting for impurities)

diluted to 100 mL in methanol. This solution has a shelf life of six months.

4. Mixed Intermediate 1: This solution has a shelf life of six (6) months if stored in a freezer (≤ -70°C).

Table 2:

Analyte	Conc. of Parent Solution	Volume Used (µL)	Final Volume in Methanol *(mL)*	Final Concentration
Ciprofloxacin	200 µg/mL	25.0	25.0	200 ng/mL
Danofloxacin	200 µg/mL	25.0	25.0	200 ng/mL
Sarafloxacin	200 µg/mL	25.0	25.0	200 ng/mL
Norfloxacin	200 µg/mL	25.0	25.0	200 ng/mL
Enrofloxacin	200 µg/mL	25.0	25.0	200 ng/mL
Difloxacin	200 µg/mL	25.0	25.0	200 ng/mL
Oxytetracycline	2.00 mg/mL	25.0	25.0	2.00 µg/mL
Doxycycline	2.00 mg/mL	25.0	25.0	2.00 µg/mL
Tetracycline	2.00 mg/mL	25.0	25.0	2.00 µg/mL
Chlortetracycline	2.00 mg/mL	25.0	25.0	2.00 µg/mL
Sulfamerazine	500 µg/mL	250	25.0	5.00 µg/mL
Sulfadiazine	500 µg/mL	250	25.0	5.00 µg/mL
Sulfachloropyridine	500 µg/mL	250	25.0	5.00 µg/mL
Sulfathiazole	500 µg/mL	250	25.0	5.00 µg/mL
Sulfaquinoxaline	500 µg/mL	250	25.0	5.00 µg/mL
Sulfamethazine	500 µg/mL	250	25.0	5.00 µg/mL
Sulfadimethoxine	500 µg/mL	250	25.0	5.00 µg/mL
Sulfacetamide	500 µg/mL	250	25.0	5.00 µg/mL
Sulfadoxine	500 µg/mL	250	25.0	5.00 µg/mL
Sulfamethoxypyridazine	500 µg/mL	250	25.0	5.00 µg/mL
Sulfamethoxazole	500 µg/mL	250	25.0	5.00 µg/mL
Sulfaethoxypyridazine	500 µg/mL	250	25.0	5.00 µg/mL
Sulfapyridine	500 µg/mL	250	25.0	5.00 µg/mL
Lincomycin	2.00 mg/mL	25.0	25.0	2.00 µg/mL
Monensin	100 µg/mL	25.0	25.0	100 ng/mL

5. Mixed Intermediate 2 (ICV): This solution has a shelf life of six (6) months if stored in a freezer (≤ -70°C). *(This solution should be prepared from separate stock standards than the mixed intermediate standard listed above).*

Table 3:

Analyte	Conc. of Parent Solution	Volume Used (µL)	Final Volume in Methanol (mL)	Final Concentration
Norfloxacin	200 µg/mL	25.0	25.0	200 ng/mL
Tetracycline	2.00 mg/mL	25.0	25.0	2.00 µg/mL
Sulfamethazine	500 µg/mL	250	25.0	5.00 µg/mL
Lincomycin	2.00 mg/mL	25.0	25.0	2.00 µg/mL

Multi-Residue Extraction Procedure:

A. Honey composite/sample (2 grams +/- 0.03) is weighed into 50 mL polypropylene tubes. Control honey is used for quality control and calibration curve points.

B. All samples are fortified with 20.0 µL of the mixed intermediate internal standard solution.

C. Matrix spikes and the initial calibration verification (ICV) are fortified with 40.0 µL of a mixed intermediate standard.

D. Calibration standards are fortified as listed in the table below:

Table 4:

Extracted Calibration Curve	Volume of Mixed Intermediate Standard (µL)
Cal Std-1	0
Cal Std-2	20.0
Cal Std-3	40.0
Cal Std-4	80.0
Cal Std-5	100
Cal Std-6	200

E. 10 mL of water is added to each sample and vortexed/shook until sample is dissolved into the solution.

F. 10 mL of acetonitrile and 200 µL of glacial acetic acid are added to each tube and the tubes are agitated with a Geno/Grinder for 2 minutes.

G. Approximately 5 grams of NaCl is added to each tube and the tubes are shaken with a Geno/Grinder (2 minutes at a rate of 1500 strokes per minute).

H. Centrifuge samples at approximately 4300 g for 10 minutes at 5 °C.

I. The (upper) organic layer is transferred to a clean 15 mL centrifuge tube. The 15 mL tube is placed in a nitrogen evaporator at approximately 50°C.

J. 10 mL of acetonitrile is added an additional time to each of the 50 mL tubes and the tubes are shaken with a Geno/Grinder for 2 minutes.

K. Transfer the organic layer to the original 15 mL tubes and continue evaporation.

L. Evaporate samples to approximately < 1 mL. If a salt precipitant is present, add an additional 7 mL of acetonitrile to each tube, vortex (~15 seconds), and centrifuge (4000 g for 10 minutes at 5 °C). Decant into clean 15 mL tube and continue evaporation to dryness. If a salt precipitant is not present, then proceed evaporating to dryness.

M. Add 50 µL of methanol to each tube, sonicate (5 minutes), and vortex (~15 seconds).

N. Add 450 µL of 0.1% Formic Acid in water to each tube, sonicate (5 minutes), and vortex (~15 seconds).

O. Transfer sample to autosampler vial for analysis.

Sample Concentrations:

Table 5:

	ENRO ng/g	SARA ng/g	CIPRO ng/g	DANO ng/g	DFLX ng/g	NOR ng/g
Cal Std # 1	0.00	0.00	0.00	0.00	0.00	0.00
Cal Std # 2	2.00	2.00	2.00	2.00	2.00	2.00
Cal Std # 3	4.00	4.00	4.00	4.00	4.00	4.00
Cal Std # 4	8.00	8.00	8.00	8.00	8.00	8.00
Cal Std # 5	10.0	10.0	10.0	10.0	10.0	10.0
Cal Std # 6	20.0	20.0	20.0	20.0	20.0	20.0

	DC ng/g	TC ng/g	CTC ng/g	OTC ng/g
Cal Std # 1	0.00	0.00	0.00	0.00
Cal Std # 2	20.0	20.0	20.0	20.0
Cal Std # 3	40.0	40.0	40.0	40.0
Cal Std # 4	80.0	80.0	80.0	80.0
Cal Std # 5	100	100	100	100
Cal Std # 6	200	200	200	200

	SMZ ng/g	SMR ng/g	SDM ng/g	SDZ ng/g	SQX ng/g
Cal Std # 1	0.00	0.00	0.00	0.00	0.00
Cal Std # 2	50.0	50.0	50.0	50.0	50.0
Cal Std # 3	100	100	100	100	100
Cal Std # 4	200	200	200	200	200
Cal Std # 5	250	250	250	250	250
Cal Std # 6	500	500	500	500	500

	SEP ng/g	SMX ng/g	SMP ng/g	SPD ng/g	SDX ng/g
Cal Std # 1	0.00	0.00	0.00	0.00	0.00
Cal Std # 2	50.0	50.0	50.0	50.0	50.0
Cal Std # 3	100	100	100	100	100
Cal Std # 4	200	200	200	200	200
Cal Std # 5	250	250	250	250	250
Cal Std # 6	500	500	500	500	500

	SCP ng/g	STZ ng/g	SAA ng/g	LIN ng/g
Cal Std # 1	0.00	0.00	0.00	0.00
Cal Std # 2	50.0	50.0	50.0	20.0
Cal Std # 3	100	100	100	40.0
Cal Std # 4	200	200	200	80.0
Cal Std # 5	250	250	250	100
Cal Std # 6	500	500	500	200

Instrument Configuration for Positive Ionization Multi-Residue Analysis:

LC-MS/MS System and Operating Conditions for Positive Ionization Only:

Chromatography (utilizing Agilent Poreshell EC-C18 column (4.6 mm×50 mm X 2.7 µm) chromatographic column):

Table 6:

Minutes	Flow Rate (µL/min)	0.1% formic acid in water	0.1% formic acid in acetonitrile
0.00	500	95%	5.0%
0.50	500	95%	5.0%
6.00	500	50%	50%
6.10	500	1.0%	99%
8.10	500	1.0%	99%
8.20	500	95%	5.0%
11.0	500	95%	5.0%

Column Temp: 25°C
Injection Volume: 5 µL
Autosampler Temp: 5°C

Mass Spectrometry
Typical voltages and settings for the MS: Spray
Voltage: + 5500 V
Curtain Gas: 10 psi Source
Heater: 650°C
Declustering Potential: + 70 V
Entrance Potential: + 10 V I o n
Source Gas 1: 40 psi
Ion Source Gas 2: 30 psi

MRM Acquisition Parameters:

Table 7:

Name	Precursor m/z	Product m/z	Scheduled MRM Time (min)
[1] Ciprofloxacin (CIP)	332.1	231.2 *	5.00
Ciprofloxacin (CIP)	332.1	288.4	5.00
[1] Danofloxacin (DANO)	358.1	283.0 *	5.15
Danofloxacin (DANO)	358.1	314.1	5.15
[1] Sarafloxacin (SAR)	386.1	299.2 *	5.70
Sarafloxacin (SAR)	386.1	342.3	5.70
[1] Norfloxacin (NOR)	320.1	276.1	5.00
Norfloxacin (NOR)	320.1	233.0 *	5.00
[1] Enrofloxacin (ENRO)	360.1	245.2 *	5.30
Enrofloxacin (ENRO)	360.1	316.3	5.30
[1] Difloxacin (DFLX)	400.4	299.0 *	5.70
Difloxacin (DFLX)	400.4	356.1	5.70
[2] Oxytetracycline (OTC)	461.1	337.2	5.00
Oxytetracycline (OTC)	461.1	426.3 *	5.00
[2] Tetracycline (TC)	445.100	154.2 *	5.00
Tetracycline (TC)	445.100	410.3	5.00
[2] Doxycycline (DC)	445.105	410.3 *	6.20
Doxycycline (DC)	445.105	428.3	6.20

[2] Chlortetracycline (CTC)	479.1	154.2	6.10
Chlortetracycline (CTC)	479.1	462.3 *	6.10
[3] Sulfamerazine (SMR)	265.0	92.3 *	4.90
Sulfamerazine (SMR)	265.0	108.3	4.90
[3] Sulfadiazine (SDZ)	251.0	92.3	4.40
Sulfadiazine (SDZ)	251.0	156.2 *	4.40
[3] Sulfachlorpyridazine (SCP)	285.0	92.3	6.10
Sulfachlorpyridazine (SCP)	285.0	108.3 *	6.10
[3] Sulfathiazole (STZ)	256.0	92.3	4.70
Sulfathiazole (STZ)	256.0	156.2 *	4.70
[3] Sulfaquinoxaline (SQX)	301.0	92.3	7.00
Sulfaquinoxaline (SQX)	301.0	108.3 *	7.00
[3] Sulfamethazine (SMZ)	279.1	92.3 *	5.30
Sulfamethazine (SMZ)	279.1	156.2	5.30
[3] Sulfadimethoxine (SDM)	311.0	92.3	7.04
Sulfadimethoxine (SDM)	311.0	108.3 *	7.04
[3] Sulfacetamide (SAA)	215.0	108.0 *	4.10
Sulfacetamide (SAA)	215.0	92.3	4.10
[3] Sulfaethoxypyridazine (SEP)	295.0	156.0*	6.30
Sulfaethoxypyridazine (SEP)	295.0	92.3	6.30
[3] Sulfamethoxazole (SMX)	254.0	156.0*	6.40
Sulfamethoxazole (SMX)	254.0	92.3	6.40
[3] Sulfamethoxypyridazine (SMP)	280.9	92.1*	5.90
Sulfamethoxypyridazine (SMP)	280.9	156.0	5.90
[3] Sulfapyridine (SPD)	250.0	92.3*	4.70
Sulfapyridine (SPD)	250.0	156.0	4.70
[3] Sulfadoxine (SDX)	310.9	156.0*	6.20
Sulfadoxine (SDX)	310.9	92.3	6.20
[4] Lincomycin (LIN)	407.2	126.2 *	4.40
Lincomycin (LIN)	407.2	359.2	4.40
Monensin (MON)	693.5	461.4	10.00
Monensin (MON)	693.5	657.4	10.00
Sulfamethazine $^{13}C_6$ (SMZ $^{13}C_6$)	285.3	124.1	5.30
Demeclocycline (DEME)	465.1	448.1	5.75
Ciprofloxacin $^{13}C_3$ (CIPRO $^{13}C_3$)	336.1	291.1	5.00
Lincomycin d_3 (LIN d_3)	410.3	129.2	4.40

*Notes the quantitation ion.

[1] Ciprofloxacin $^{13}C_3$ was utilized as the internal standard for quantitation.

[2] Demeclocycline was utilized as the internal standard for quantitation.

[3] Sulfamethazine $^{13}C_6$ was utilized as the internal standard for quantitation.

[4] Lincomycin d_3 was utilized as the internal standard for quantitation.

DC and TC are isomers. Precursor 445.105 for DC was chosen to distinguish from TC because the software utilized prohibits duplicate precursor and product ions.

Data Interpretation:

All the targeted drug residues included in this method made use of an internal standard to improve quantitation. The designated analyte/internal standard is listed in Table 7. The calibration curves yielded a regression (R^2) ≥0.99. For positive confirmation all product ions must be detected, the associated chromatographic peak must exhibit a retention time within ± 5% of the average retention time of the calibration standards, and the product ion ratios must be within 10% of the average product ion ratios obtained from the calibration standards (9).

Analysis of Reference Materials and Commercial Products:

Reference materials were obtained from commercially available materials and previously analyzed products. Samples were quantitated using matrix-matched extracted standards that were previously screened and determined to be free of the targeted residues. It should also be noted that one incurred residue sample was analyzed to verify that the methodology could quantitate and confirm residues in matrix.

Limits of Detection and Quantitation Studies:

The method detection limits (MDL) and limits of quantitation (LOQ) for each analyte were determined on the basis of replicate analyses (n=7). The MDL of each analyte was calculated by the multiplication of the standard deviation by the student's t-value at the 99% confidence level. The LOQ was calculated by multiplying the standard deviation by ten.

Results and Discussion

Method Optimization:

The first step in the method optimization process was to expand the scope of chemical residues assayed previously with LIB 4650. Our initial efforts were focused on maximizing signal response of the five additional sulfonamide compounds. Additionally, the levels of concern for almost all residues assayed changed over the last few years. Therefore, there was a need to optimize all the other chemical residues previously analyzed in order to provide adequate sensitivity and peak shape.

The next modification was the use of a different chromatographic column. The original column used in LIB 4560 had limitations with regards to ruggedness and reproducibility with this specific analysis. Over the past years, there have been substantial improvements in HPLC columns. A few different column manufacturers, and column sizes (particle size, internal diameter, and length) were tested with regards to peak height, peak asymmetry and resolution. After careful evaluation, the Agilent Poreshell provided the best overall chromatography during our research, as well as exhibiting a relatively low backpressure at a flow rate of 0.500 mL/min.

Once the instrument acquisition method was developed, our attention shifted to the extraction method. Multiple extraction techniques were evaluated during the method modification process, including the use of solid-phase extraction (SPE). Although these

alternative procedures for extraction provided acceptable results, there was no clear advantage demonstrated over the liquid-liquid salt-assisted extraction process outlined in LIB 4560. However, it was discovered that a Geno/Grinder agitated the honey samples more effectively than the multi-vortexer used previously. Furthermore, the agitation time could be reduced from 10 minutes to 2 minutes when using the Geno/Grinder.

Method Validation: Three vastly different honey matrices were evaluated (manuka, clover, and Acacia) when performing the method validation. Validation was performed utilizing the U.S. Food and Drug Administration guidance for industry for the mass spectrometry confirmation and identification of animal drug residues, and the FDA guidelines for chemical validation (9, 10). The validation procedure consisted of a total of 42 matrix spikes and 13 matrix blanks. Method accuracies and precisions, using a matrix extracted calibration curve with internal standard correction for selected analytes, were acceptable for the fortified honey (Table 8).

All 35 matrix spikes analyzed met the required confirmation criteria for all residues of interest. No false positives were observed in the 13 matrix blanks that were analyzed. In addition to the 42 assayed matrix spikes, sulfathiazole was confirmed in the incurred honey sample. Previous analysis of the incurred residue utilizing LIB 4560 found sulfathiazole at 44.8 ng/g. The result in the current study found sulfathiazole at 42.2 ng/g, which was within the validated uncertainty level. It should also be noted that all statistical limit of quantitation values (LOQ) from the validation were below the required TTL for analysis.

CONCLUSION:

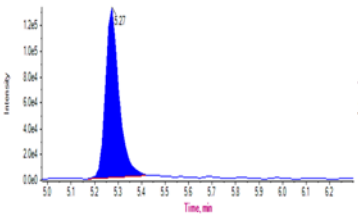
LIB 4560 is an effective multi-class, multi-residue, quantitative confirmatory LC-MS/MS method for honey. However, the method has become outdated and needed major revisions to accommodate additional analytes and revised levels of concern for most all drug residues assayed. Additional modifications to the method included the use of a different analytical column to improve chromatography, and the use of a Geno/Grinder to provide better sample and solvent interactions. The method described herein can accurately quantitate 24 different drug residues from 4 classes of drugs. Additionally, monensin can be confirmed but not quantitated with this method. The sample extraction and cleanup procedure is relatively simple and quick, all the while being extremely effective. The additional compounds added had no effects on sample throughput when compared to LIB 4560. This makes the method a viable option for regulatory laboratories performing analysis of honey for multi-drug residues.

Table 8

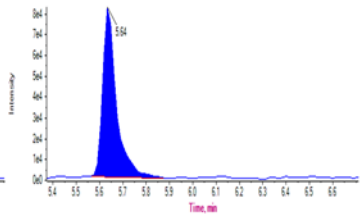
Analyte	MDL ng/g	LOQ ng/g	Average recovery ng/g (Target concentration)	%RSD
Enrofloxacin	0.268	0.852	1.88 (2.00)	4.54
Sarafloxacin	0.559	1.78	2.07 (2.00)	8.59
Ciprofloxacin	0.396	1.26	1.76 (2.00)	7.16
Danofloxacin	0.380	1.21	1.86 (2.00)	6.49
Difloxacin	0.315	1.00	1.98 (2.00)	5.07
Norfloxacin	0.449	1.43	2.16 (2.00)	6.62
Sulfaethoxypyridazine	5.60	17.8	20.6 (20.2)	8.67
Sulfamethoxazole	2.92	9.29	22.6 (25.0)	4.12
Sulfamethoxypyridazine	4.63	14.7	22.5 (25.2)	6.62
Sulfapyridine	3.67	11.7	25.2 (25.0)	4.64
Sulfathiazole	8.44	26.9	49.7 (50.0)	5.41
Sulfamethazine	6.23	19.8	52.4 (50.0)	3.78
Sulfamerazine	4.61	14.7	50.0 (50.0)	2.94
Sulfadimethoxine	12.3	39.1	49.4 (50.0)	7.91
Sulfadiazine	7.54	24.0	45.2 (50.0)	5.31
Sulfachloropyridazine	10.0	31.8	46.6 (50.0)	6.83
Sulfaquinoxaline	10.1	32.0	48.8 (50.0)	6.57
Sulfacetamide	14.5	46.1	46.5 (50.0)	9.90
Sulfadoxine	3.67	11.7	19.1 (25.0)	4.64
Lincomycin	2.58	8.21	10.6 (10.0)	8.21
Doxycycline	1.66	5.30	7.79 (10.0)	6.80
Tetracycline	1.54	4.90	8.20 (9.95)	5.97
Chlortetracycline	1.11	3.54	9.48 (9.90)	3.73
Oxytetracycline	1.35	4.30	12.9 (10.0)	3.33

Table 9: Chromatograms of Fortified Blank at the Lowest Calibration Standard

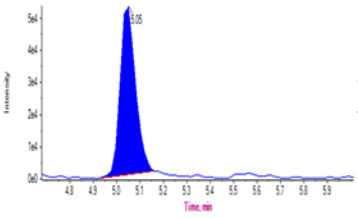
Enrofloxacin



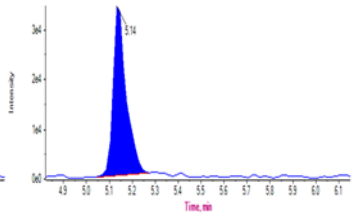
Sarafloxacin



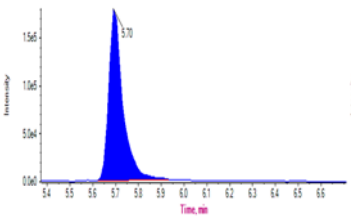
Ciprofloxacin



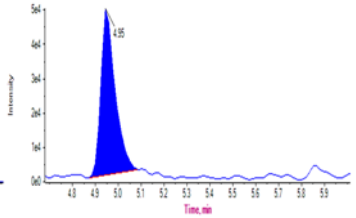
Danofloxacin



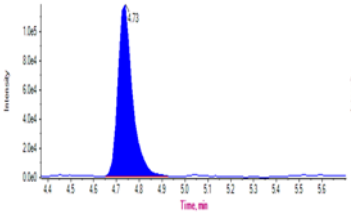
Difloxacin



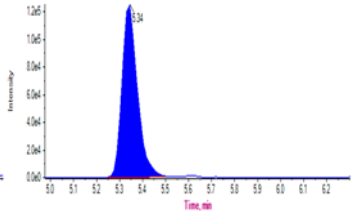
Norfloxacin



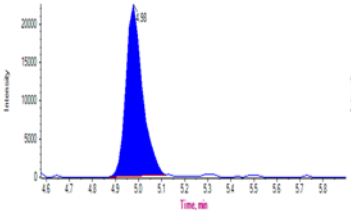
Sulfathiazole



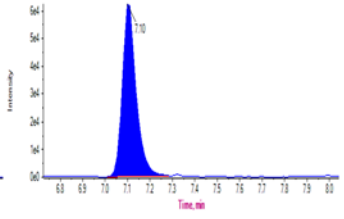
Sulfamethazine



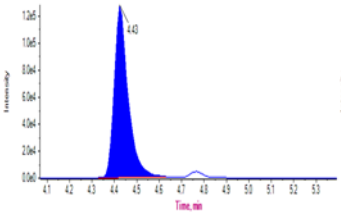
Sulfamerazine



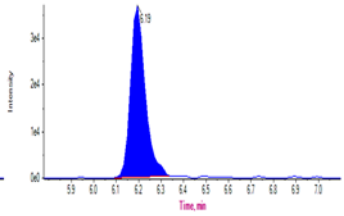
Sulfadimethoxine



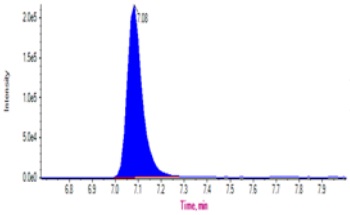
Sulfadiazine



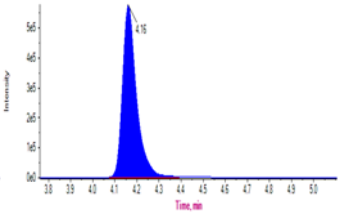
Sulfachloropyridazine



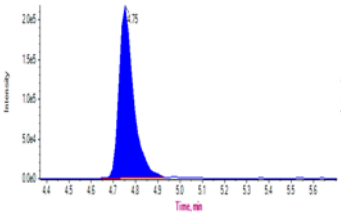
Sulfaquinoxaline



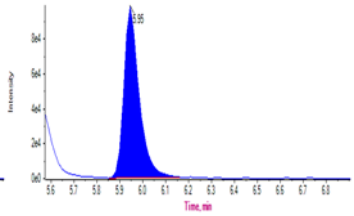
Sulfacetamide



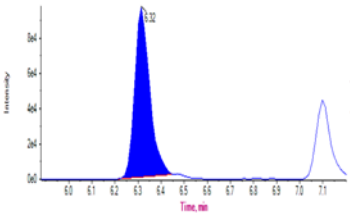
Sulfapyridine



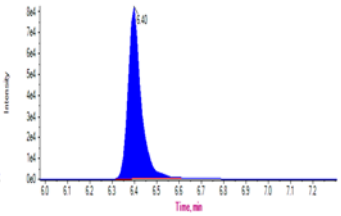
Sulfamethoxypyridazine



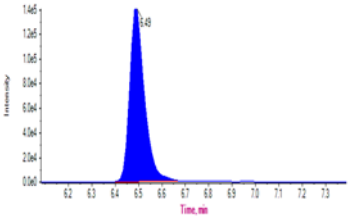
Sulfadoxine



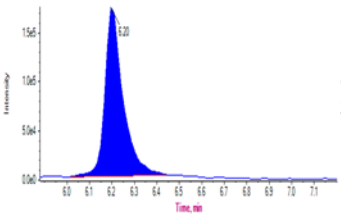
Sulfaethoxypyridazine



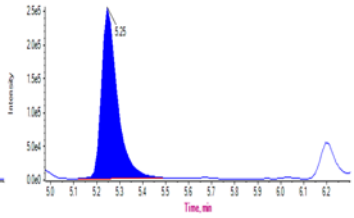
Sulfamethoxypyridazine



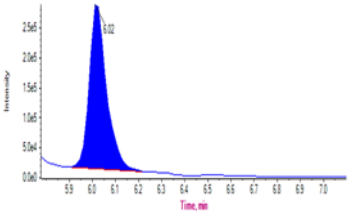
Doxycycline



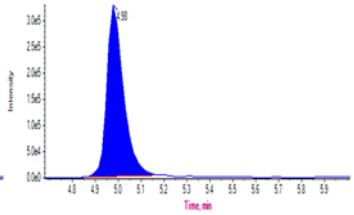
Tetracycline



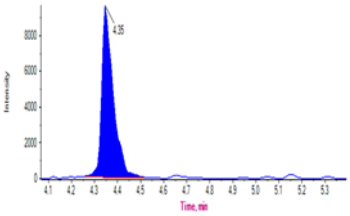
Chlortetracycline



Oxytetracycline



Lincomycin



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